



Research Article

Automated Screening for Oxidative or Methylation-Induced DNA Damage in Human Cells

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Abstract

The assessment of genotoxicity upon exposure to chemical and environmental agents plays an important role in basic research as well as in pharmaceutical, chemical, cosmetic and food industry. Low sensitivity and lack of inter-laboratory comparability are considered problematic issues in genotoxicity testing. Moreover, commonly used mutagenicity assays lack information about early and specific genotoxic events.

Previously, we developed an automated version of the “Fluorimetric detection of Alkaline DNA Unwinding” (FADU) assay as a high-throughput screening method for the detection of DNA strand breaks in living cells. Here, we report an enzyme-modified version of the cell-based FADU assay (emFADU) for the determination of oxidative and methylation lesions in cellular DNA. Our method is based on the use of formamidopyrimidine DNA glycosylase or human alkyladenine DNA glycosylase for the detection of chemically-induced nucleobase modifications in lysates of immortalized cell lines, growing in suspension or as adherent cells, and in peripheral blood mononuclear cells. We could show that upon treatment with sub-cytotoxic doses of known genotoxins, oxidative and methylation lesions are readily detectable.

This fast, inexpensive, and convenient method could be useful as a high-content screening approach for the sensitive and specific assessment of genotoxicity in human cells. Thus, when implemented in the early compound development in an industrial setting, the emFADU assay could help reduce the number of animals used for toxicity testing. Furthermore, as we established the method for different cell types, this new assay may provide an opportunity for population studies and/or mechanistic research into DNA repair pathways.

1 Introduction

Every day, a human cell must cope with a variety of DNA insults, including DNA strand breaks (SB), apurinic/aprimidinic (AP) sites and nucleobase lesions (Lindahl, 1993). Therefore, highly efficient DNA repair mechanisms have evolved to safeguard the integrity of the human genome. Unrepaired DNA damage could lead to cell death, resulting in accelerated aging, or mutations, increasing the risk of cancer development (Bernstein et al., 2002; Hoeijmakers, 2009). Besides endogenous DNA damage, our genome is constantly challenged by a variety of exogenous genotoxic sources, e.g., tobacco smoke, air pollution, processed meat, and conceivably unknown chemicals in the environment, highlighting the necessity of genotoxicity testing (Bouvard et al., 2015; Ciccia and Elledge, 2010; Cross and Sinha, 2004). Furthermore, ongoing product development in pharmaceutical, chemical, cosmetic and food industry requires sensitive, robust, and cost-effective meth-

ods for risk assessment and classification of chemicals. When establishing new test methods, it is imperative to implement the 3R principle where possible. Ideally such tests should be animal-free and should rely on human-relevant biological systems (Beken et al., 2016; Knudsen et al., 2019).

The comet assay is the gold standard in many laboratories for genotoxicity measurements. With its high sensitivity and broad scope of application, it is an attractive technique for analyzing DNA damage induction and repair in a broad variety of cells. Nevertheless, a problematic issue is the lack of inter-laboratory comparability (Ersson et al., 2013). Work on standardization of the comet assay protocol has decreased the inter-experimental and inter-laboratory variability of the assay (Cassano et al., 2020), but also shown its drawbacks (Brunborg et al., 2018; Enciso et al., 2018). The CometChip[®] platform provides a high-throughput DNA damage analysis in a 96-well format, but no automated version has been described so far (Sykora et al., 2018). Additionally,

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costs per run remain high (> 200€ per chip), limiting its use especially for big sample sizes.

As an alternative to the comet assay, we have developed an automated version of the “Fluorimetric detection of Alkaline DNA Unwinding” (FADU) assay (Moreno-Villanueva et al., 2009, 2011). First described by Birnboim and Jevcak (1981), the method uses a highly alkaline pH for unwinding of genomic DNA in a cell lysate, starting at chromosome ends and sites of replication. DNA SBs serve as additional starting points for DNA unwinding. Therefore, increased numbers of SBs lead to increased unwinding, which can be quantified by measuring the amount of DNA remaining double stranded during the alkaline unwinding phase of the assay, as detected via SYBR™ Green I staining. Based on the use of a liquid handling device and a 96-well format, we have been able to reduce operator bias and increase sample throughput. In a previous study, we could show that the FADU principle can be used to detect chemically induced formamidopyrimidine DNA glycosylase (Fpg)-sensitive sites in purified plasmid DNA (Müller et al., 2013). The glycosylase recognizes and excises oxidative DNA lesions, like 8-oxo-2'-deoxyguanosine (8-oxodG), leaving an apurinic/aprimidinic (AP) site that can be processed by Fpg's endonuclease function, resulting in a DNA SB (O'Connor and Laval, 1989; Tchou et al., 1991). In the present work, we could also show that the FADU can be used to detect methyl methanesulfonate (MMS) induced methylation lesions in purified plasmid DNA by using the human alkyladenine DNA glycosylase (hAAG) in combination with AP endonuclease 1 (APE1) (Fig. S1¹).

Already more than 20 years ago, glycosylases were used to assess DNA damage in human cells. This was implemented in alkaline elution and alkaline unwinding to detect oxidative DNA damage (Hartwig et al., 1996; Pflaum et al., 1997). However, the methods had to be performed manually and are not suitable for analyzing larger numbers of samples at high throughput. Here, we describe a novel method for the detection of genotoxicity in human cells. This was implemented by modifying the automated FADU assay for the detection of glycosylase-specific DNA lesions to assess the amount of oxidative DNA lesions as well as methylation lesions. We found that subcytotoxic doses of hydrogen peroxide (H₂O₂), potassium bromate (KBrO₃) and MMS lead to a significant increase in nucleobase modifications, thus demonstrating the proof-of-principle to increase the sensitivity of genotoxicity testing with our new technique. Regarding workload, assay duration and cost efficiency, we show that the emFADU is an attractive alternative to the CometChip® platform.

2 Materials and methods

2.1 Cell culture

THP-1 cells were cultivated in RPMI 1640 medium supplemented with 10% fetal calf serum (FCS; Biochrome) and 1% penicillin-streptomycin (PenStrep; Gibco). Cells were kept at 37°C in a humidified atmosphere with 5% CO₂. Cells were cryopreserved

to create stock cultures. Cells were routinely tested for mycoplasma contamination (once per year and prior to every master stock preparation).

Venous blood was obtained from apparently healthy human donors aged 23-29 (two thirds women, one third men). Selection of volunteers was carried out in accordance with the Declaration of Helsinki, and ethical approval was obtained from the Ethics Committee of the University of Konstanz. Signed informed consent was obtained from each subject. Peripheral blood mononuclear cells (PBMCs) were isolated using Biocoll density centrifugation. Briefly, 10 mL whole blood was drawn with citrate as anti-coagulant. The blood was diluted with an equal volume of phosphate buffered saline (PBS; Biochrome), layered on top of 15 mL Biocoll separation solution (Biochrome), and centrifuged at 900 g for 15 min at RT in a swing-out rotor without break. PBMCs were harvested from the gradient and washed in PBS by centrifugation at 300 g for 10 min at 4°C. After washing, PBMCs were resuspended in pre-warmed RPMI 1640 medium supplemented with 10% FCS and 1% PenStrep.

HeLa Kyoto cells were cultivated in DMEM (Dulbecco's Modified Eagle's Medium; Gibco), supplemented with 10% FCS and 1% PenStrep. Cells were kept at 37°C in a humidified atmosphere with 5% CO₂. Cells were routinely tested for mycoplasma contamination (once per year and prior to every master stock preparation).

Cultivation of cells was established and conducted with the use of FCS. For future studies we aim to investigate alternatives to the use of FCS in order to replace the use of animal-derived products.

2.2 Genotoxic treatment

For X-irradiation, 300 µL cell suspension (THP-1: 6.7 x 10⁵ cells/mL; PBMCs: 13.4 x 10⁵) were irradiated in reaction tubes on ice using an X-RAD 225 - Precision X-ray irradiator. Cells were washed twice with 900 µL ice-cold PBS and resuspended in 300 µL PBS containing 300 µM deferoxamine mesylate salt (DFA; CAS: 138-14-7, Sigma-Aldrich) and 300 µM 4 hydroxy-TEMPO (TEMPOL; CAS: 2226-96-2, Sigma-Aldrich).

For genotoxic treatment of THP-1 cells and PBMCs, 500 µL cell suspension (THP-1: 6.7 x 10⁵ cells/mL; PBMCs: 13.4 x 10⁵) was transferred to a 24-well plate for each sample. The genotoxin was pre-diluted in growth medium, and cells were treated with various concentrations at 37°C in a humidified atmosphere with 5% CO₂. The time of incubation for H₂O₂ (CAS: 7722-84-1, Sigma-Aldrich) was set to 30 min, and to 120 min for KBrO₃ (CAS: 7758-01-2, Sigma-Aldrich) or MMS (CAS: 66-27-3, Sigma-Aldrich). After treatment, 300 µL cell suspension was transferred to a reaction tube and used for cell harvest (300 g, 4°C, 10 min), washed twice with ice-cold 900 µL PBS, and resuspended in 300 µL PBS containing 300 µM DFA and 300 µM TEMPOL.

For genotoxic treatment of HeLa cells, 15 µL cell suspension (3.3 x 10⁵ cells/mL) was seeded per well in a Falcon® 96-well Clear Round Bottom TC-treated Cell Culture Microplate. After 2 h, 85 µL culture medium was added to avoid desiccation. The next day, the genotoxin was pre-diluted in growth medium and cells

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were treated with various concentrations at 37°C in a humidified atmosphere with 5% CO₂. For each condition, 12 wells were treated equally, i.e., 3 replicates each for T values (= total DNA), P values (= physiological DNA SBs), F values (= Fpg-sensitive sites), and hA values (= hAAG/APE1-sensitive sites). The time of exposure to KBrO₃ and MMS was set to 120 min. After treatment, all wells were washed twice with 200 µL ice-cold PBS.

2.3 Detection of DNA strand breaks and glycosylase-specific DNA lesions

For the detection of DNA base lesions (oxidative and methylation lesions) in cells, the automated FADU assay (Moreno-Villanueva et al., 2009) was modified in order to increase the sensitivity and extend the scope of application. Besides the T value, representing the total amount of DNA, and the P value, reflecting the extent of DNA strand breaks, two additional parameters for enzyme-specific DNA lesions were established. Due to incubation with lesion-specific glycosylases, these values reflect the numbers of directly induced DNA strand breaks plus enzyme-induced strand breaks. In particular, the F value reflects the extent of DNA strand breaks and Fpg-sensitive sites. The hA value reflects the extent of DNA strand breaks and hAAG/APE1-sensitive sites. All automated steps of the emFADU, performed by a TECAN Genesis RSP 100 liquid handling device, are illustrated in Figure 1A.

Cell transfer and lysis

The samples of suspension cells had to be transferred into 96-well plates. For each sample of THP-1 cells or PBMCs, 12 wells of a 96-well plate master block with V-shaped bottom (Greiner-Bio One GmbH, Frickenhausen, Germany), which was cut horizontally to hold 1 mL volume, were filled with 15 µL cell suspension (3 replicates each for T = total DNA, P = physiological DNA SBs, F value = Fpg-sensitive sites, hA = hAAG/APE1-sensitive sites). 15 µL lysis buffer (400 mM guanidine thiocyanate; CAS: 593-84-0, Sigma-Aldrich; 30 mM EDTA, Sigma-Aldrich; 0.2% N-laurylsarcosine; CAS: 137-16-6, Sigma-Aldrich; 300 µM DFA and 300 µM TEMPOL, adjusted to pH 12 with NaOH, CAS: 1310-73-2, Sigma-Aldrich) was added. Cell suspension, lysis buffer and the well plate were all kept at 4°C.

HeLa cells were already seeded, treated, and washed in a 96-well plate. The supernatant was removed and 7.5 µL PBS (supplemented with 300 µM DFA and 300 µM TEMPOL) was added. 7.5 µL lysis buffer was added. Cell suspension, lysis buffer, and the well plate were all kept at 4°C. For the following steps, all volumes described for suspension cells were reduced to 50%. 12 equally treated wells were divided into 3 replicates for each value (T, P, F and hA).

Dilution and enzyme incubation

After 12 min of lysis at 4°C, 270 µL dilution buffer (2 mM HEPES; CAS: 1365-25-9, Sigma-Aldrich; 300 µM DFA and 300 µM TEMPOL) was added on top of the lysate of each well at a dispensing rate of 10 µL/s. For data points with enzyme addition, the dilution buffer was supplemented with lesion-specific enzymes: 0.2 U/mL Fpg (M0240S, New England BioLabs), 0.8 U/mL hAAG (M0313S, New England BioLabs) + 0.05 U/mL

APE1 (M9282S, New England BioLabs). After 3 min diffusion, the 96-well plate was transferred to 37°C for a 1-h incubation.

Alkaline DNA unwinding and neutralization

The plate was transferred back to the cooling device and cooled down to 4°C for 3 min. First, the replicates of the T value received 150 µL neutralization solution (14 mM β-mercaptoethanol; CAS: 60-24-2, Merck; 1 M D(+)-glucose monohydrate; CAS: 14431-43-7, Sigma-Aldrich). 50 µL alkaline solution (160 mM guanidine thiocyanate, Sigma-Aldrich; 12 mM EDTA; 0.08% N-laurylsarcosyl; 300 mM NaOH) was added to all wells on top of the solution at a dispensing rate of 10 µL/s. After 5 min diffusion, the temperature was set to 30°C for 1 h to allow DNA unwinding. Then, the temperature was set to 20°C to reach RT within 30 min. Thereafter, wells for P, F and hA values were neutralized with 150 µL neutralization solution.

SYBRTM Green staining and fluorescence read-out

For the determination of double-stranded DNA, 50 µL SybrGreenTM I (S7563, Invitrogen) solution (1:2,700 SYBRTM Green in H₂O) was added to each well and the content was mixed 6 times. Fluorescence intensity (excitation: 485 nm, emission: 535 nm) was measured by using a TECAN infiniteTM 200. The relative fluorescence intensity for each value was calculated by normalizing the mean of all technical replicates to the mean of the corresponding T values. With increasing numbers of DNA strand breaks, the amount of double stranded DNA and thus the resulting fluorescence intensity decreases.

2.4 Annexin V APC/PI flow cytometry

For the determination of apoptosis induction, THP-1 cells were stained with Annexin V APC (A35110, Invitrogen) and propidium iodide (PI; P3566, Invitrogen) and analyzed via flow cytometry. Briefly, 1.8 mL cell suspension (5 × 10⁵ cells/mL) was treated with genotoxins as described in Section 2.2. After the washing steps, cells were resuspended in RPMI medium and incubated for 16 h at 37°C in a humidified atmosphere with 5% CO₂. Cells were harvested (300 g, 4°C, 10 min) and resuspended in 500 µL Annexin binding buffer. For each sample, 2 FACS vials were filled with 100 µL cell suspension for one stained and one unstained control sample. One drop of Annexin V APC Ready FlowTM Reagent and 5 µL PI were added to the stained sample. After 15 min incubation at RT, 400 µL Annexin binding buffer was added. Samples were analyzed via BD FACSLyricTM Research System. Gates were set as illustrated in Figure S1¹.

2.5 AlamarBlueTM cell viability assay

For the analysis of cell viability, 100 µL THP-1 cell suspension (5 × 10⁵ cells/mL) was seeded in triplicates for each sample in a 96-well plate and 2 µM camptothecin (CPT; CAS: 2114454, Sigma-Aldrich), 200 µM KBrO₃, 20 µM H₂O₂ or 50 µM MMS were added to the cell suspension. Cells were incubated for 16 h at 37°C in a humidified atmosphere with 5% CO₂. 10 µL AlamarBlueTM cell viability reagent (DAL1025, Invitrogen) was added to each well. After 4 h of incubation, fluorescence intensity (excitation: 535 nm, emission: 590 nm) was measured in a TECAN

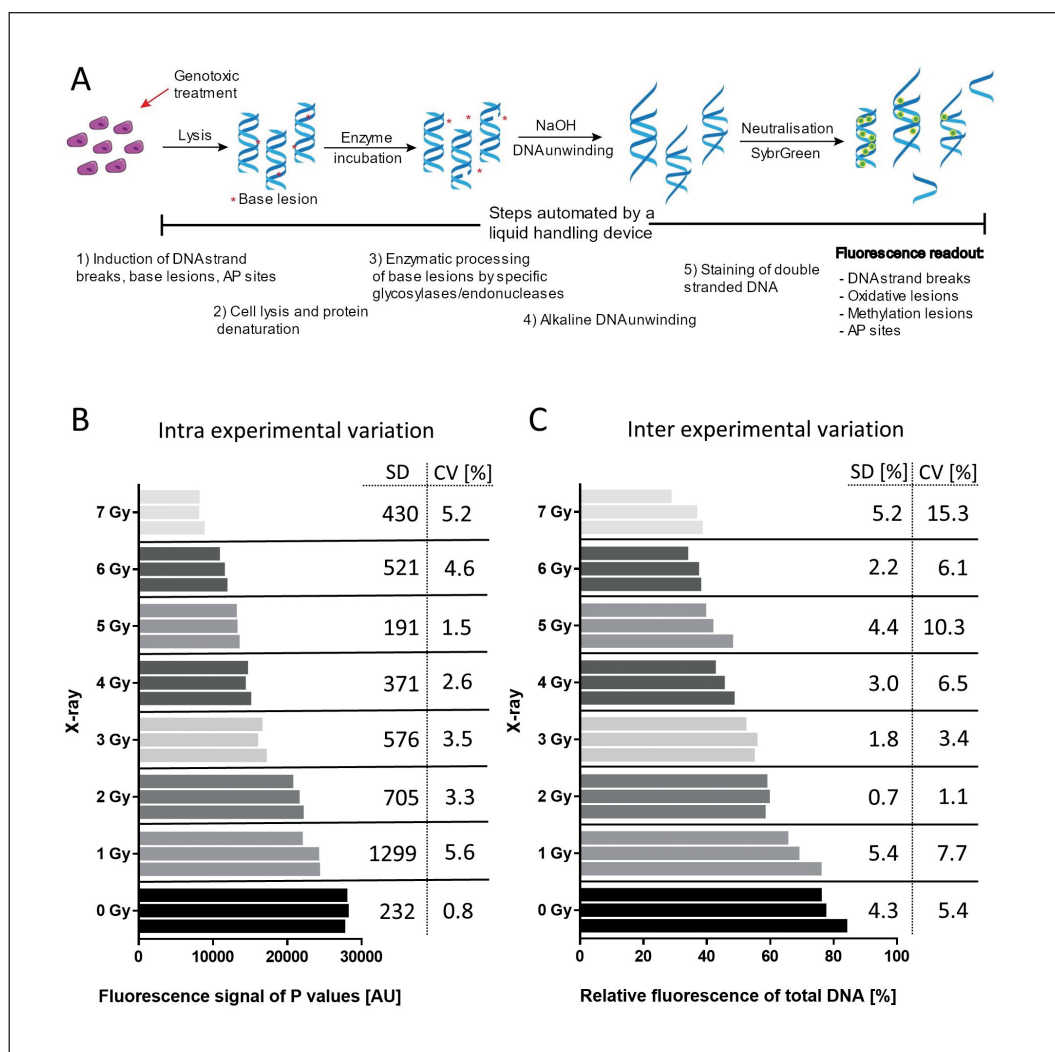


Fig. 1: The enzyme-modified FADU assay (A) Essential steps in the emFADU assay.

(B,C) THP-1 cells were irradiated with increasing X-ray doses, and DNA strand breaks were measured via emFADU assay.

(B) Intra-experimental variation of 3 technical replicates. Fluorescence signal of 3 P values with the corresponding standard deviation and the coefficient of variation are shown. (C) Inter-experimental variation of 3 independent experiments with their corresponding standard deviation and coefficient of variation are shown.

infinite™ 200. Fluorescent intensity of each sample was normalized to the untreated control.

2.6 Detection of DNA damage in plasmid DNA

Fpg-sensitive sites and 8-oxodG were determined upon treatment with 3-morpholinopyridone (Sin 1, CAS 16142-27-1, Calbiochem) via emFADU assay and LC-MS/MS in purified plasmid DNA, respectively (Müller et al., 2013). For the detection of methylation lesions upon treatment with MMS via FADU in purified plasmid DNA, 8 U hAAG and 0.5 U APE1 were used instead of Fpg. For determination of 7-methylguanine (7mG), its ion transition of 166 > 149 was detected, as described by Hu et al. (2012). In parallel to the detection of 8-oxodG and 7mG, the number of nucleosides was determined and used for normalization. As external standards, 8-oxodG (CAS: 88847-89-6, Sigma-Aldrich), 7mG (CAS: 578-76-7, Sigma-Aldrich), dA (CAS: 16373-93-6, Sigma-Aldrich), dG (CAS: 312693-72-4, Sigma-Aldrich), dT (CAS: 50-89-5, Sigma-Aldrich), and dC (CAS: 951-77-9, Sigma-Aldrich) were used.

2.7 Statistical analysis

Statistical analysis was conducted using GraphPad Prism 6 software. For the analysis of experiments with one parameter, a one-way ANOVA followed by Dunnett's multiple comparisons test was performed (Fig. 2A,E; Fig. S1A¹). For the analysis of experiments with two parameters, a two-way ANOVA followed by Sidak's multiple comparisons test was performed (Fig. 2B-D,F; 3A-D). P values are reported by asterisks (*p < 0.05, **p < 0.01, ***p < 0.001).

3 Results

3.1 Detection of DNA strand breaks, oxidative lesions, and methylation lesions in THP-1 cells

As previously described, the FADU method can be used to detect oxidative DNA lesions in purified plasmid DNA (Müller et al., 2013). We reproduced these findings by measuring the formation of Fpg sensitive sites upon Sin-1 treatment of plasmid DNA (Fig.

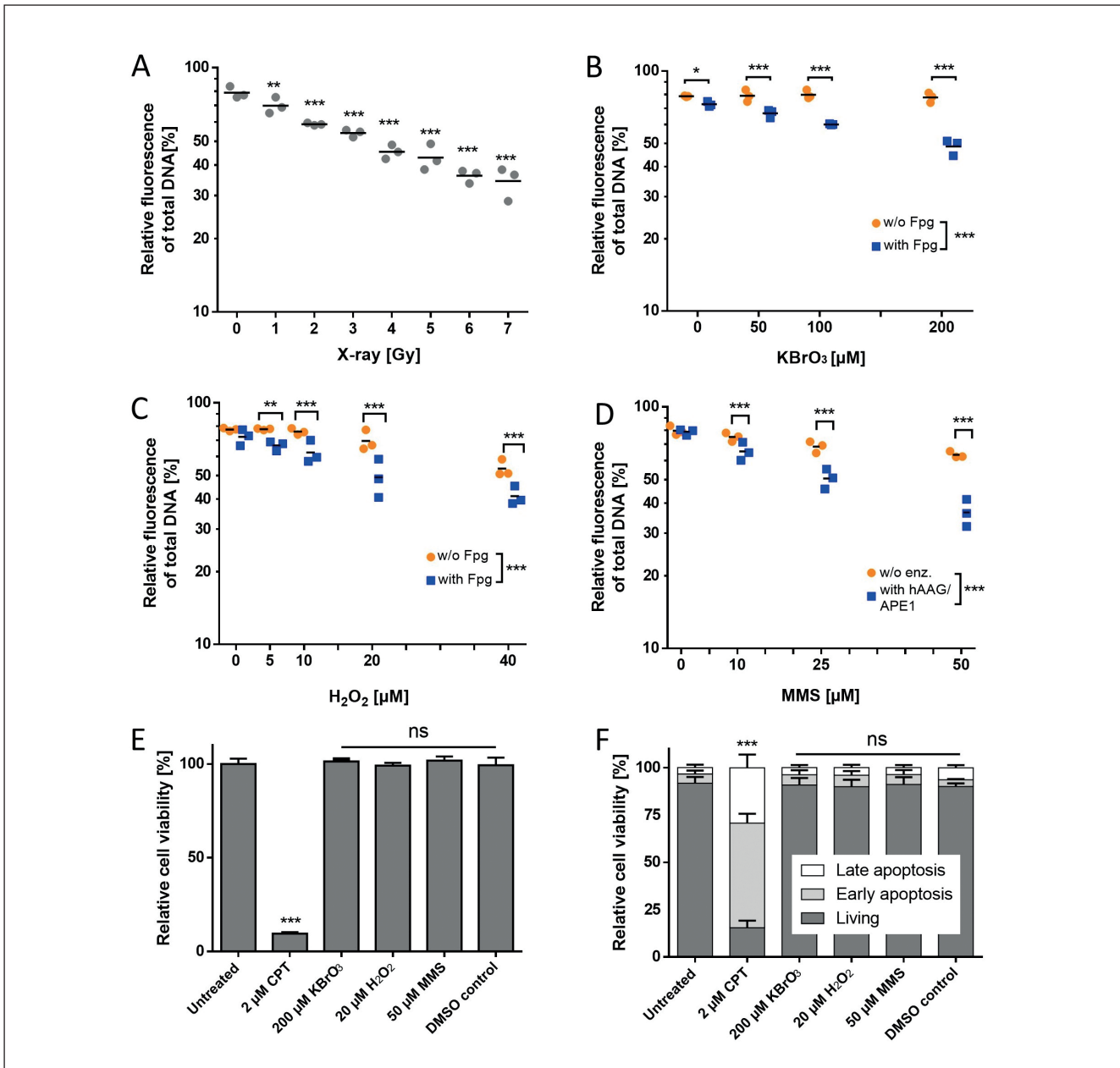


Fig. 2: Detection of DNA strand breaks, oxidative lesions, and methylation lesions in THP-1 cells

(A) THP-1 cells were irradiated on ice with X-ray doses as indicated. DNA SBs were measured via the emFADU assay. Three independent experiments with 3 technical replicates per data point were performed. Statistical analysis: One-way ANOVA with Dunnett's multiple comparisons test (** $p < 0.01$, *** $p < 0.001$). (B,C) THP-1 cells were treated for 2 h with KBrO₃ or for 30 min with H₂O₂ as indicated. DNA SBs and Fpg-sensitive sites were measured via the emFADU assay. (D) THP-1 cells were treated for 2 h with the indicated concentration of MMS. DNA SBs and hAAG/APE1-sensitive sites were measured via the emFADU assay. (B-D) For each data set, 3 independent experiments with 3 technical replicates per data point were performed. Statistical analysis: Two-way ANOVA with Sidak's multiple comparisons test (** $p < 0.01$, *** $p < 0.001$). (E,F) THP-1 cells were treated with the indicated genotoxins (CPT: 16 h, KBrO₃/MMS: 2 h, H₂O₂: 30 min). (E) 16 h after treatment, cells were stained with AlamarBlue™. Fluorescence intensity was measured after 4 h. Three independent experiments with 3 technical replicates per data point were performed. Statistical analysis: One-way ANOVA with Dunnett's multiple comparisons test compared to untreated control (*** $p < 0.001$, ns: not significant). (F) Cells were stained with Annexin V APC and PI 16 h after treatment. Fluorescence intensities of individual cells were analyzed via flow cytometry. Three independent experiments were performed. Statistical analysis: Two-way ANOVA with Sidak's multiple comparisons test compared to untreated control (*** $p < 0.001$; ns, not significant).



S1A; S2A,B¹). We could show that this approach is also suitable for the assessment of chemically induced methylation lesions as well as AP sites (Fig. S1A; S2C,D¹). Figure S1A¹ shows that the number of hAAG- and APE1 specific lesions increases upon treatment with 10 mM MMS, reflecting the induction of methylation lesions and AP sites, respectively. As indicated, the incubation with hAAG only did not result in the formation of DNA SBs. For verification of the enzyme specificity, we determined the number of 8-oxodG and 7mG via LC-MS/MS in parallel to the FADU experiments in plasmid DNA. Treatment of plasmid DNA with increasing concentrations of Sin-1 led to an increase of Fpg-sensitive sites and an increase of 8-oxodG (Fig. S2A,B¹). Treatment of plasmid DNA with increasing concentrations of MMS led to an increase of hAAG/APE1-sensitive sites and 7mG (Fig. S2C,D¹). Having shown the specificity of Fpg and hAAG for oxidation- and methylation-induced DNA damage, respectively, we then transferred this to a cellular setting.

As described in Section 2.3 and illustrated in Figure 1A, we present here a novel automated screening method for the detection of DNA strand breaks and glycosylase-specific base modifications in human cells. We used the cell line THP-1, which is derived from an acute monocytic leukemia, to establish the method. As the principle of the FADU method relies on the detection of DNA strand interruption-induced DNA unwinding, we first used X-rays to induce DNA SBs. Cells were irradiated on ice and DNA SBs were measured via the emFADU assay protocol. To show the intra-experimental variation of the assay, we analyzed technical triplicates of P values. As shown in Figure 1B, the coefficient of variation ranged from 0.8 to 5.6%. To analyze the inter-experimental variation, we conducted three independent experiments. Results in Figure 1C show that the standard deviation of three experiments is below 5.4% for all X-ray doses. However, the coefficient of variation of the highest dose (7 Gy) was 15.3%, indicating that the induction of heavy DNA damage leads to an increased sample variation between experiments. We also analyzed the inter- and intra-experimental variation of F and hA values and found a standard deviation comparable to the P value (data not shown).

Next, we analyzed the genotoxicity of X-rays, KBrO₃, H₂O₂ and MMS in THP-1 cells via the emFADU assay. Increasing X-ray doses led to a decrease of the fluorescence signal, indicating an induction of SBs (Fig. 2A). THP-1 cells were treated for 2 h with various concentrations of KBrO₃ for induction of oxidative lesions. The treatment did not lead to the induction of SBs but to a significant induction of Fpg-sensitive sites already at a concentration of 50 μM (Fig. 2B). We could also show that low concentrations of H₂O₂ lead to an increase of Fpg-sensitive sites, whereas higher concentrations lead to the formation of SBs without a further increase of Fpg-sensitive sites, which indicates that in this case the upper limit of detection has almost been reached due to saturation (Fig. 2C), i.e., if the DNA is heavily damaged, the unwinding is very fast, and it is not possible to record any additional damage. Further, THP-1 cells were treated with MMS to induce DNA methylation lesions, which could be measured by using a combination of hAAG and APE1. Results in Figure 2D show a slight induction of SBs and a significant increase of hAAG/APE1-sensitive sites upon MMS treatment.

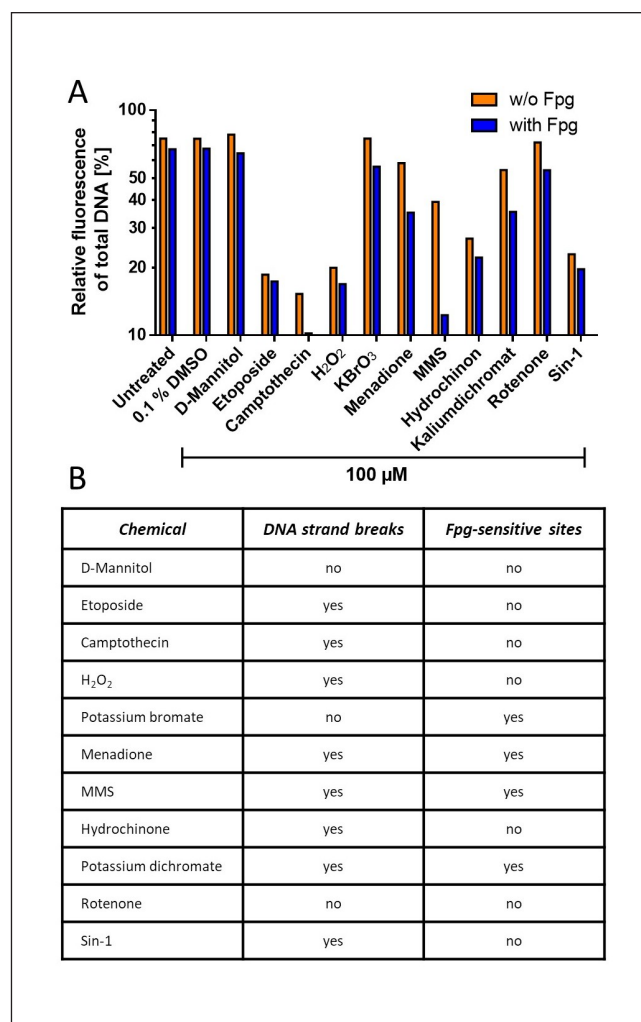


Fig. 3: Genotoxicity screening

(A) 5 μL of an anonymized genotoxin solution (10 mM) was added to 500 μL THP-1 cell suspension. After 1 h incubation at 37°C, DNA SBs and Fpg-sensitive sites were measured via the emFADU assay. For T, P and F value, three technical replicates were used (n = 1). (B) Positive induction of DNA SBs was determined via Formula 1; positive induction of Fpg-sensitive sites was determined via Formula 2 (Section 3.2).

To determine whether the applied genotoxin doses are cytotoxic, we measured cell viability via AlamarBlue™ assay and Annexin V APC/PI flow cytometry 16 h after treatment. The topoisomerase I inhibitor CPT was used as a positive control. 200 μM KBrO₃, 20 μM H₂O₂ and 50 μM MMS, respectively, did not affect viability of THP-1 cells, whereas incubation with CPT led to a massive reduction of viable cells (Fig. 2E). The applied genotoxic doses showed no induction of apoptosis compared to the significant apoptosis induction upon CPT treatment (Fig. 2F).

Taken together, the data presented in Figure 2 show that the emFADU assay is suitable for the detection of SBs, oxidative and methylation lesions induced by subcytotoxic concentrations of KBrO₃ (200 μM), H₂O₂ (20 μM) and MMS (50 μM).

3.2 Establishment of a prediction rule for genotoxicity

To establish a preliminary prediction rule for positive genotoxins, we calculated the mean standard deviation of all data points of all experiments shown in Figure 2 (each was analyzed in three independent experiments). The mean standard deviation of the X-ray experiments was 3.49% (see Fig. 1C, 2A). The mean standard deviation of all data points (Fig. 2A-D) was 3.52%, indicating that the addition of enzymes did not increase assay variation substantially. This mean standard deviation σ_{assay} was used to define a result as positive if its relative fluorescence differs at least by three times σ_2 from the untreated control. Therefore, a substance X is defined as a DNA strand break or Fpg-sensitive site inducing agent if $y(X) \geq 0$.

Formula 1 for DNA strand breaks:

$$y_{\text{SB}}(X) = \left(\frac{P}{T}\right)_{\text{ctr}} - \left(\frac{P}{T}\right)_X - 3\sigma_{\text{assay}}$$

Formula 2 for Fpg-sensitive sites:

$$y_{\text{Fpg}}(X) = \left(\frac{P}{T}\right)_X - \left(\frac{F}{T}\right)_X - \left(\frac{P}{T}\right)_{\text{ctr}} + \left(\frac{F}{T}\right)_{\text{ctr}} - 3\sigma_{\text{assay}}$$

In a first proof-of-principle experiment, 10 known genotoxins were used in a blinded experiment. Additionally, D-mannitol, a known non-genotoxin was included. All substances were employed at a concentration of 100 μM in THP-1 cells for 1 hour at 37°C in a humidified atmosphere. As shown in Figure 3, 9 of the 10 genotoxins were identified correctly and 4 substances were identified as inducers of Fpg-sensitive sites.

3.3 Detection of DNA strand breaks, oxidative lesions, and methylation lesions in PBMCs and HeLa cells

Having shown the functionality of the emFADU assay as a genotoxicity screening method in immortalized suspension cells, we further explored its applicability to PBMCs. PBMCs were isolated from fresh blood of healthy donors. First, we confirmed that the new protocol of the emFADU assay, like the original FADU protocol, is suitable for the detection of X-ray induced SBs (Fig. 4A). Moreover, treatment with 1 mM KBrO_3 led to a significant induction of Fpg-sensitive sites (Fig. 4B), whereas treatment with 25 μM MMS led to the induction of both Fpg- and hAAG/APE1-specific sites.

In addition, we extended our approach to the assessment of genotoxicity in adherent cells. To do this, HeLa cells were seeded the day before genotoxic treatment and all assay volumes were reduced by 50% as the commercial 96 well plates only hold 300 μL per well. H_2O_2 treatment resulted in a dose-dependent decrease in the fluorescence signal (Fig. 4C). Furthermore, as shown in Figure 4D, treatment with 1 mM KBrO_3 and 50 μM MMS also led to glycosylase-specific DNA lesions in HeLa cells.

4 Discussion

The automated FADU assay was developed as a very sensitive and fast method for the analysis of DNA SBs in human cells. The use of a liquid handling device reduces operator bias and increases sample throughput. Therefore, the automated FADU as-

say is an attractive alternative to the commonly used comet assay (Moreno-Villanueva, 2009). Whereas glycosylase-modified versions of the comet assay have already been described, the FADU assay was so far limited to DNA SBs (Collins, 2014). With the enzyme-modified version of the FADU assay (emFADU), we developed a screening method for the parallel assessment of SBs, oxidative lesions, and methylation lesions in human cells. The detection of oxidants and methylating agents could improve the sensitivity of the FADU. Furthermore, this version provides mechanistic information.

First, a cell-free approach was used to describe the functionality and specificity of Fpg, hAAG and APE1. Figure S1A¹ clearly shows that Fpg cleaves Sin-1-induced DNA lesions, whereas hAAG alone is not able to cleave MMS-induced DNA lesions. Therefore, the combination with the endonuclease APE1 is necessary to convert the resulting AP sites into detectable DNA SBs. Moreover, Figure S1A¹ shows that MMS induces APE1-sensitive sites, indicating the spontaneous depurination of DNA methylation lesions. Attempts were made to measure the spontaneous depurination in cells, too, but no formation of AP sites was detectable in MMS-treated THP-1 cells in first experiments. Therefore, we assume that the depurination of methylation lesions is slower in cellular DNA compared to purified plasmid DNA. We then verified the specificity of Fpg, hAAG and APE1 via the detection of oxidation- and methylation-induced DNA damage in plasmid DNA using the FADU assay together with LC-MS/MS analysis (Fig. S2¹). We could show that the peroxyntrite generator Sin-1 leads to an increase of Fpg-sensitive sites and 8-oxodG in plasmid DNA. Additionally, MMS treatment led to an increase in hAAG/APE1-sensitive sites and, in one exemplary experiment, we could show the induction of 7mG in parallel. We were also able to detect the induction of 7mG in MMS-treated THP1 cells via LC-MS/MS (data not shown), but, most probably due to spurious oxidation during DNA purification, it was not possible to detect any induction of 8-oxodG in cellular DNA via mass spectrometry.

For cell-based experiments, we used 96 well plates and triplicates for each of the 4 different values (T = total DNA, P = SBs, hA = SBs + hAAG/APE1-sensitive sites, F = SBs + Fpg-sensitive sites). Although not all parameters were analyzed in every experiment, it was shown in Figure 4 that DNA strand breaks, Fpg-sensitive sites and hAAG/APE1-sensitive sites can be measured in parallel. Thus, it is possible to analyze all parameters of 8 different samples within one plate in a 3-hour run. Depending on the liquid handling system, it is possible to run multiple plates in parallel for high-throughput screening. Moreover, having shown that the intra-experimental variation is quite low ($CV < 6\%$), it should be possible to omit technical replicates (Fig. 1B) so that more samples can be measured per plate.

For the detection of Fpg-sensitive sites, we treated cells with KBrO_3 . It was previously shown that KBrO_3 selectively leads to 8-oxodG in cellular DNA in a glutathione (GSH) dependent manner (Murata et al., 2001). We hypothesize that a high level of GSH in THP-1 cells allows for the high sensitivity to KBrO_3 (Fig. 2B) compared to PBMCs and HeLa cells, where up to 20 times higher doses were necessary for the induction of Fpg-sensitive sites (Fig. 4B,D). This sensitivity was never described before and could become important in analyzing bromate contami-

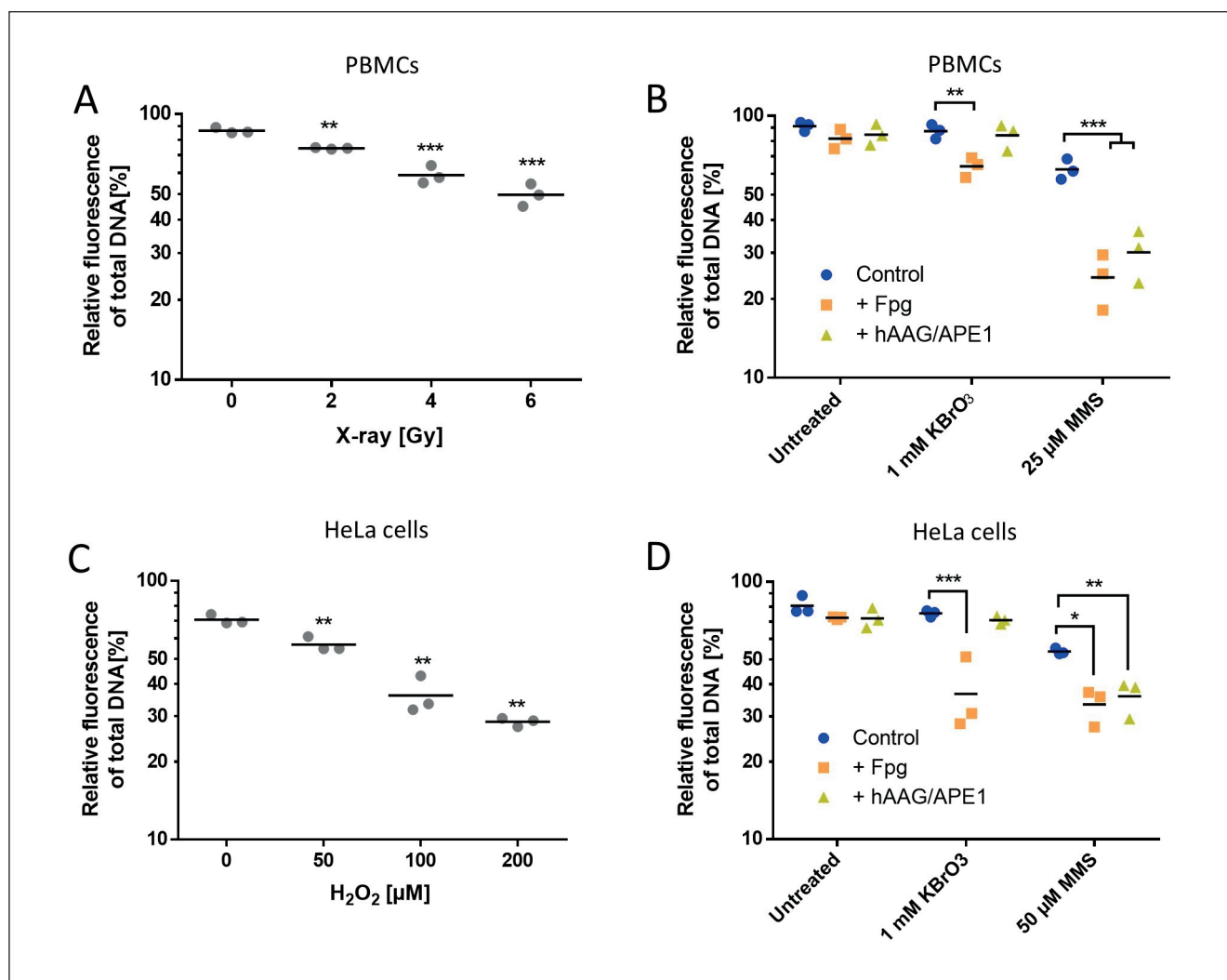


Fig. 4: Detection of DNA strand breaks, oxidative lesions, and methylation lesions in PBMCs and HeLa cells

(A,B) PBMCs were isolated from peripheral blood of healthy human donors. (A) PBMCs were irradiated on ice with X-ray doses as indicated. DNA SBs were measured via the emFADU assay. (B) PBMCs were treated with KBrO₃ or MMS for 2 h. DNA SBs, Fpg-sensitive sites, and hAAG/APE1-sensitive sites were measured via the emFADU assay. (C) HeLa cells were treated with H₂O₂ for 30 min. DNA SBs were measured via the emFADU assay. (D) HeLa cells were treated with KBrO₃ or MMS for 2 h. DNA SBs, Fpg-sensitive sites and hAAG/APE1-sensitive sites were measured via the emFADU assay. (A,C) Three independent experiments with 3 technical replicates per data point were performed. Statistical analysis: One-way ANOVA with Dunnett's multiple comparisons test compared to untreated control (**p < 0.01, ***p < 0.001). (B,D) Three independent experiments with 3 technical replicates per data point were performed. Statistical analysis: Two-way ANOVA with Sidak's multiple comparisons test (*p < 0.05, **p < 0.01, ***p < 0.001).

nated samples, which is of great interest, for example in the now more commonly used potentially genotoxic ozonation of water (Aljundi, 2011; Wu et al., 2019). We showed that KBrO₃ treatment selectively leads to Fpg-sensitive sites without direct induction of SBs. Therefore, KBrO₃ served as a positive control for subsequent experiments.

We used MMS, which was shown to primarily induce 7-methylguanine and 3-methyladenine, to establish the detection of methylation lesions. Within DNA, these bases are efficiently removed by hAAG (Beranek, 1990; Wyatt and Pittman, 2006). Since the monofunctional hAAG does not bind to AP sites, we combined

it with APE1, which recognizes and cleaves AP sites, leaving a DNA SB (Abner et al., 2001; Levin and Demple, 1990). While not formally investigated in this study, we assume that our method should be suitable for the assessment of chemically-induced AP sites by the use of APE1 only, but, as described previously, we were not able to detect the formation of AP sites in MMS treated THP-1 cells. In line with the literature (Speit et al., 2004), Figure 4B,D shows that Fpg recognizes both oxidative and methylation lesions. Since human 8-oxoguanine-DNA glycosylase recognizes only oxidative DNA lesions like 8-oxodG and formamidopyrimidine, Fpg could be replaced with this more specific glycosyl-

lase (Sidorenko et al., 2009). However, by measuring Fpg- and hAAG/APE1-sensitive sites in parallel, we can easily distinguish between oxidative and methylation lesions.

At this point, it must be mentioned that any residual chemical that interferes with the fluorescence signal could alter the readout. For example, doxorubicin shows autofluorescence and would lead to an artificial increase in the fluorescence signal. Nevertheless, such interferences are easily detectable by the comparison of T values of the control sample and chemically treated samples. Additionally, inhibitors of the enzymes used here could lead to false-negative results. To exclude such interference, the enzyme activity could be measured via the use of pre-treated plasmid DNA by the FADU assay.

To prove that the emFADU assay can be used as a screening method for genotoxic substances, we conducted one exemplary experiment with 10 genotoxins and one non-genotoxin. A difference of the value of 10.57% compared to the control sample, i.e., three times the mean assay standard deviation, was the criterion for definition as a positive hit. This very conservative method identified 9 genotoxins correctly. Although rotenone, a commonly known genotoxin and oxidant, was not detected as a genotoxic substance, potassium bromate was just identified as an oxidizing agent. Furthermore, low concentrations of hydrogen peroxide were shown to induce Fpg-sensitive sites (Fig. 2C), which are not detectable after high-dose exposure (Fig. 3). This shows that exposure time and compound concentration are indeed important issues in genotoxicity testing.

Having shown the proof-of-principle of the emFADU assay, further experiments are planned to assess the sensitivity and specificity of the method. We plan to analyze a large sample set of known genotoxins and non-genotoxins, as well as unclassified substances. In parallel, cytotoxicity studies should be conducted. As shown in Figure 2, due to different reaction mechanisms, the optimal treatment time for different substances varies. Therefore, the best procedure needs to be investigated with a larger sample set. As a next step, the emFADU assay shall be transferred to other laboratories to investigate reproducibility. Here, we presume a high reproducibility due to the use of a liquid handling device.

Due to the high number of chemicals released via drugs, cosmetics, pesticides or food additives, the need for risk assessment testing is undeniable. Different tests for mutagenicity like the Ames test, micronucleus test or chromosomal aberration test have different levels of sensitivity and specificity, leading to false positive and false negative results (Kumar et al., 2018). Here, we provide the basis for a new tool to assess the genotoxic potential of chemicals in a high-throughput format. This could gain importance especially in the early development of compounds and could help to reduce the huge number of animals used in routine toxicity testing. Moreover, besides mutagenicity, the emFADU assay provides useful additional information with regards to specific kinds of DNA damage.

Taken together, based on the automation via a liquid handling system, we have developed a fast and convenient method to assess oxidative and methylation-induced nucleobase lesions in DNA. As described, one run is done in a 96-well plate and takes three hours with costs of less than 10€ per 96-well plate for all consumables. In contrast, one CometChip® is more than 20 times

more expensive. Additionally, our emFADU assay provides higher throughput with minimal workload. We have also demonstrated its applicability to human PBMCs, thus enabling the use of the emFADU assay in population studies. However, the usefulness of our novel technique to reflect the real-life setting in patient-derived PBMCs needs to be confirmed. Besides cells in suspension, we showed that adherent cells, such as HeLa, can also be used to study genotoxicity. Moreover, with the ability to distinguish between several kinds of DNA damage, the new assay should facilitate the study of DNA repair mechanisms in living cells to address the function of specific proteins involved in repair pathways.

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Conflict of interest

The authors declare that they have no conflict of interest.

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