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Abstract

Even if the comet assay has been widely used for decades, there is still a need for controlled studies and good mathematical models to assess the variability of the different versions of this assay and in particular to assess potential intra-experimental variability of the high-throughput comet assay.

To address this point, we further validate a high-throughput comet assay that uses hydrophilic polyester film (Gelbond®). Experiments were performed using human peripheral blood mononuclear cells (PBMC) either untreated or treated with different concentration of MMS (Methyl Methanesulfonate). A positive control for the Fpg (Formamidopyrimidine DNA glycosylase)-modified comet assay (Ro 19-8022 with light) was also included. To quantify the sources of variability of the assay, including intra-deposit variability, instead of

summarizing DNA damage on 50 cells from a deposit by the mean or median of their percentage DNA tail, we analyzed all logit-transformed data with a linear mixed model.

The main source of variation in our experimental data is between cells within the same deposit, suggesting genuine variability in the response of the cells rather than variation caused by technical treatment of cell samples. The second source of variation is the inter-experimental variation (day-to-day experiment); the coefficient of this variation for the control was 13.6 %. The variation between deposits in the same experiment is negligible. Moreover, there is no systematic bias due to the position of samples on the Gelbond® film nor the position of the films in the electrophoresis tank.

This high-throughput comet assay is thus reliable for various applications.

Introduction

The comet assay is based on electrophoresis in agarose of single nucleoids giving a comet-like image with the intensity of the tail depending on the frequency of breaks within the nuclear genome. Such breaks cause changes in DNA supercoiling and altered migration of DNA loops (Collins et al. 1997). The standard alkaline comet assay enables the detection of single strand breaks, double strand breaks plus alkali-labile sites. Various modifications of the standard comet assay have been developed, introducing the use of lesion-specific repair enzymes in order to detect a broader range of damage by digestion of nucleoids with enzymes that convert specific lesions into DNA breaks. For example, the use of formamidopyrimidine DNA glycosylase (Fpg) enzyme reveals typically oxidized bases (Dusinska and Collins 1996).

The assay is being used in many different fields of applications, such as genotoxicity testing, ecotoxicology, DNA repair, and human biomonitoring [for review (Azqueta and Collins 2013)]. Different intra- and inter-laboratory studies have been performed using the standard alkaline comet assay as well as the Fpg-modified comet assay [see (Forchhammer et al. 2010; Johansson et al. 2010; Forchhammer et al. 2012; Ersson et al. 2013) as examples]. The results of these intra- and inter- laboratory studies pointed out the weak sides of the assay and the impact of variation of critical steps of the comet assay protocol such as % of melting agarose and electrophoresis parameters (field and duration). For example, the ECVAG trials ((Forchhammer et al. 2010; Johansson et al. 2010)) highlighted the necessity of standardized protocols in order to reduce the variability between laboratories and also proposed the use of reference standards. Recently, the comet assay was accepted as an international regulatory test for *in vivo* testing of chemical genotoxicity with the OECD Guideline TG-489. For the *in vitro* comet assay, further international efforts and inter-laboratory studies are required in

order to evaluate the robustness of the method and to propose a standardized protocol, which may eventually lead to an international guideline.

Some laboratories use standard glass slides as substrate for one or two agarose samples on each slide to perform the comet assay; in this case, the assay has a low throughput and sample manipulation tend to be time consuming. To reach medium and high-throughput, modified protocols were proposed recently [For review, see (Brunborg et al. 2014)]. Moreover, around 15 % of people using the comet assay encounter problems with gels coming off the glass support (Koppen et al. 2017). In order to improve the processing of gels and reduce gel detachment, the use of large hydrophilic polyester films (Gelbond®) was proposed for a high-throughput comet assay supporting 96 minigels (Gutzkow et al. 2013). A collaborative study was set up to develop a sophisticated statistical analysis to model the fixed and random effects of the assay, with the ultimate purpose to improve the validation of a high-throughput comet assay with its parallel processing of large numbers of samples.

We investigated in detail the sources of potential variability of the high-throughput comet assay, in both the alkaline and the Fpg-modified versions. Different experiments were set up using human peripheral blood mononuclear cells (PBMC), treated with increasing concentrations of methyl methanesulfonate (MMS) to induce different levels of DNA damage. Treatment with the photosensitizer compound Ro 19-8022 (for (R)-1-((10-choloro-4-oxo-3-phenyl-4H-benzo(a)quinolizin-1-yl)carbonyl)-2-pyrrolidine-methanol) with visible light was also used, inducing lesions which are recognized *via* different mechanisms including cleavage of modified bases with an exogenous enzyme. All the data were analyzed statistically using a linear mixed model (LMM) to identify the different sources of variability.

Materials and methods

Isolation of mononuclear cells

Peripheral Blood Mononuclear cells collected from one volunteer [Établissement Français du Sang, (EFS), written consent for the use of blood samples for the research protocol obtained according to the regulation for blood transfusion of the French blood organization EFS, Toulouse (France)]) were Ficoll-separated from whole blood and aliquoted ($1*10^6$ cells/aliquot) prior to cryopreservation in liquid nitrogen as described previously (Lebailly et al. 1998). These conditions of preparation and conservation of cells are suitable for long-term conservation and do not in themselves affect the comet assay results (Dusinska and Collins 2008).

Treatment of PBMC with MMS

For each experiment, aliquots of PBMC were defrosted, washed with phosphate-buffered saline (PBS) with 10% serum, centrifuged (200g, 10 min) and resuspended in PBS at a concentration of 120 000 cells/mL. Cells were either untreated (negative controls) or treated with MMS (from 12.5 µM to 500 µM, prepared in PBS) for 1 h, or for 1 h 30 min (only for 500 µM MMS) at 37°C to induce different level of DNA damage detectable with the alkaline comet assay. Afterwards, cells were kept on ice to prevent DNA repair.

Treatment of PBMC with light-activated Ro 19-8022

To induce DNA base oxidations detectable with the Fpg-enzyme (gift from Serge Boiteux, CNRS, France), defrosted PBMC (120 000 cells/mL) were placed on ice and treated with the compound Ro 19-8022 (Gift from Hoffman Laroche Ltd) (at 1 µM in PBS) during 2 min 30 sec under visible light (1000 W-halogen). Ro 19-8022 plus visible light exposure is an appropriate positive control for the Fpg-modified comet assay (Collins 2014). Cells were then pelleted for 10 min at 200xg at 4°C.

Comet assay

Cells were gently resuspended to a final concentration of 0.7 %, in low-melting point agarose (Sigma "Low Gelling Temperature") prepared with PBS. Pre-cut sheets of polyester film (Gelbond® film) were used as substrate as previously described (Gutzkow et al. 2013), with minor modifications: 40 gels, instead of 96 minigels, were deposited on sheets each of 85 x 125 mm (40 deposits, Figure 1). Films were placed on a standard metal plate (placed on ice to ensure solidification of agarose deposits in few seconds) and hooked onto plastic frames with stainless-steel metal feet (custom-made) as seen in supplementary Figure S1. A paper with the outlined positions for the deposits were placed underneath the film to ensure appropriate positioning of each gel. Cell samples were mixed with a mono-channel repeat dispenser pipette allowing four successive gel deposits (15 µL each). Paper was removed and each film was immersed in cold lysis solution once all samples had been deposited.

Lysis, unwinding and electrophoresis of the samples.

After 18 h in lysis solution (2.5 M NaCl, 0.1 M Na_2EDTA, 10 mM Tris, 1 % Triton X-100, 10 % DMSO) at 4°C, each film was transferred either directly into freshly prepared alkaline unwinding and electrophoresis solution (for the alkaline comet assay), or processed by an Fpg-enzyme treatment step or enzyme buffer treatment step for 1 h at 37°C. The enzyme buffer for Fpg-modified comet assay was 40 mM HEPES, 0.5 mM Na_2EDTA, 0.1 M KCl and 0.2 mg/mL BSA, pH 8. The Fpg enzyme enables the detection of oxidative DNA damages as described previously (European Standards Committee on Oxidative DNA Damage 2003). The unwinding took place in cold electrophoresis solution (0.3 M NaOH, 1 mM Na_2EDTA, pH > 13) for 40 min. Electrophoresis were carried out in cold (4°C - 8°C) fresh electrophoresis solution, in a tank with a power supply giving 28 V (resulting in 0.8 V/cm on the platform where the films were kept), for 24 min. The circulation of the electrophoresis solution in the tank was performed using a peristaltic pump (100 mL/min). As illustrated in Figure 1, three films can be processed at the same time using a large electrophoresis tank [Econo-Submarine

(20 cm*30 cm, CBS, USA)]. After electrophoresis, films were immersed for 2 x 5 min in PBS for neutralization, followed by fixation in 100% ethanol for 1.5 h and drying. Films were kept in a dry and dark place until further analysis.

Prior to staining, films were cut in two pieces (named "Plate" as shown in Figure 1), each half-film was glued onto a large glass slide and rehydrated in TE buffer (10 mM Tris-HCl, 1 mM Na_2EDTA, pH 8.0), containing the DNA stain SYBR® Gold (Life Technologies) at 10 000 X dilution for 20 min, followed by rinsing in distilled water. Each experiment contains 12 to 191 technical replicates.

Scoring of comets

Films were analyzed with an epifluorescence microscope equipped with an automated platform (Nikon NiE, Nikon Instruments Europe B.V.) and coupled to a camera (DS-Q1Mc) and the software (Nikon NiS Element Advanced Research, Nikon Instruments Europe B.V.) to automatically capture images. In these images, for each cell, the level of DNA damage was evaluated using a semi-automated scoring system, by measurement of the intensity of all tail pixels divided by the total intensity of all pixels in head and tail of comet, by means of the software "Lucia comet assay" (Laboratory Imaging, Prague Czech Republic). This measure is hereafter designated as "percentage tail DNA".

Statistical analyses

For each deposit, the median of 50 percentage tail DNA measurements was computed. For each experiment, the mean of the median percentage tail DNA across all deposits was computed. Then, for each level of treatment, the coefficient of variation between these means was computed as the ratio of standard deviation to the mean, to assess the inter-experimental variation.

Study of components of experimental variability using a linear mixed model

We used a linear mixed model to further analyze the experimental variability. Beforehand, in order to improve the symmetry of the data, they were logit-transformed by $Y = \text{logit}(p) = \log\left(\frac{p}{1-p}\right)$ where $p \in [0,1]$ is the percentage of DNA in the tail of the comet.

To estimate the components of the experimental variability we used the linear mixed model

$$Y_{ijkl} = Z\,\beta + \mu_i + \nu_{ij} + \omega_{ijk} + \gamma_j + \delta_k + \varepsilon_{ijkl}$$

where the index i is for the experiment, j is for the plate, k is for the deposit, and l is for the cell; thus Y_{ijkl} is the logit-transformed percentage tail DNA of the l-th cell of the k-th deposit on plate number j of the i-th experiment. The matrix Z is a matrix of dummy variables for treatments levels (i.e. variables that take the value 0 or 1 to indicate the absence or presence of the treatment), and β is a vector of fixed effects (one for each treatment level). All other terms on the right hand side are random effects: μ_i is the experiment effect, ν_{ij} is the plate effect (nested in the effect factor), ω_{ijk} is the deposit effect (nested in experiment and plate factors), γ_j is a systematic plate effect (e.g. an effect created by the position of the plate in the experiment), δ_k is a systematic deposit effect (e.g. an effect created by the rank or the position of the deposit), and, finally, ε_{ijkl} is the between cell variation (nested in experiment, plate, and deposit). The variance of all random effects was estimated with the R package lme4 (Bates et al. 2015).

Results

Dose-response relationship of MMS using alkaline comet assay

PBMC samples were treated with six different concentrations of MMS in the range 0 to 500 µM during 1 h. A clear dose-response relationship was obtained (Table 1, Figures 2, 3 and 4), with median DNA damage ranging from 0.1 % to 11.3 % DNA in the comet-tail.

These results, obtained from seven independent experiments, were suitable for further detailed investigation of sources of variability of the results at different levels of DNA damage. Moreover, in order to study the variability of the assay in the case of high levels of damage, further experiments were performed with 500 µM of MMS during 1 h 30 m of incubation of PBMC instead of 1 h. As expected, these conditions induced a higher level of DNA damage i.e 77.7 ± 1.8 % (Mean ± SD) tail DNA (Figure 3).

Ro 19-8022 effects using the Fpg-modified comet assay

PBMC were treated with a single concentration of light activated- Ro 19-8022, as described in the materials and methods section, in order to induce different types of DNA damage, mainly oxidized bases which are recognized by the Fpg enzymic treatment. Under standard alkaline conditions, Ro 19-8022 plus light induced very low levels of DNA damage (without Fpg, as represented in Figure 5A). In the presence of Fpg, the level of DNA damage was high, as illustrated in Figure 5A. The net level of Fpg-sensitive sites was obtained by subtracting the damage obtained in the absence of Fpg from Fpg-exposed comets (Figure 5B), quantifying mainly oxidized bases.

Analysis of inter-experimental variations

The experiments were independently performed on seven separate days during a 7 months period, in one of the participating laboratories. For each experiment and each treatment group, the mean of the median from each deposit (technical replicate) was calculated as described in the materials and methods section. For each level of treatment, the coefficient of variation (CV) of these means was calculated as SD/mean. It reflects the variation between experiments, i.e. the day-to-day variation.

Linear Mixed Model

The LMM allowed us to analyze all individual cell determinations. For the untreated group, this represents 9549 determinations from 191 gel deposits laid on 32 independent plates in seven independent experiments. For each group, the distribution of the data is represented in supplementary Figure S2. These distributions are skewed, however after logit transformation they are approximately symmetric (Figure 2 and Supplementary Figure S3 for Ro 19-8022) and suitable for analysis by means of the linear mixed model (LMM). The variance of all effects is reported in Table 2.

These results discard the relevance of possible systematic effects of the position of the deposit on the plate or of the number of the plate (related to the chronology of the processing of plate during the experiment and the position of the plate during the electrophoresis). The main variation is due to the differences of damage level between the cells within each deposit (residual component), followed by the variation between experiments (i.e day-to-day variation) (experiment component), which is much weaker. There are also weaker deposit and plate effects ("experiment:plate:deposit" and "experiment:plate"). The magnitude of systematic plate and deposit effects ("plate" and "deposit") is negligible as compared to all other effects.

Discussion

Since 1999, various modifications and adaptations of the comet assay have been developed to increase the throughput, notably based on parallel sample processing [for review (Brunborg et al. 2014)]. These techniques include either modifications of glass formats, substitution of glass slides with polyester films, or the use of more advanced technologies such as cell microarrays and microfluidics. Such advanced technologies have a strong appeal but have so far showed limited spreading (few publications and applications from independent laboratories over the years), likely to be due to some technical complexity and/or requirement of specific and expensive equipment. In contrast, the high-throughput comet assay method

described in 2013 (Gutzkow et al. 2013) using a Gelbond® film format is highly versatile and flexible and can be used to analyze DNA in a variety of cell types and tissues. Moreover, for a laboratory already equipped for the standard glass slide comet assay, no specific equipment is required and the use of home-made plastic frame film holders being facultative. Using X-ray radiation on human PBMC, the authors showed that the use of Gelbond® film as a substrate allows a similar dynamic range for detecting DNA damage as for glass slides. Moreover, another study implying this laboratory and another one, using a single dose of MMS (250 μM) on TK-6 cells was also performed (Azqueta et al. 2013) for further validation of the method and inter-laboratory comparison of the results obtained.

In this context, our collaborative study assessed setting up the high-throughput method using Gelbond® film to replace the standard comet assay. The aim was to further validate this high-throughput method, thereby contributing to its wider use. We were easily successful in adapting our standard glass slide protocol (Lebailly et al. 2015) to the high-throughput method.

The inter-experimental variation of the experiment is low, for the negative control (untreated cells; very low absolute levels of DNA damage), the CV was 13.6 % (Table 1). This is consistent with previous reports [for example (Forchhammer et al. 2010; Ersson et al. 2013)]. For MMS-treated cells, the CVs were higher and could be explained by a potential day-to-day variation of the MMS activity. However, all the data are within the limits of agreement of Mean ± 2 SD (Supplementary Figure S4) that is commonly accepted for the comet assay (De Boeck et al. 2000).

To go further in the validation of the method by analyzing the source of variability of the comet assay, statistical analyses were performed after transformation of the data. Although the comet assay has been used for three decades, it is still challenging to choose the

appropriate way of carrying out data analysis because most of the distributions of the comet measures within a sample are not normally distributed but rather asymmetric, skewed or multimodal. The heterogeneity of the distribution curves of the data has two major implications. Firstly, the use of the median is more appropriate than the mean and is frequently adopted as the preferred analysis of the data (Table 1). Secondly, the use of some standard parametric and non-parametric method for statistical analysis is not recommended. In this case, a transformation of the data is required. For example logarithmic, logistic, arcsin or root square transformation have been proposed (Collins et al. 1997; Wiklund and Agurell 2003; Moller 2006; Lovell and Omori 2008). However, no consensus is possible concerning the choice of the most appropriate transformation method since it depends on the distribution curve of each set of data, which vary a lot between cell types and the application of the comet assays (e.g. biomonitoring, *in vitro*, *in vivo*). In our study, the logit transformation of the data appeared to be the most appropriate and allowed the use of the linear mixed model to analyze the data.

Many reports focus on day-to-day variation of the comet assay or inter-laboratory variability (Forchhammer et al. 2010; Ersson et al. 2013). The originality of our work is in studying both day-to-day variation and intra-experimental variability. Thanks to the linear mixed model, we were able to decipher the sources of variability of the assay. Our results demonstrate that even if many samples (120 samples) are processed at once, neither the deposit position nor the plate position during experiments have an impact on the results. The main sources of variability are the differences of damage levels within a deposit, underlining the importance of scoring a sufficient number of cells (at least 50 cells) within a replicate as also previously highlighted and recommended (Lovell et al. 1999; Lovell and Omori 2008). The second source of variability is the day-to-day variation, represented by the coefficient of variation which is in agreement with previous reports accepted for standard comet assay method (De

Boeck et al. 2000). Our validation work has been performed with the alkylating chemical compound MMS; it could be of interest to carry out such validation studies using X-ray radiation inducing single strand breaks, double strand breaks and alkali labile sites.

In conclusion, detailed mathematical analysis based on LMM, yields supplementary validation of the high-throughput comet assay. In this way the method has been adopted to our laboratory as well; it is likely that the versatility and cost-efficiency will be recognized also by other laboratories.

Statement of author contributions

HP and EBR designed the study. MC and EBR performed the experiments with technical advice of KBG and GB. HP and EBR analysed the data, prepared draft figures and tables. HP and EBR prepared the manuscript draft with important intellectual input from LH, KBG and GB. All authors approved the final manuscript.

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The authors have no conflicts of interest and declare no competing financial interests.

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Tables

TABLE 1. DNA damage of human peripheral blood mononuclear cells induced by 1 h of treatment with MMS [% Tail DNA, mean and Standard Deviation (SD) of median from 3-7 independent experiments, CV: Coefficient of variation. For each technical replicate, median from 50 cells scored (% Tail DNA), all technical replicates summarized within each experiment (39 to 191 technical replicates). Statistical analysis was performed with Wilcoxon's signed rank test, * P value < 0.01.]

	Mean (SD)	CV (%)
Untreated	0.09 (0.01)	13.6
MMS 12.5 µM	0.14* (0.03)	21.4
MMS 25 µM	0.18* (0.09)	49.8
MMS 50 µM	0.26* (0.05)	21.5
MMS 100 µM	0.76* (0.11)	14.8
MMS 250 µM	7.00* (3.84)	54.9
MMS 500 µM	11.29* (2.14)	18.9

TABLE 2. Total variance and variance decomposition of all measurements (the treated column summarizes data from all MMS-treated groups including a fixed effect of the MMS concentration). Data from 3-7 independent experiments.

	Untreated	Treated	All Groups
Total variance : $\mathrm{var}(Y_{ijkl})$	3.305	2.437	2.660
Number of determinations	9 549	26 850	36 399
Variance decomposition			
Residual : $\mathrm{var}(\varepsilon_{ijkl})$	3.179	2.220	2.472
Experiment : $\mathrm{var}(\mu_i)$	0.055	0.143	0.096
Experiment:plate:deposit : $\mathrm{var}(\omega_{ijk})$	0.067	0.062	0.077
Experiment:plate : $\mathrm{var}(\nu_{ij})$	0.010	0.030	0.027
Deposit : $\mathrm{var}(\delta_k)$	0.005	0.026	0.018
Plate : $\mathrm{var}(\gamma_j)$	0.000	0.011	0.018

Figures legends

Fig. 1. A. Pattern of gels deposit (named "D") on a Gelbond® film [prior to staining, each film was further cut into 2 pieces and glued on a glass slide (named "plate") for subsequent analysis with the microscope]. **B**. Illustration of the position of the Gelbond® films during the electrophoresis step.

Fig 2. DNA damage in human peripheral blood mononuclear cells (PBMC) induced by MMS treatments. Histogram of the distribution of logit-transformed tail DNA (from 3-7 independent experiments).

Fig 3. Dose-response relationship of MMS on human peripheral blood mononuclear cells (1 h of treatment, except for the right box: 1 h 30 of treatment). Box plots representation of tail DNA (data from Figure 2). Boxes are limited by first and third quartiles separated by median; thin horizontal lines represent minimum and maximum values in 10-90th percentile range. Points are the extreme values.

Fig 4. Frequency polygons of the data from Figure 2.

Fig. 5. Level of DNA damage in human peripheral blood mononuclear cells (PBMC). **A.** PBMC were treated with 1 μM Ro 19-8022 plus visible light (2 min 30 s), DNA damage is shown for samples that have been incubated with buffer containing Fpg ("+FPG") or with buffer alone ("-FPG"). (Mean ± SD from 6 independent experiments). Statistical analysis was performed with Wilcoxon's signed rank test, * P value < 0.001, for each treatment, comparison of +FPG group *versus* –FPG group. **B.** Fpg-sensitive sites was obtained by subtracting the level of DNA damage revealed without Fpg from damage obtained with Fpg, to calculate mainly oxidatively damaged DNA named "Fpg-sensitive sites". Statistical analysis was performed with Wilcoxon's signed rank test, # P value < 0.001, comparison of Fpg-sensitive sites of Ro 19-8022 group *versus* untreated group.

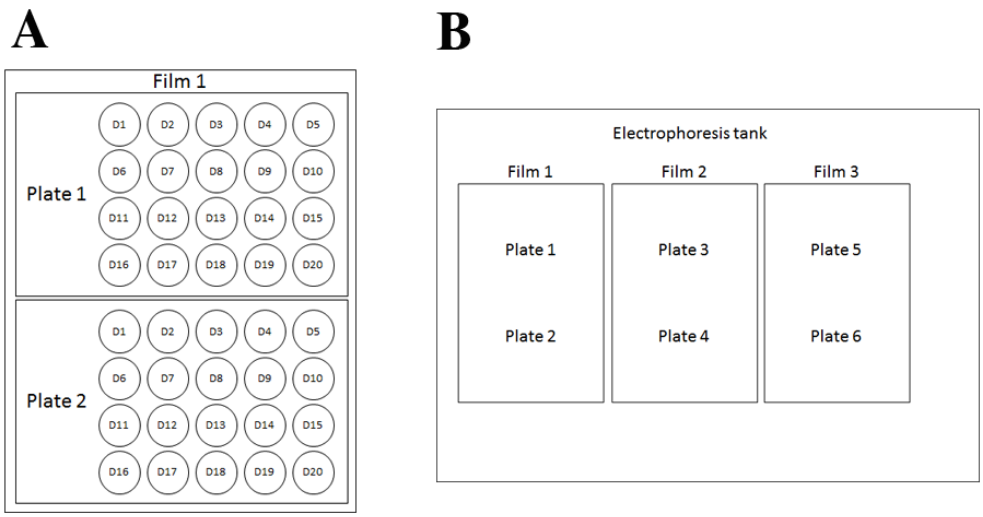
A
Film 1
Plate 1
D1 D2 D3 D4 D5
D6 D7 D8 D9 D10
D11 D12 D13 D14 D15
D16 D17 D18 D19 D20
Plate 2
D1 D2 D3 D4 D5
D6 D7 D8 D9 D10
D11 D12 D13 D14 D15
D16 D17 D18 D19 D20
B
Electrophoresis tank
Film 1
Film 2
Film 3
Plate 1
Plate 2
Plate 3
Plate 4
Plate 5
Plate 6

Fig. 1

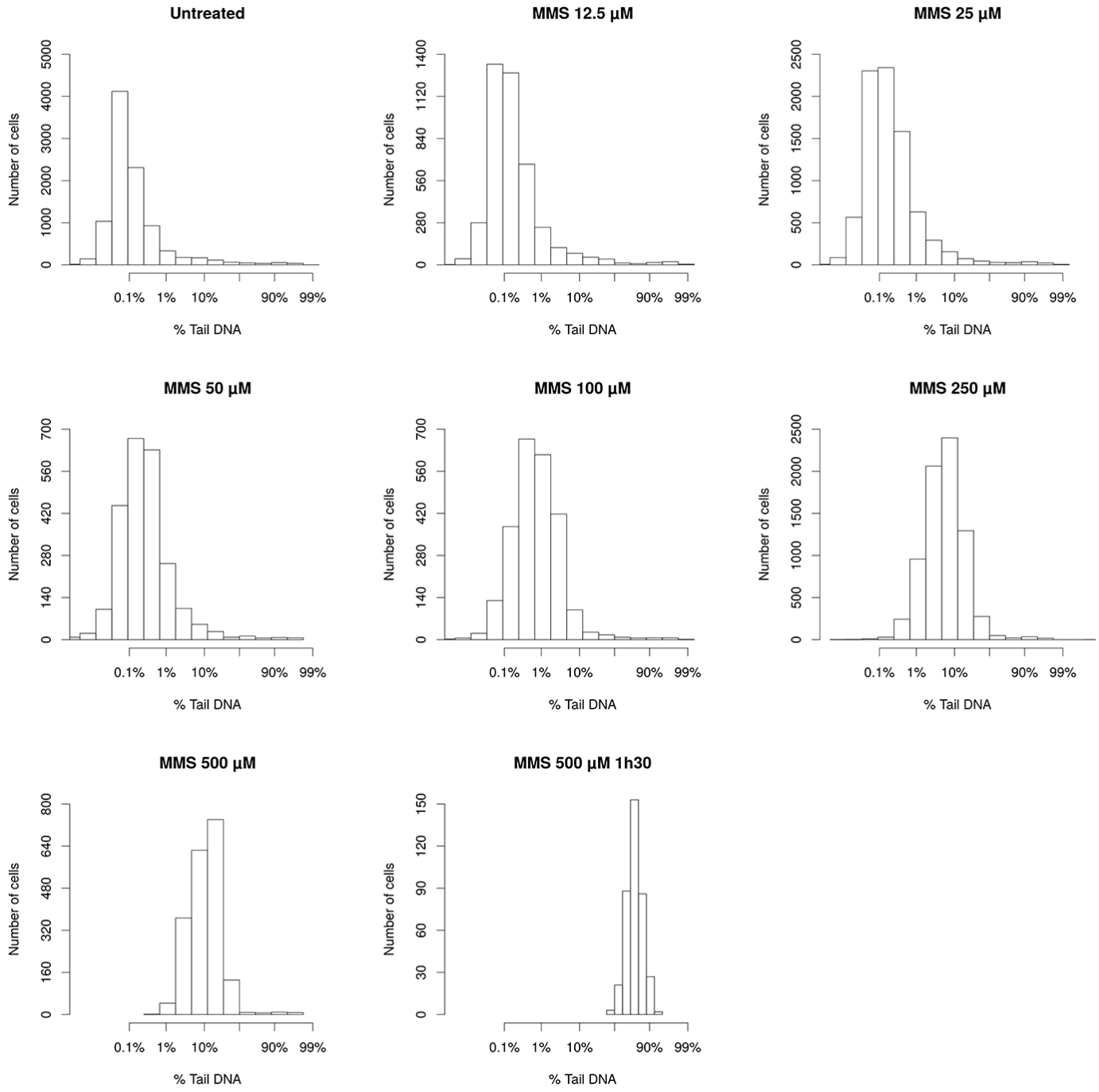
Untreated
Number of cells
0
1000
2000
3000
4000
5000
0.1%
1%
10%
90%
99%
% Tail DNA
MMS 12.5 µM
Number of cells
0
280
560
840
1120
1400
0.1%
1%
10%
90%
99%
% Tail DNA
MMS 25 µM
Number of cells
0
500
1000
1500
2000
2500
0.1%
1%
10%
90%
99%
% Tail DNA
MMS 50 µM
Number of cells
0
140
280
420
560
700
0.1%
1%
10%
90%
99%
% Tail DNA
MMS 100 µM
Number of cells
0
140
280
420
560
700
0.1%
1%
10%
90%
99%
% Tail DNA
MMS 250 µM
Number of cells
0
500
1000
1500
2000
2500
0.1%
1%
10%
90%
99%
% Tail DNA
MMS 500 µM
Number of cells
0
160
320
480
640
800
0.1%
1%
10%
90%
99%
% Tail DNA
MMS 500 µM 1h30
Number of cells
0
30
60
90
120
150
0.1%
1%
10%
90%
99%
% Tail DNA

Fig. 2

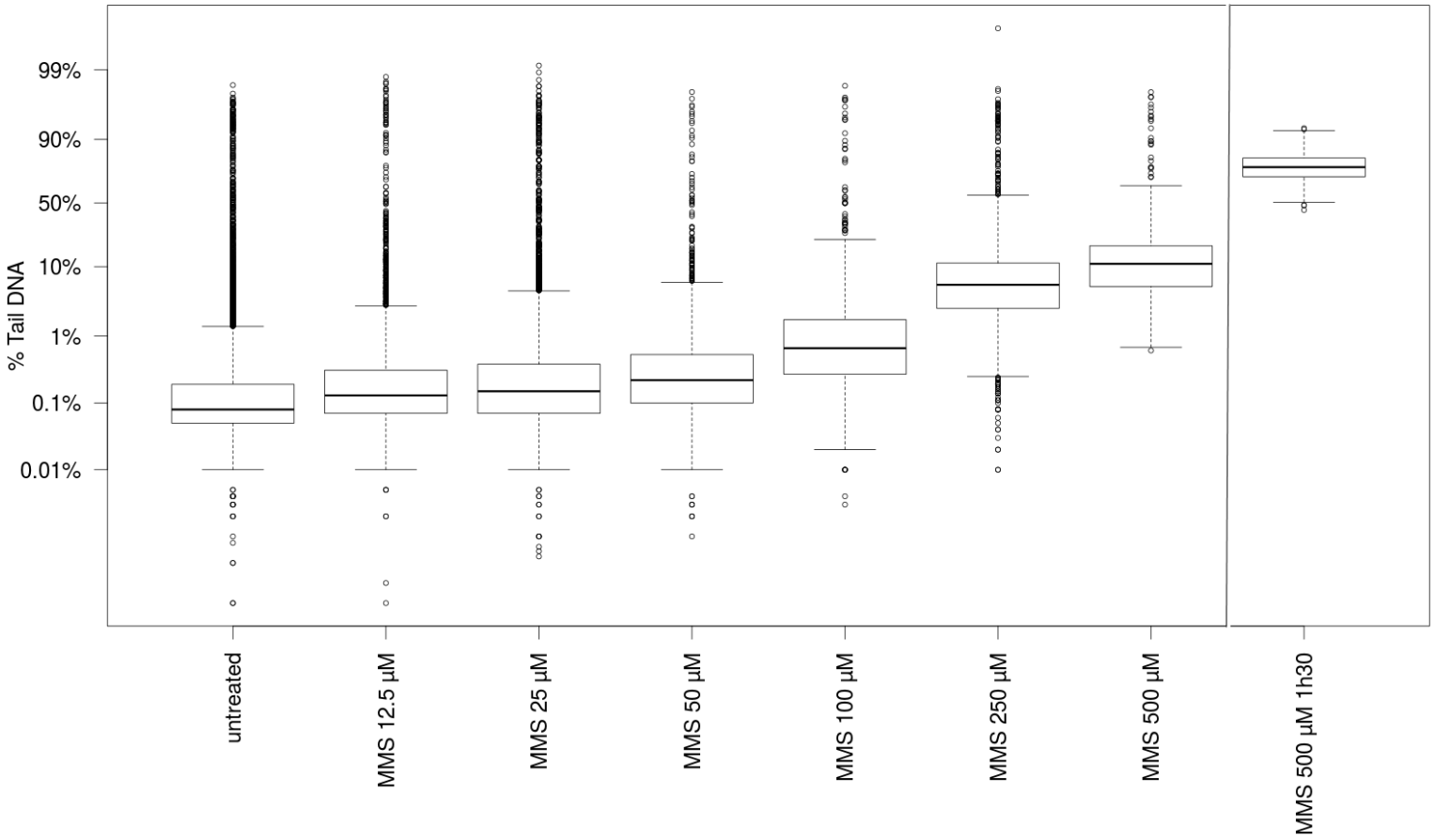
% Tail DNA
99%
90%
50%
10%
1%
0.1%
0.01%
untreated
MMS 12.5 µM
MMS 25 µM
MMS 50 µM
MMS 100 µM
MMS 250 µM
MMS 500 µM
MMS 500 µM 1h30

Fig. 3

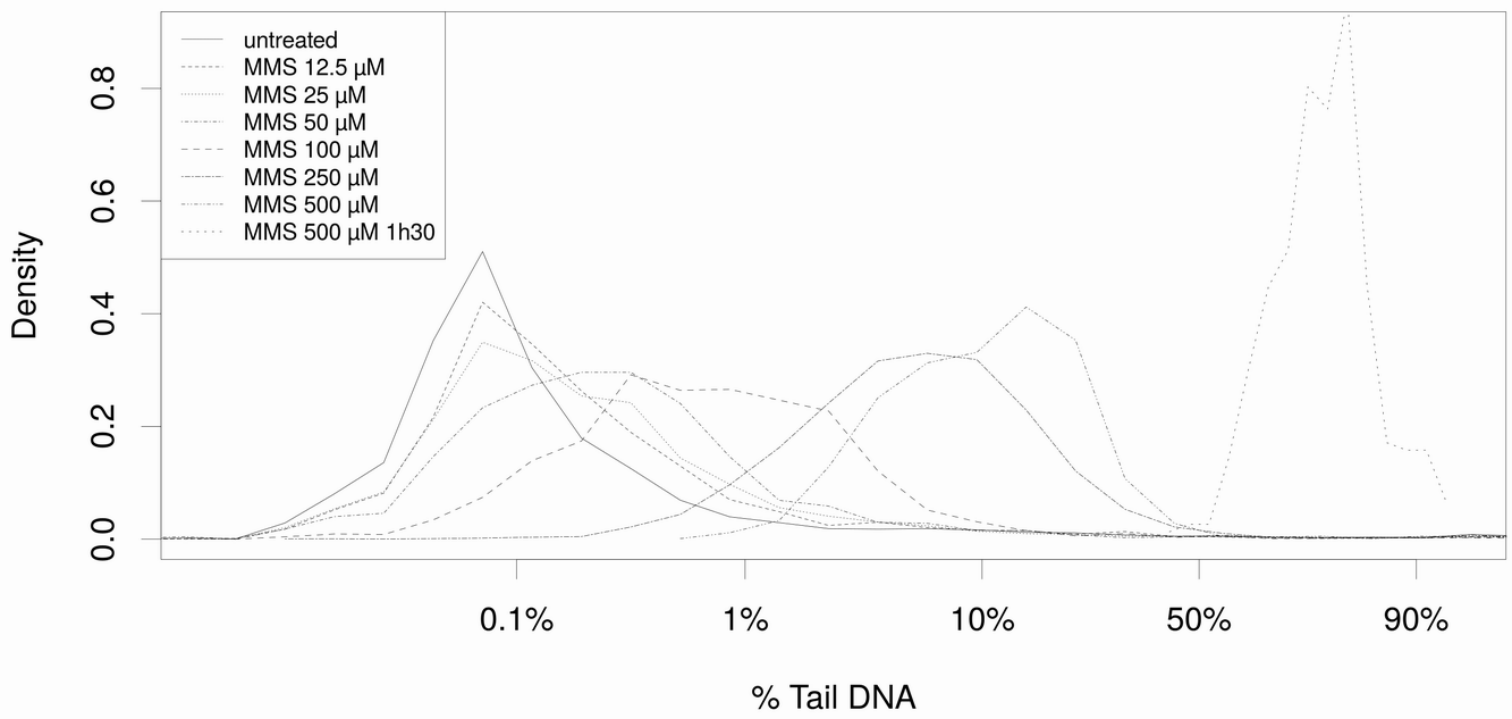
untreated
MMS 12.5 µM
MMS 25 µM
MMS 50 µM
MMS 100 µM
MMS 250 µM
MMS 500 µM
MMS 500 µM 1h30
Density
0.0
0.2
0.4
0.6
0.8
0.1%
1%
10%
50%
90%
% Tail DNA

Fig. 4

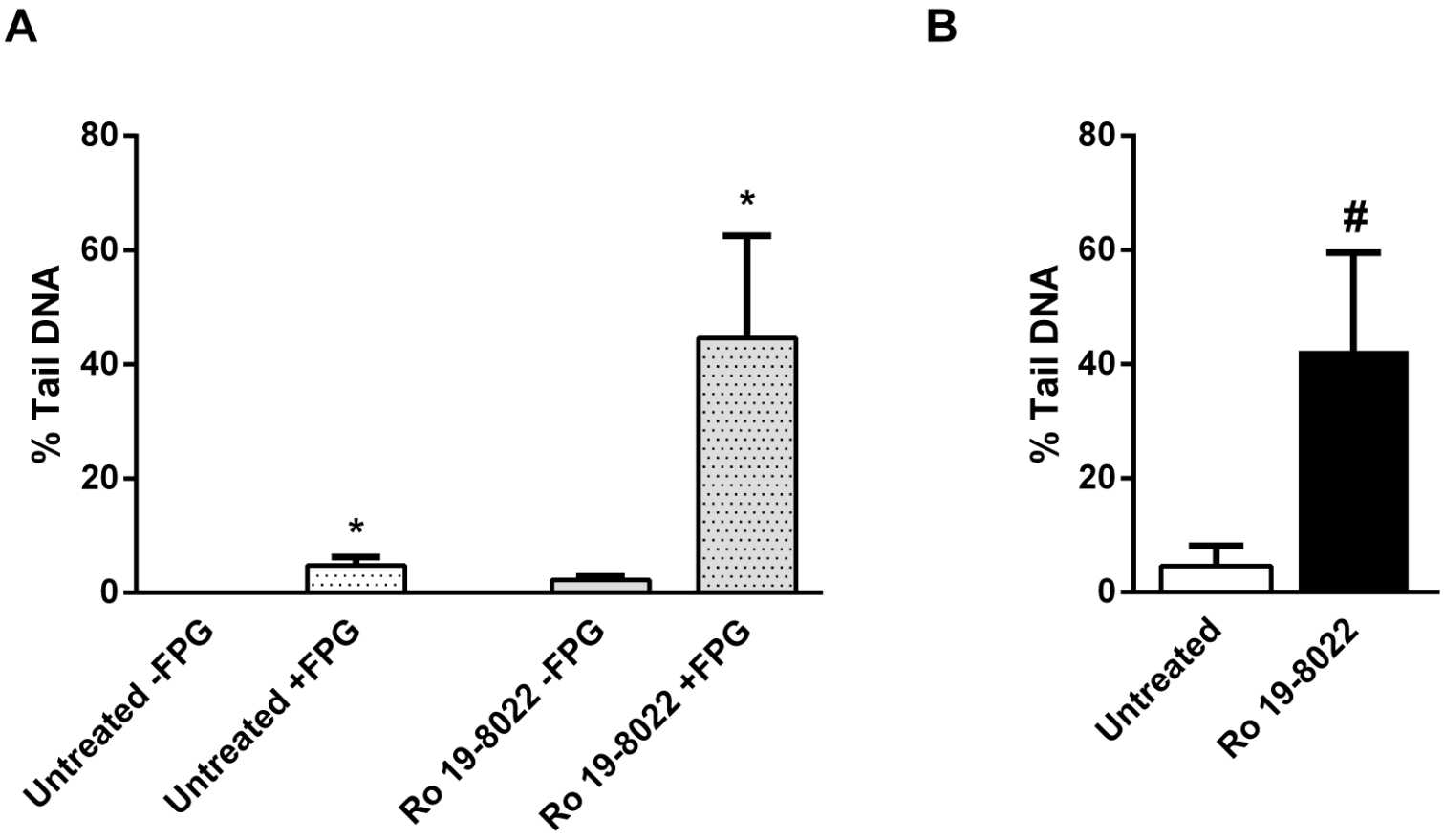
A
B
80
60
40
20
0
% Tail DNA
*
*
#
Untreated -FPG
Untreated +FPG
Ro 19-8022 -FPG
Ro 19-8022 +FPG
Untreated
Ro 19-8022

Fig. 5