Introducing a true internal standard for the Comet assay to minimize intra- and inter-experiment variability in measures of DNA damage and repair

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ABSTRACT

The Comet assay (CA) is a sensitive/simple measure of genotoxicity. However, many features of CA contribute variability. To minimize these, we have introduced internal standard materials consisting of 'reference' cells which have their DNA substituted with BrdU. Using a fluorescent *anti*-BrdU antibody, plus an additional barrier filter, comets derived from these cells could be readily distinguished from the 'test'-cell comets, present in the same gel. In experiments to evaluate the reference cell comets as external and internal standards, the reference and test cells were present in separate gels on the same slide or mixed together in the same gel, respectively, before their co-exposure to X-irradiation. Using the reference cell comets as internal standards led to substantial reductions in the coefficient of variation (CoV) for intra- and inter-experimental measures of comet formation and DNA damage repair; only minor reductions in CoV were noted when the reference and test cell comets were in separate gels. These studies indicate that differences between individual gels appreciably contribute to CA variation. Further studies using the reference cells as internal standards allowed greater significance to be obtained between groups of replicate samples. Ultimately, we anticipate that development will deliver robust quality assurance materials for CA.

INTRODUCTION

The Comet assay (also known as single cell gel electrophoresis) is a straightforward and highly sensitive method for measuring DNA damage and repair at the level of individual cells (1–4). Various versions of the assay enable the detection of a variety of DNA lesions with ease and speed including, single-strand breaks (SSBs) (both frank breaks and incomplete excision repair sites) plus alkali labile sites (ALSs), DNA–DNA and DNA–protein crosslinks and specific classes of base lesions (5,6). Due to its high sensitivity and simplicity, the Comet assay is being increasingly exploited as a laboratory measure of genotoxicity both *in vitro* and *in vivo*. Importantly, the Comet assay only requires a low number of cells. Consequently, the assay is now considered a powerful and useful tool in assessing genotoxicity in human biomonitoring and clinical studies (7–9).

Due to its greater sensitivity, the alkaline version of the Comet assay (ACA) is the most commonly used form of the assay. ACA measures SSBs and breaks formed from ALSs as well as specific base lesions if combined with specific endonucleases (10,11), and is sensitive enough to detect clinically relevant levels of damage (12–14). Briefly, for ACA, cells embedded in agarose gels on microscope slides are lysed in the presence of high salt concentration and detergents to generate 'nucleoids'. These bodies consist of loops of negatively supercoiled DNA anchored to a residual proteinaceous nuclear matrix network. The agarose-embedded nucleoids are then subjected to high pH, to allow DNA unwinding, and subsequent brief alkaline electrophoresis. Upon electrophoresis, nucleoid DNA is attracted to the anode, but only those

loops containing a break, which relaxes the supercoiling, are free to unwind and migrate in the direction of electrophoresis to form comet-like bodies; the comet 'head' containing undamaged DNA and the comet 'tail' containing the damaged/relaxed DNA. Following electrophoresis, the slides are neutralized, stained with a DNA binding dye and the comets visualized by fluorescence microscopy. Individual comet images may be recorded and these images analysed for a variety of densitometric and geometric parameters by purpose-designed image analysis software. The extent of comet tail formation is proportional to the amount of DNA damage present, with '% Tail DNA' (%TD) and 'Olive tail moment' being regarded as parameters that well reflect DNA damage, particularly radiation-induced DNA damage (3,6,15). Alternatively, comets can be visually classified into groups, according to the comet's appearance, reflecting their damage level (6). In the present study we report %TD as it shows a linear relationship to break frequency (6), is relatively unaffected by threshold settings, and has a wider dynamic range compared to other measures. It also gives a very clear indication of what the comets actually look like.

Despite the widespread use of the assay, only a small number of studies have addressed the issue of experimental variation. Indeed, many features of the assay affect intra-assay variability and inter-assay reproducibility. These include any *in vitro* or *ex vivo* cell exposure and key stages of the Comet protocol such as slide preparation, cell lysis and electrophoresis conditions (including homogeneity of the agarose layers, electrical field inhomogeneity inside the tank, buffer variations), and also comet analysis (5). In attempts to reduce/minimize such variation, several clinical and human biomonitoring studies have included supposed 'internal' standards in which untreated or treated 'reference' cells were analysed alongside the test cells as 'negative' and 'positive' controls, respectively (16–19). However, in all these studies the reference cells are present in separate gels to the test cells, so it is more appropriate to consider these as 'external' or 'parallel' standards, rather than true internal standards, as they will not account for inter-gel variations.

To take into account inter-gel variation and ultimately to be able to compare measurements from different electrophoretic runs, as would be necessary when large numbers of samples need to be analysed, it would be ideal to integrate a true internal standard into the assay. In the current study we introduce a true internal standard for the Comet assay. The internal standard materials consist of reference cells which have had their DNA thymidine substituted with BrdU. The post-electrophoresis comets, derived from these reference cells (reference cell comets), can be readily distinguished from the test cell comets present in the same gel, at the time of comet analysis, using a fluorescently tagged *anti*-BrdU antibody together with an appropriate additional barrier filter. The unambiguous identification/distinguishing of the test and reference cell comets enable the reference cell comets to be selectively analysed in an extra round of analysis. In experiments to evaluate the reference cell comets as both external and internal standards, the

reference and test cells were either present in separate gels (on the same slide) or mixed together in the same gel, respectively, before their co-exposure to X-irradiation and subsequent ACA analysis. Accordingly, the test cell data are either normalized using the reference cell comets in the separate gel acting as an external/parallel standard, or normalized using the reference cell comets in the same gel acting as a true internal standard.

MATERIALS AND METHODS

Chemicals

Chemicals, reagents and tissue culture medium were all purchased from Sigma (Poole, UK). Foetal calf serum (FCS) and Alexafluor®488-tagged *anti*-BrdU antibody were obtained from Invitrogen (Paisley, UK).

Cells

H460 and A549, human lung carcinoma cell lines, were purchased from ATCC. The cells were cultured in RPMI 1640 medium supplemented with 10% FCS and incubated at 37°C in 5% carbon dioxide (CO_2). The cells were maintained in exponential growth by sub-culturing into fresh medium every three or four days. No antibiotics were added to the medium. Cells were tested and confirmed as mycoplasma contamination free.

For preparation of the cells for use as BrdU-containing reference cells, the growth medium from actively growing cells (at ~60% confluence) was replaced with fresh growth medium containing 25 μM BrdU and incubated for 48 h at 37°C in 5% CO_2. The cells were then serum starved, to arrest cells in the G_0/G_1 phase, by replacing the BrdU-supplemented medium with RPMI 1640 medium containing 0.5% FCS and the cells then incubated at 37°C in 5% CO_2 for 24 h prior to harvesting; the arrest was confirmed by flow cytometry and was performed to sharpen up the comet assay response [cells arrested in G1 generate comet measures with dramatically reduced variation (A. Rapp, unpublished data)]. These steps were performed under low light, as BrdU is light-sensitive.

Alkaline Comet assay

Radiation-induced DNA damage (SSB and ALS) was assessed using a modified version of ACA whereby the cells were irradiated 'set' in agarose gels on microscope slides. This modified version of the comet assay, described fully by Moneef and co-workers (12), increases the assay's sensitivity by minimising the opportunity for repair of induced damage prior to cell lysis (20).

Slide preparation and Irradiation. For the assessment of intra-experimental variability, individual slides were prepared consisting of two gels, one gel containing both non-BrdU test cells (15 000 cells) and BrdU-labelled reference cells (15 000 cells) (co-embedded in the same gel), the other gel containing just BrdU-labelled reference cells (30 000 cells). For the assessment of inter-experimental variability, two sets of slides were prepared; the first set consisting of one gel containing both BrdU-labelled reference cells (15 000 cells) and non-BrdU test cells (15 000

cells) (co-embedded in the same gel), and the second set consisting of two gels, one containing non-BrdU test cells (30 000 cells) and the other just BrdU-labelled reference cells (30 000 cells).

For the preparation of the individual slides, pellets containing the requisite 30 000 cells were suspended in 80 μl of 0.6% low melting point agarose, then dispensed onto a clear microscope slide precoated with dried 1% normal melting point agarose, and allowed to solidify under a cover slip on ice. The slides were then irradiated on ice using a Pantak X-ray machine (dose rate of 1 Gy/min). For measures of immediate damage, duplicate slides were irradiated with doses of 4, 6 or 10 Gy. For measures of damage repair, duplicate slides were irradiated with 10 Gy and 'repair incubated' (see next). All these steps were conducted under low light to prevent additional DNA damage.

Lysis and eletrophoresis. For studies of immediate damage, the irradiated cell slides were immediately placed in cold lysis buffer (2.5 M NaCl, 100 mM Na_2EDTA, 10 mM Tris, pH 10 and 1% Triton X-100 added fresh, 4°C) overnight. For repair studies, the 10 Gy irradiated cell slides were incubated in growth medium at 37°C for 5, 10, 15, 30 and 45 min then placed in lysis buffer overnight. After lysis, the slides were washed twice in ice-cold distilled water for 10 min, incubated in ice-cold alkali buffer (300 mM NaOH, 1 mM NaEDTA, pH > 13) for 20 min followed by electrophoresis in the same buffer at 30 V (0.88 V/cm) and 300 mA for a further 20 min. The slides were rinsed with neutralisation buffer (0.4 M Tris–HCl, pH 7.5) for 20 min followed by washing with ice-cold distilled water for 10 min and left to dry in a 37°C incubator. All these procedures were carried out on ice and under low light.

After drying, the slides were re-hydrated for 30 min, stained with a freshly made solution of 2.5 μg/ml propidium iodide (PI) for 20 min and then washed with distilled water for a further 30 min. Those slides containing both BrdU and non-BrdU cells were further re-hydrated for 3 h in the dark. Forty microlitres of the *anti*-BrdU antibody solution (1:20 in PBS) was transferred to each gel and incubated in a humidity chamber for 45 min. The slides were immediately scored as the fluorescence dye tagged to the antibody fades relatively quickly.

Comet image capture and data analysis. Comet images were visualized using an Olympus fluorescence BH2 microscope fitted with an excitation filter of 515–535 nm (Alexa488 is not optimally excited with these conditions (ex: 515–535), but still the comets are visible) and a barrier filter of 590 nm, at 200× magnification. An additional barrier filter (XF3084 (535AF45), Omega Optical, Brattleboro, VT, USA) was used to distinguish the BrdU labelled cells from the non-BrdU cells, but the images were captured/recorded with the XF3084 filter removed. Comet images were captured by an on-line charge-coupled device (CCD) camera and analysed using the Komet Analysis software (version 5.5) from Andor Technology (Belfast, UK).

%TD was selected as the parameter that best reflects DNA damage (6) and was reported as the median value (m%TD) to minimize the effect of anomalous values. Fifty randomly chosen, non-overlapping reference or test comets were analysed per gel, with two gels being analysed per data point. Data normalization was undertaken as follows: comparison of the individual median reference cell comet value (derived from a single gel) to a determined average reference cell comet response (derived from the average of all the median scores for reference comets from different gels at a single dose or repair time point) generates a series of individual correction factors that can then be applied to normalize the corresponding individual median test cell comet values in the same or 'associated' separate gel (the separate gel on the same slide). In this way, the test cell data are either normalized using the reference cell comets in the same gel, as an internal standard, or normalized using the reference cell comets in the separate gel, as an external standard. The means of the normalized and non-normalized test data were determined and the coefficient of variation noted as an indicator of the data's correspondence to the mean.

Statistical analysis. To determine the significance of differences between groups of replicate samples the obtained results were analysed by the statistical software package Minitab 15. The significance of difference was determined by the non-parametric Mann–Whitney test.

RESULTS AND DISCUSSION

Figure 1a depicts a schematic illustration of the fluorescence microscope mirror and filter arrangement, indicating the location of the additional barrier filter. Figure 1b and c depict identical images for a single field of view of comets, derived from irradiated co-embedded BrdU and non-BrdU labelled H460 cells, co-stained with PI and the *anti*-BrdU antibody and visualized either without (Figure 1b) or with (Figure 1c) the additional filter. It can be seen that the post-electrophoresis BrdU-labelled reference cell comets can be readily distinguished from the non-BrdU test cell comets by insertion of the additional barrier filter into the emitted light path; the additional barrier filter prevents the 617 nm wavelength light emitted from PI from reaching the eyepiece/camera, but allows the 519 nm wavelength emitted from the Alexafluor®488 dye tagged to the *anti*-BrdU antibody to pass through. Using the additional barrier filter to distinguish the BrdU-containing cells allows for the reference and test cell comets to be scored separately. Comet images were captured/recorded with the barrier filter removed and under these conditions the fluorescence emitted from the Alexafluor®488 dye is also seen, but as can be seen in Figure 1c it is much weaker and does not significantly interfere with the measurements. Preliminary experiments using solely BrdU-labelled reference cells and solely non-BrdU test cells revealed the Alexafluor®488 dye tagged *anti*-BrdU antibody to be entirely specific for the BrdU-labelled reference cell comets (data not presented).
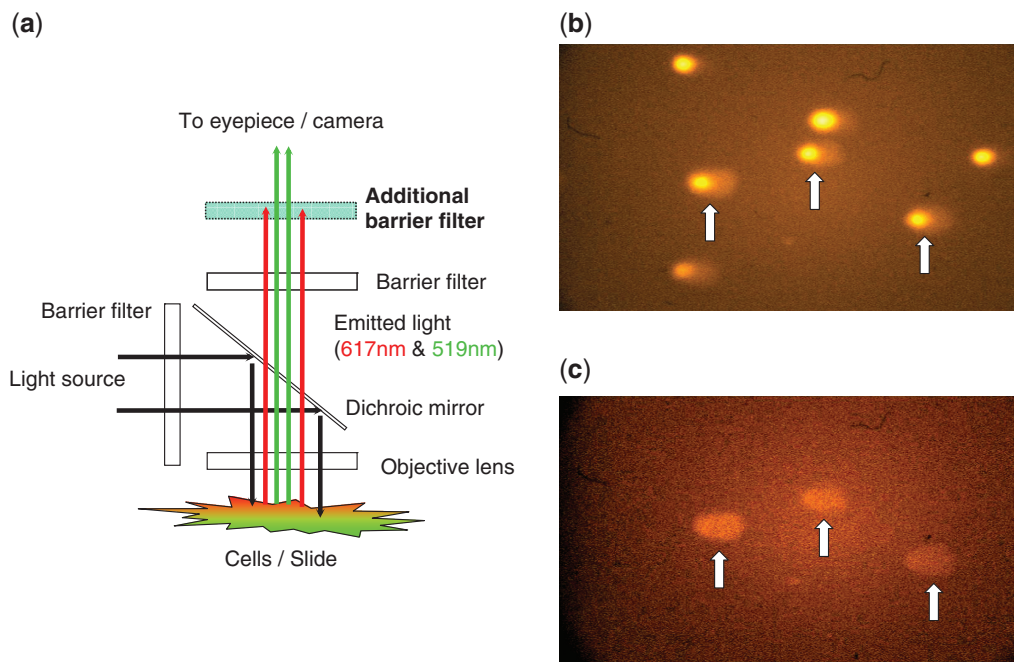
Figure 1. (a) Schematic diagram of the fluorescence microscope filter and mirror arrangement, using the additional barrier filter to discriminate BrdU-containing reference cell comets from the non-BrdU containing test cell comets; (b) Image of comets observed without the additional barrier filter, showing predominately PI fluorescence from both test and reference cell comets; (c) Identical image of comets observed with the additional barrier filter, showing fluorescence from the *anti*-BrdU antibody bound to the reference cell comets only.

Intra- and inter-experimental variability in ACA measures of DNA damage and repair

To evaluate the reference cell comets as a means of reducing intra- and inter-experimental variability, we conducted a series of experiments in which the reference and test cells were either present in separate gels (on the same slide) or mixed together in the same gel, before their co-exposure to X-irradiation and subsequent ACA analysis. In this way, the test cell data are either normalized using the reference cell comets as internal standards, or as external standards.

Figure 2 shows the results of a single experiment consisting of 18 individual replicate measures of initial comet formation (m%TD, as determined by ACA analysis of A549 cells after 6 Gy X-irradiation) in which non-BrdU test cells and BrdU-labelled reference cells were either co-embedded in the same gel (Figure 2a) or were present in separate gels on the same slide (Figure 2b). Figure 2c compares the average test cell comet response derived from the individual test cell comet values before and after the latter's normalization using the correction factors calculated from the BrdU-labelled reference cell comets acting either as internal standards or as external/parallel standards.

Comparing Figure 2a and b it can be seen that the profile of the test cell comets' variable response better mirrors the reference cell comets' variable response when the two cell types were together in the same gel (Figure 2a) [compared to when they were present in separate gels on the same slide (Figure 2b)] with the reference cell comets generating higher measures of comet formation. The reason for the reference cell comets displaying

greater measures of comet formation is because BrdU substitution increases the level of radiation-induced strand breaks in cellular DNA (21).

Figure 3 shows the results of three independent dose response experiments (Figure 3a + b; c + d; e + f) (m%TD; as determined by ACA analysis of H460 cells after 0–10 Gy X-irradiation) in which the non-BrdU test cells and BrdU-labelled reference cells were either present in separate gels on the same slide (Figure 3a, c and e) or were co-embedded in the same gel (Figure 3b, d and f) before their co-exposure to X-irradiation and subsequent ACA analysis. Again, from Figure 3 it can be seen that the BrdU-containing reference cells, for the most part, generated higher measures of comet formation (notably after 4 and 6 Gy irradiation), with a more consistent difference between the measures being observed when the reference and test cells were together in the same gel, as compared to when they were present in separate gels on the same slide.

Figure 4 shows the averaged test cell dose responses derived from the individual test cell comet values, before (Figure 4a and c) and after (Figure 4b and d) the latter's normalization using the correction factors calculated from the responses of the BrdU-labelled reference cell comets acting either as internal standards or acting as external standards.

In addition to assessing the impact and value of using the prepared reference cells as both internal and external standards on measures of immediate DNA damage, we also investigated their impact on measures of DNA damage repair. Three independent repair response experiments were undertaken in which the test cells and
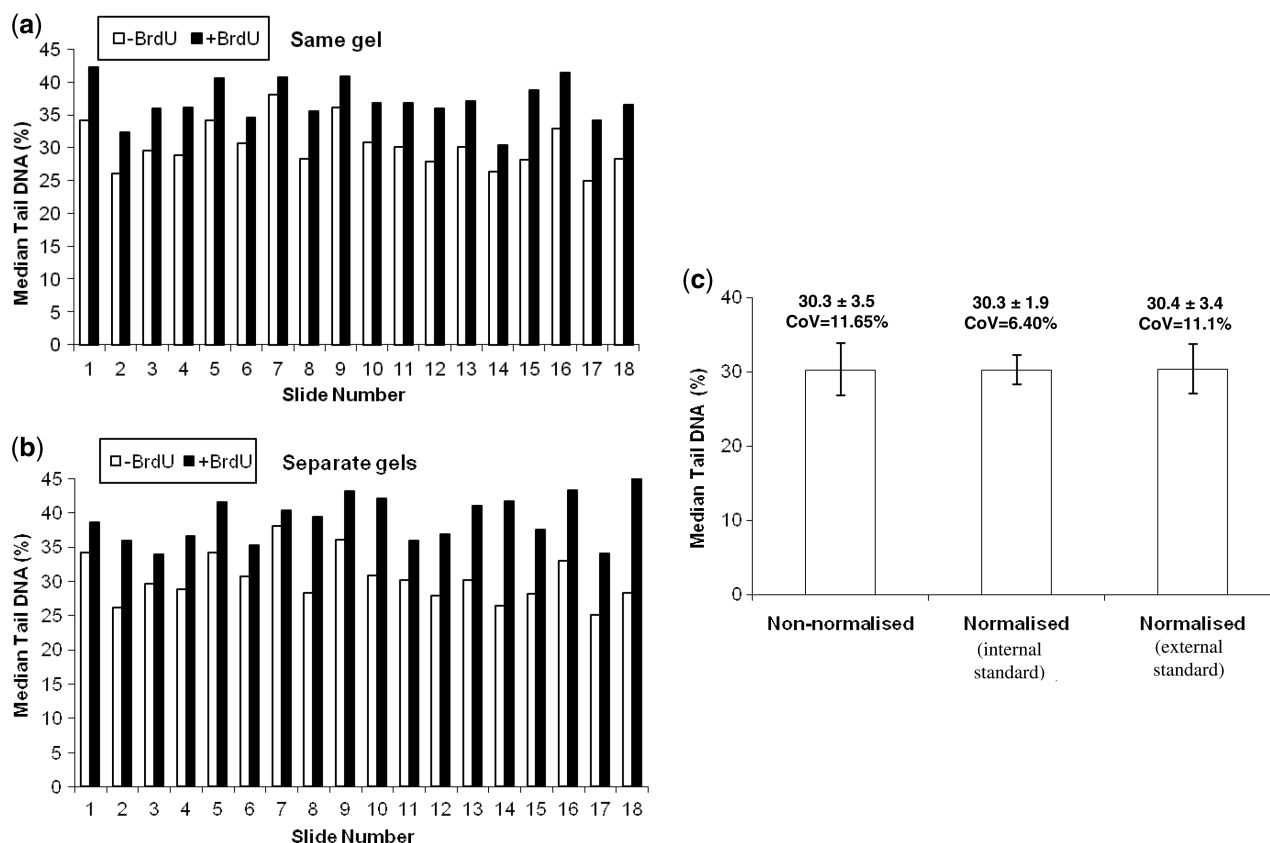
Figure 2. The extent of initial comet formation (m%TD) in a single experiment of 18 replicate slides, in which the BrdU-containing reference cells and the non-BrdU test cells were either prepared in the same gel (**a**) or in separate gels on the same slide (**b**), prior to 6 Gy X-irradiation. (**c**) Comparison of the averaged test cell response (m%TD ± SD), derived from the individual test cell comet values before and after normalization using correction factors derived from the BrdU-labelled reference cell comets acting as either internal or external standards.

reference cells were either present in separate gels on the same slide or co-embedded in the same gel before their co-exposure to X-irradiation, repair incubation and subsequent ACA analysis. Figure 5 shows the averaged relative test cell repair responses derived from the individual test cell values before (Figure 5a and c) and after (Figure 5b and d) the latter's normalization using the BrdU-labelled reference cell comets either as internal standards or as external standards. The data in Figures 4 and 5 indicates that the CoVs were markedly reduced when normalization was based on reference standards in the same gel.

The inclusion of an external standard, in which the test and reference cells are in separate gels but present in the same experiment, may take into account inter-experiment variability arising from differences in cell lysis and electrophoresis conditions, and could to some extent account for variability arising from cell exposure and comet analysis, but will not account for inter gel differences. However, the inclusion of a true internal standard, in which the reference and test cells are present together in the same gel, ensures that both cell types are exposed to exactly identical conditions; consequently, with internal standard cells experiencing the exact same conditions as the test cells, they will have a greater

capacity to account for, and reduce, protocol-induced variability. Accordingly, for intra- and inter-experimental measures of radiation-induced comet formation and DNA damage repair we have obtained substantial (~2-fold) reductions in the CoV when the reference and test cells were in the same gel. However, when the reference and test cells were in separate gels, at best, only minor/moderate reductions in CoV were noted. This indicates that differences between individual gels significantly contribute to experimental variation in the Comet assay, even when present on the same slide.

Improved statistical significance in comparing groups of replicate samples

To determine whether the reference cells, acting as internal standards, could be used to improve estimates of significance between groups of replicate samples, we further analysed the data used to evaluate the impact of the reference cell comets on intra-experiment measures of immediate DNA damage (see Figure 2). For this we took the uncorrected data presented in Figure 2a, and for both the BrdU-containing cells and the non-BrdU-containing cells, we consecutively averaged the results of, firstly, all 18 samples; then 17 samples (samples 1–17 inc.); then 16 samples (1–16 inc.) and so on. For the averaged
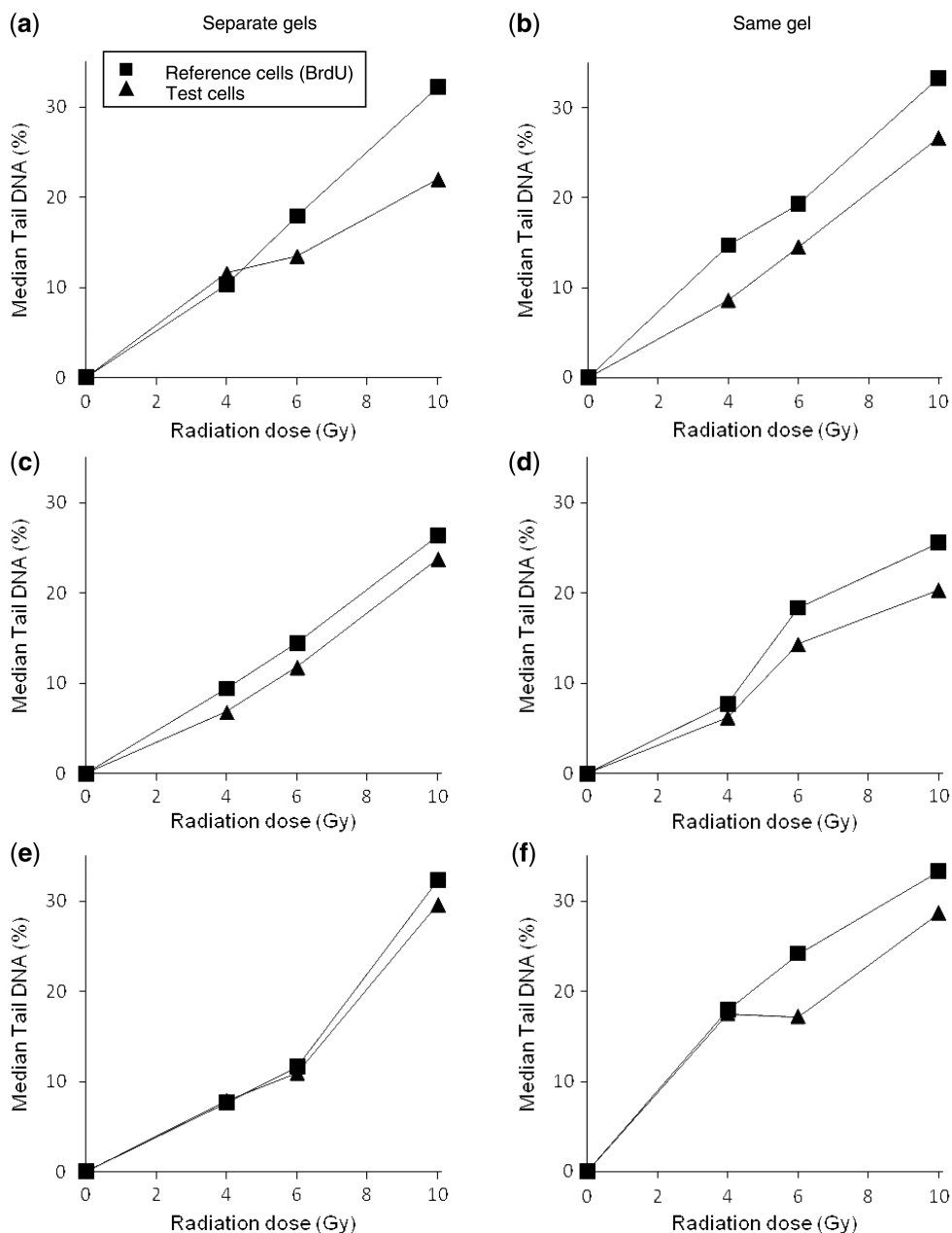
Figure 3. Three independent dose response experiments (**a** + **b**, **c** + **d** and **e** + **f**) in which the BrdU-containing reference cells and non-BrdU-containing test cells were either prepared in separate gels on the same slide (a, c and e) or prepared together in the same gel (b, d and f), prior to their co-exposure (on slides and on ice) to X-irradiation.

uncorrected data of the first 11 samples (samples 1–11 inc.) the statistical significance between the measures of DNA damage for the BrdU-containing and non-BrdU-containing cells was $P < 0.005$ ($P = 0.0039$) (Figure 6). However, in using the BrdU-containing cells as internal standards to correct the non-BrdU-containing cells (as previously undertaken) and vice versa (i.e. using the non-BrdU-containing cells as internal standards to correct the BrdU-containing cells), the statistical significance of the difference is substantially increased to $P < 0.0001$. Hence using an internal standard can greatly increase the level of significance obtained between groups of replicate samples

when assessing the same number of samples. Alternatively, using the internal standard permits a smaller number of samples to be analysed ($n = 6$) whilst maintaining an equivalent statistical significance ($P = 0.0022$). The latter use of the internal standard material would be of benefit in situations when the sample is precious or when sample numbers are limiting (i.e. clinical samples).

Whilst we have demonstrated that the inclusion of an internal standard leads to substantial improvements in data quality, as it stands, the internal standard presents certain disadvantages and further development is needed. For instance, its inclusion does require further comet
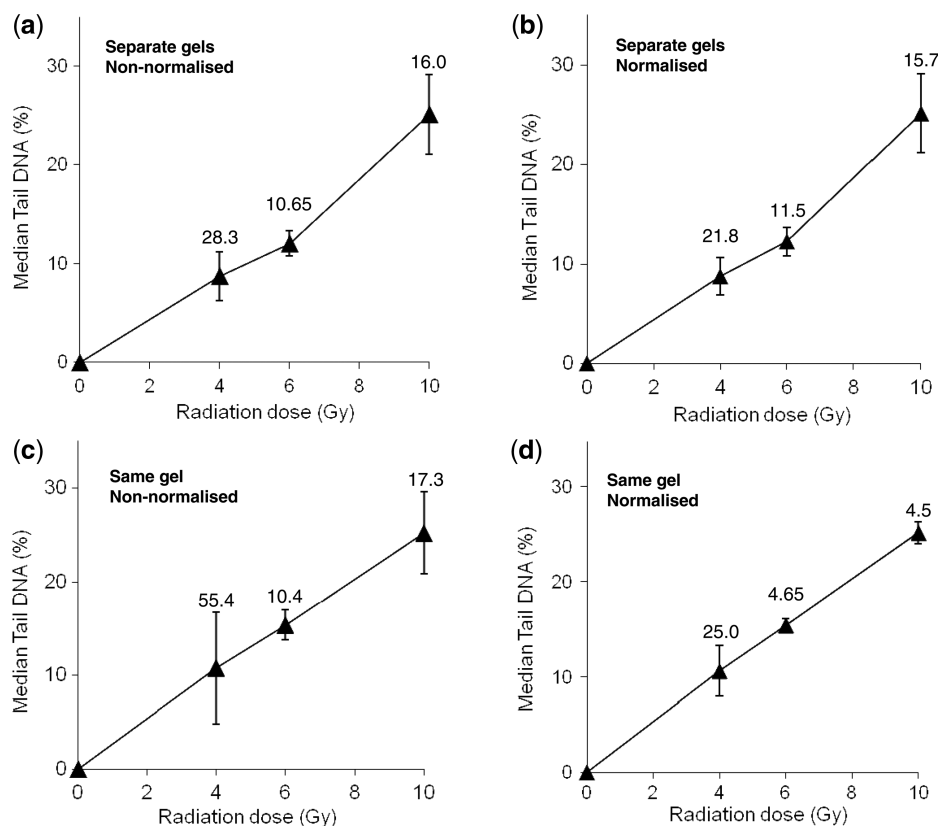
Figure 4. The extent of radiation-induced comet formation in the test cells from the three independent dose response experiments; (**a**) and (**b**) show the averaged test response (m%TD ± SD) obtained when the test and reference cells were in separate gels, before and after normalization, respectively (using correction factors derived from the BrdU-labelled reference cell comets acting as external standards); (**c**) and (**d**) show the averaged test response obtained when the test and reference cells were in the same gel, before and after normalization, respectively (using correction factors derived from the BrdU-labelled reference cell comets acting as internal standards). The number above each data point is the corresponding coefficient of variation.

scoring; however, it may be feasible that fewer reference cell comets could actually be analysed (i.e. 20% of the test cell comets analysed) to achieve data normalization, and as automated Comet assay systems become increasingly available, any additional time required for further scoring will be less of a hindrance. With regards to further development, for long-term and/or large comet assay-based human biomonitoring studies, robust and stable internal standard materials are required; a single cell line should be chosen and a standard preparative protocol validated to negate batch-to-batch variability. Furthermore, the approach described by Rapp and co-workers (data presented at the 5th Comet Assay Workshop, Aberdeen, August 29–30, 2003, 'An internal fragment length standard for the Comet-Assay' Rapp *et al.*, Dept. for Single Cell and Single Molecule Techniques, Institute fur Moleulare Biotechnology Jena, Beutenbergstr. 11, 07745 Jena, Germany), in which cells are encapsulated in agarose microbeads (one cell per bead) and their DNA fragmented in a controlled manner, holds promise as a means of developing robust internal standard materials suitable for long-term/large human biomonitoring studies.

In summary, we report the early stage development and integration of a true internal standard for the Comet assay consisting of BrdU substituted reference cells. The comets derived from these reference cells can be readily distinguished from the test cell comets present in the same gel. Using the reference cells as internal standards we have obtained substantial (>2-fold) reductions in the coefficient of variation (CoV) for intra- and inter-experimental measures of radiation-induced comet formation and DNA damage repair; but only minor reductions in CoV were noted when the reference cells were used as external/parallel standards. These studies indicate that differences between individual gels, even when present on the same slide, markedly contribute to experimental variation in the Comet assay. Having both the reference and test cells together in the same gel provides a means of reducing variation in comet measures caused by differences/inconsistencies in the preparation of the slides, cell exposure, nucleoid electrophoresis and comet analysis. Finally, we have shown that using the reference cells as internal standards permits greater significance to be obtained between groups of replicate samples when the same number of samples are analysed; alternatively, it was demonstrated that the same level of significance can be achieved using smaller numbers of samples. Ultimately, we anticipate that further development will deliver widely applicable 'off the shelf' quality
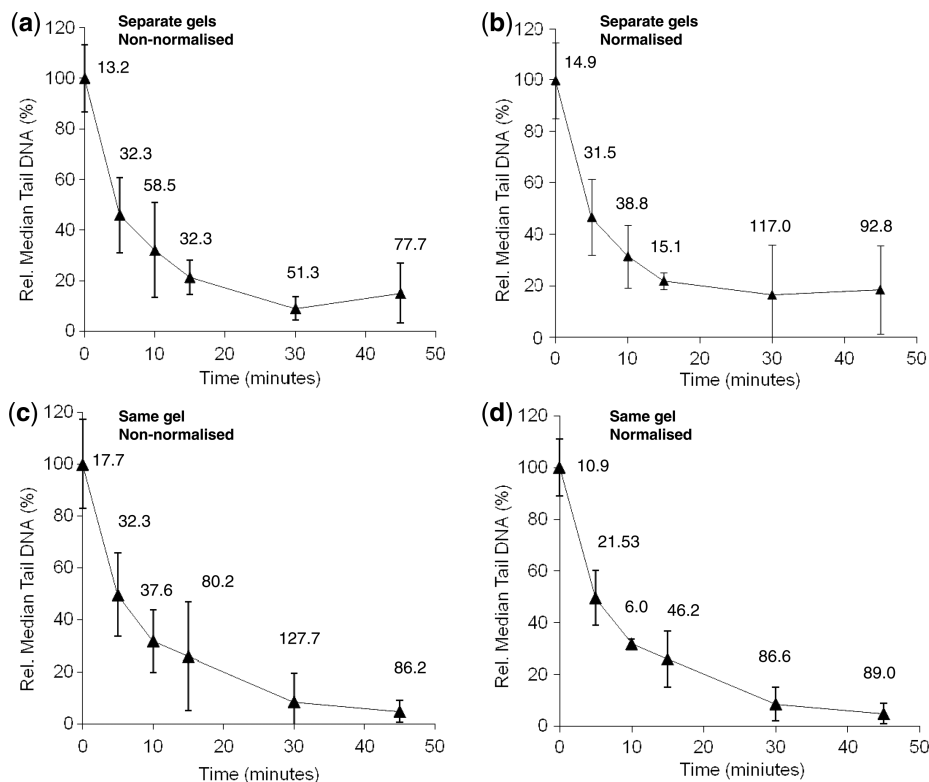
Figure 5. The extent of radiation-induced DNA damage repair in the test cells from the three independent experiments; (**a**) and (**b**) show the averaged test response (m%TD ± SD) obtained when the test and reference cells were in separate gels, before and after normalization, respectively (using correction factors derived from the BrdU-labelled reference cell comets acting as external standards); (**c**) and (**d**) show the averaged test response obtained when the test and reference cells were in the same gel, before and after normalization, respectively (using correction factors derived from the BrdU-labelled reference cell comets acting as internal standards). The number above each data point is the corresponding coefficient of variation.
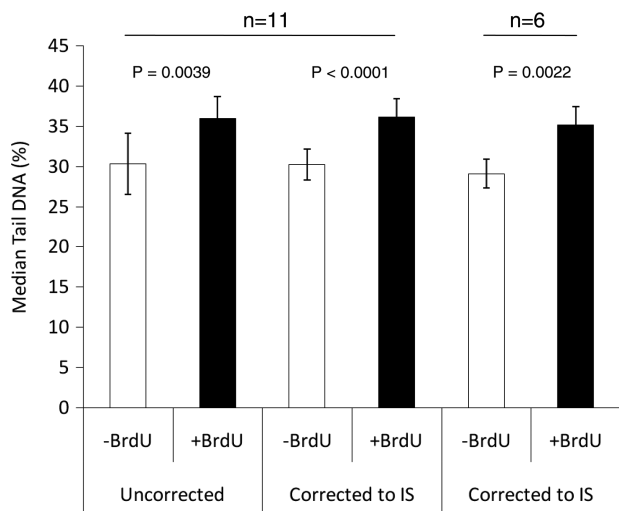
Figure 6. The extent of initial comet formation (m%TD) in replicate slides, in which the BrdU-containing reference cells and the non-BrdU test cells were present in the same gel prior to 6 Gy X-irradiation. For the averaged uncorrected data of the first 11 samples ($n = 11$) the statistical significance between the measures of DNA damage for the BrdU-containing and non-BrdU-containing cells was $P < 0.005$ ($P = 0.0039$). However, in using the BrdU-containing cells as internal standards to correct the non-BrdU-containing cells and *vice versa* (see text) the statistical significance of the difference is increased to $P < 0.0001$. Alternatively, using the internal standard permits a smaller number of samples to be analysed ($n = 6$) whilst maintaining an equivalent statistical significance ($P = 0.0022$).

assurance (QA) materials for investigators using the Comet assay.

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