Original Article

Comparison of different immunoassays for γH2AX quantification

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Background: One of the most sensitive methods for DNA double-strand break (DSB) detection is the assessment of phosphorylated histone protein 2AX (γH2AX), which can be visualized as γH2AX focus surrounding the damage site. Quantification of γH2AX offers a variety of potential diagnostic applications being on the brink of introduction into clinical routine. Therefore, further assay optimization and automation is required to enable standardized, high-throughput γH2AX analysis. To quantify γH2AX levels, different immunoassay techniques can be performed, each showing specific characteristics regarding assay sensitivity and sample processing. Although microscopic foci quantification is considered the most sensitive approach, data of direct comparison of multiple γH2AX immunoassay techniques are rare. In the current study we compared γH2AX quantification by different methods, including automated fluorescence microscopy, flow cytometry as well as immunoblotting. Further, we discussed assay-specific advantages and disadvantages.

Methods: Isolated human peripheral blood mononuclear cells (PBMCs) were exposed to various concentrations of the DNA DSB-inducing, cytostatic drug etoposide for one hour. Subsequently, γH2AX levels were assessed by flow cytometry, immunoblotting and automated microscopy.

Results: Automated fluorescence microscopic foci quantification revealed the lowest limit of detection (LoD) (0.53 µM etoposide). More than 10-fold higher etoposide concentrations were required to distinguish γH2AX values form background signal by fluorescence intensity-based methods like flow cytometry. Immunoblotting showed the poorest LoD of all three techniques.

Conclusions: In contrast to flow cytometry and immunoblotting, automated fluorescence γH2AX foci quantification showed the lowest LoD. This low LoD allows the assessment and follow-up of patients with respective antitumor therapy. Thus, to apply the most suitable γH2AX analysis, specific assay characteristics must be considered.

Keywords: DNA double-strand breaks; γH2AX; immunoassay; microscopy; flow cytometry

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Introduction

Cellular biomolecules are constantly harmed by various endogenous and exogenous substances. In contrast to damaged proteins and lipids undergoing general recycling processes based on degradation and synthesis, altered DNA is repaired by different, lesion specific repair mechanisms throughout lifetime instead (1). One of the most severe types of DNA lesions are DNA double-strand breaks (DSB). After DSB formation, rapid phosphorylation of the core histone variant H2AX at serine 139 is induced in adjacent

chromatin, leading to the formation of a γH2AX focus (2). Initially, γH2AX was detected by two-dimensional gel electrophoresis, but generation of specific antibodies enabled the development of more sensitive γH2AX immunoassays (2-4). Thereby, γH2AX has been established as one of the most sensitive biomarkers for DNA DSB detection offering multiple fields of application (5-8). Apart from basic research, γH2AX has the potential to be used as diagnostic biomarker in clinics, e.g., for monitoring of radio- or chemotherapy, for biodosimetry, for drug development and to study the impact of environmental toxins or the process of aging (5-7).

Within the last two decades, innumerous reports have been published applying γH2AX immunoassays, including immunocytochemical staining followed by microscopic or flow cytometric analysis, immunoblotting and enzyme-linked immunosorbent assay (ELISA). The most common approach is microscopic quantification of γH2AX foci. Here, single cells as well as single foci can be analyzed. In contrast, flow cytometry only allows overall γH2AX fluorescence intensity measurement of individual cells, but it cannot be differentiated whether the signal originated from one bright focus, many small foci or background intensity. Further, immunoblotting or ELISA only enable a general assessment of total γH2AX protein level of the whole sample, but e.g., apoptotic cells showing a pan-nuclear γH2AX expression cannot be distinguished from viable cells and may thereby influence γH2AX quantification (2).

Fluorescent microscopic foci quantification has been described as one of the most sensitive methods for γH2AX assessment, enabling detection of even one single focus (2,9-11). The aim of the current study was to verify this statement by performing automated fluorescent microscopy, as described in detail elsewhere (12-14), and to compare obtained limits of detection (LoD) with different approaches. Therefore, human peripheral blood mononuclear cells (PBMCs) were exposed to different levels of the DNA DSB-inducing cytostatic drug etoposide for one hour. Afterwards, γH2AX levels were quantified by automated microscopy, flow cytometry and immunoblotting. Here we discuss results of the γH2AX assay comparison as well as assay specific advantages and disadvantages.

Methods

Isolation and treatment of human PBMCs

Heparinized blood was obtained from healthy blood donors and PBMCs were isolated by density gradient centrifugation using Biocoll separating solution (Biochrom, Berlin, Germany). Afterwards, PBMCs were washed and suspended to a final density of 1×10^6 PBMCs per ml in Roswell Park Memorial Institute (RPMI) 1640 cell culture medium (Biochrom) containing 10% fetal bovine serum (Pan Biotech, Aidenbach, Germany), 100 U/mL penicillin and 100 µg/mL streptomycin (both Life Technologies GmbH, Darmstadt, Germany). For induction of DNA DSBs cells were transferred into 24-well cell culture plates and exposed to etoposide at a final concentration of either 0, 0.05, 0.1, 0.25, 0.5, 1, 2.5, 5, 10, 25, 50, 100 or 250 µM for 1 h at 37 °C with 7% CO_2.

γH2AX analysis using automated fluorescent microscopy

After etoposide exposure, cells were harvested, washed in phosphate-buffered saline (PBS) and transferred onto silanized glass slides. Subsequently, samples were fixed for 15 min in formaldehyde, permeabilized in 0.1% Triton X-100 and blocked in PBS containing 1% bovine serum albumin (BSA). For γH2AX detection cells, were incubated at room temperature with an anti-phosphohistone H2AX mouse monoclonal IgG primary antibody (Millipore, Schwalbach, Germany; dilution 1:2,000) for 1 h, washed in blocking buffer and additionally stained for 1 h in the dark with a polyclonal goat anti-mouse IgG antibody conjugated to Alexa-Fluor-488 (Life Technologies, Darmstadt, Germany; dilution 1:2,000). Afterwards, slides were washed in PBS and covered with 4',6-diamidino-2-phenylindole (DAPI) containing mounting medium (Medipan, Berlin/Dahlewitz, Germany).

For automated γH2AX immunofluorescence microscopy, slides were inserted into the AKLIDES cell damage system and image acquisition as well as analysis were performed as described in detail previously (12,14). In brief, DAPI-stained nuclei were selected in blue fluorescence channel according to morphological criteria using an objective with 60× magnification. After switch into the green fluorescence channel, images of γH2AX foci were obtained of five different focal planes throughout the nucleus. After investigating a minimum of 300 cells per sample, the mean number of γH2AX foci per cell as well as mean γH2AX fluorescence intensity (MFI) of selected nuclei were determined by the analysis software. Cells showing a pan-nuclear staining were recorded separately and samples containing more than 5% pan-nuclear stained PBMCs were excluded from γH2AX foci quantification.

γH2AX analysis using flow cytometry

Preparation of samples for flow cytometric γH2AX detection was performed similarly to slide preparation with minor adaptations according to the protocol by Redon *et al.* (11). One million etoposide-exposed PBMCs were transferred into a round bottom centrifuge tube and washed in PBS containing 0.5% BSA. After fixation in 1% formaldehyde, cells were incubated in 70% ice cold ethanol over night at 4 °C. Subsequently, samples were washed in PBS containing 0.5% BSA, permeabilized in 0.1% Triton X-100 and blocked in PBS containing 1% BSA. Cell staining was performed at room temperature by applying the γH2AX primary antibody (dilution 1:2,000; 1 h) and additionally the anti-mouse-Alexa-Fluor-488 secondary antibody (dilution 1:500; 1 h).

For flow cytometric analysis, PBMCs were selected according to their forward and side scatter signals using a BD LSRFortessa cell analyzer (BD Biosciences, Mountain View, CA, USA). After measuring a minimum of 20,000 PBMCs, their γH2AX level was quantified by the median fluorescence intensity (MeFI) in arbitrary units (AU) by FlowJo analyzing software (Treestar Inc., Ashland, OR, USA).

γH2AX quantification by immunoblotting

Quantification of H2AX levels by immunoblotting was based on the protocol published by Redon *et al.* with slight modifications (11). In brief, after etoposide exposure, 1×10^6 PBMCs were transferred into 1.5 mL tubes, centrifuged at 4 °C and 2,000 g for 5 min and washed in ice cold PBS containing 10 mM NaF. Afterwards, cell pellets were resuspended in 60 µL hot 1× reducing sodium dodecyl sulfate polyacrylamide (SDS) sample buffer and boiled for 10 min at 95 °C. Subsequently, samples were chilled on ice. For SDS gel electrophoresis, lysates were loaded onto a 12.5% SDS gel, following immunoblotting onto a nitrocellulose membrane. After blocking in 5% nonfat dry milk dissolved in 1× Tris buffered saline solution (TBS) for 1 h, the membrane was incubated overnight with anti-phosphohistone H2AX mouse monoclonal IgG primary antibody (Millipore) diluted 1:2,000 in TBS containing 3% BSA. After washing, membrane was incubated for 1 h with 1:10,000 diluted donkey-anti-mouse HRP-conjugated secondary antibody (Dianova, Hamburg, Germany) and washed again. Enhanced chemiluminescence (ECL) substrate (Thermo-Scientific, Rockford, USA) was applied for protein detection. Afterwards, staining was performed analog for β-actin determination. Subsequently, intensity of individual bands was determined with Kodak Image Station quantification software and quantified γH2AX levels were normalized to the corresponding intensity of the β-actin loading control.

Statistics

Data analysis was performed by GraphPad Prism software version 5.01 (Graph Pad Software, La Jolla, CA). Diagrams display the mean and standard error of the mean (SEM) of five independent experiments. Limit of detection (LoD) was determined based on the values obtain from untreated cells, as sum of the mean and three standard deviations. Afterwards, the corresponding etoposide concentration was calculated by linear interpolation.

Results

Using automated fluorescence microscopy, the mean number of γH2AX foci per cell and mean γH2AX intensity per nucleus were obtained. Representative microscopy images are shown in *Figure 1A*. As also depicted in *Figure 1B*, increasing etoposide levels induced a rise in γH2AX foci formation detectable even at low concentrations. However, PBMCs exposed to concentrations ≥50 µM exhibited enhanced foci overlap and more than 5% of cells showed pan-nuclear γH2AX staining. Therefore, these data points had to be excluded from γH2AX foci quantification and are marked with X in the diagram.

The corresponding dose-response relationship between etoposide concentration and γH2AX MFI obtained by automated fluorescence microscopy is shown in *Figure 1C*. In contrast to microscopic foci quantification of same images γH2AX intensity values exceeded baseline level only at higher etoposide doses. After a linear increase of γH2AX signal at etoposide concentrations between 2.5–50 µM (R^2=0.9862) saturation and enhanced overexposure of γH2AX intensity were observed.

Besides microscopic immunofluorescence analysis, γH2AX intensity of etoposide-exposed PBMCs was also quantified by flow cytometry. Representative histograms and the determined dose-response curve are shown in *Figure 1D,E*. Similar to the intensity analyses by automated microscopy, flow cytometric γH2AX analysis revealed γH2AX levels elevated above background only at much higher etoposide doses compared to foci quantification. At very high drug concentrations, saturation of the γH2AX

signal was observed.

As a third immunological method, we performed immunoblotting to quantify etoposide-induced γH2AX levels, as shown in *Figure 1F,G*. Similar to immunoassays based on γH2AX fluorescence intensity assessment, a low etoposide concentration did not induce a detectable rise in γH2AX levels. A linear increase of γH2AX expression was observed between a dose range of 2.5–50 µM etoposide (R^2=0.9529). PBMCs exposed to etoposide concentrations ≥100 µM had to be excluded from quantification, since the optical density (OD) of the bands exceeded the linear quantification range (indicated with X). But the use of shorter exposure times to avoid overexposure was not sufficient to visualize the weak signals from γH2AX bands of untreated samples.

Additionally, LoD were calculated for each assay as the sum of the mean and three standard deviations obtained from the negative controls. Within diagrams shown in *Figure 1* corresponding LoD is indicated as solid line. Performing interpolation the corresponding etoposide concentration was determined. Whereas γH2AX foci quantification revealed a detectable increase in γH2AX signal at etoposide concentrations ≥0.53 µM compared to untreated samples, much higher etoposide doses were necessary to obtain a positive signal for intensity-based measurements (MFI—microscopy: 6.7 µM; MeFI—flow cytometry: 8.7 µM). Also due to high standard deviations, the poorest detection limit was determined for immunoblotting (15.5 µM etoposide).

For better comparison of γH2AX levels, values obtained by each method were normalized according to untreated controls. These relative γH2AX data of all four dose-response curves are depicted in *Figure 2*. In accordance to the LoD, increasing etoposide concentrations induced the strongest gain of the γH2AX signal when assessed by microscopic γH2AX foci quantification.

Discussion

Assessment of γH2AX has been established as one of the most sensitive methods for DNA DSB detection (2). It offers a variety of potential applications in clinics e.g., for monitoring of anti-cancer therapy, determination of radio- or chemo-sensitivity/resistance, for biodosimetry or as biomarker for multiple age-related diseases (2,5-7). But due to several reasons, like the lack of standardization and insufficient data from various multi-center studies, γH2AX analysis has not yet been used as clinical biomarker in laboratory routine.

Among different assays for γH2AX detection, fluorescent microscopic γH2AX foci determination is reported the most sensitive approach (2,9,11). To evaluate the sensitivity of different immunological methods for γH2AX assessment performed in our laboratory, we compared the LoD of automated fluorescent microscopy, flow cytometry and immunoblotting. Therefore, PBMCs were exposed to increasing concentrations of the DSB-inducing cytostatic drug etoposide for one hour.

For automated γH2AX foci quantification, a LoD of 0.53 µM (0.31 µg/mL) etoposide during an exposure of one hour was calculated. In contrast, a more than 10-fold higher etoposide dose was necessary to obtain a detectable increase in γH2AX levels when determined by intensity measurements using either fluorescent microscopy or flow cytometry. Immunoblotting showed the poorest LoD. Further, the comparison of relative increase of γH2AX values after etoposide exposure confirmed the highest sensitivity for γH2AX foci quantification.

In clinical use, plasma etoposide levels of 2–3 µg/mL could be associated with hematological toxicity and antitumor activity was reported at 1–2 µg/mL (15,16). Peak levels may reach up to 18.5 µg/mL and trough levels were reported around 0.2 µg/mL (17). Thus, these levels are either well above the LoD of etoposide determined in this study with regard to antitumor activity or at least equal to the trough levels. In this context, the automated γH2AX foci quantification appears to be a well suited tool for the assessment and follow-up of DNA damage by etoposide in patients as demonstrated for partial body radiation exposure either (18). In contrast, fluorescence intensity analysis of γH2AX formation by flow cytometry or immunoblotting is not applicable for the assessment of antitumor activity due to the at least 10 times higher LoD.

But foci quantification also has limitations. Although single foci can be detected, an underestimation may occur when nuclei show elevated γH2AX levels where individual foci cannot be accurately separated anymore (19). This is also reflected by the flattening of the dose-response curve at etoposide concentrations ≥50 µM. Additionally, an increasing number of cells showed a pan-nuclear staining. Therefore, these samples were excluded form quantification. In cases of high γH2AX expression, evaluation of mean intensity or foci area seems to be more appropriate. In general, protocols of all discussed methods should be adjusted according to respective sample conditions to allow measurement within linear dynamic range.

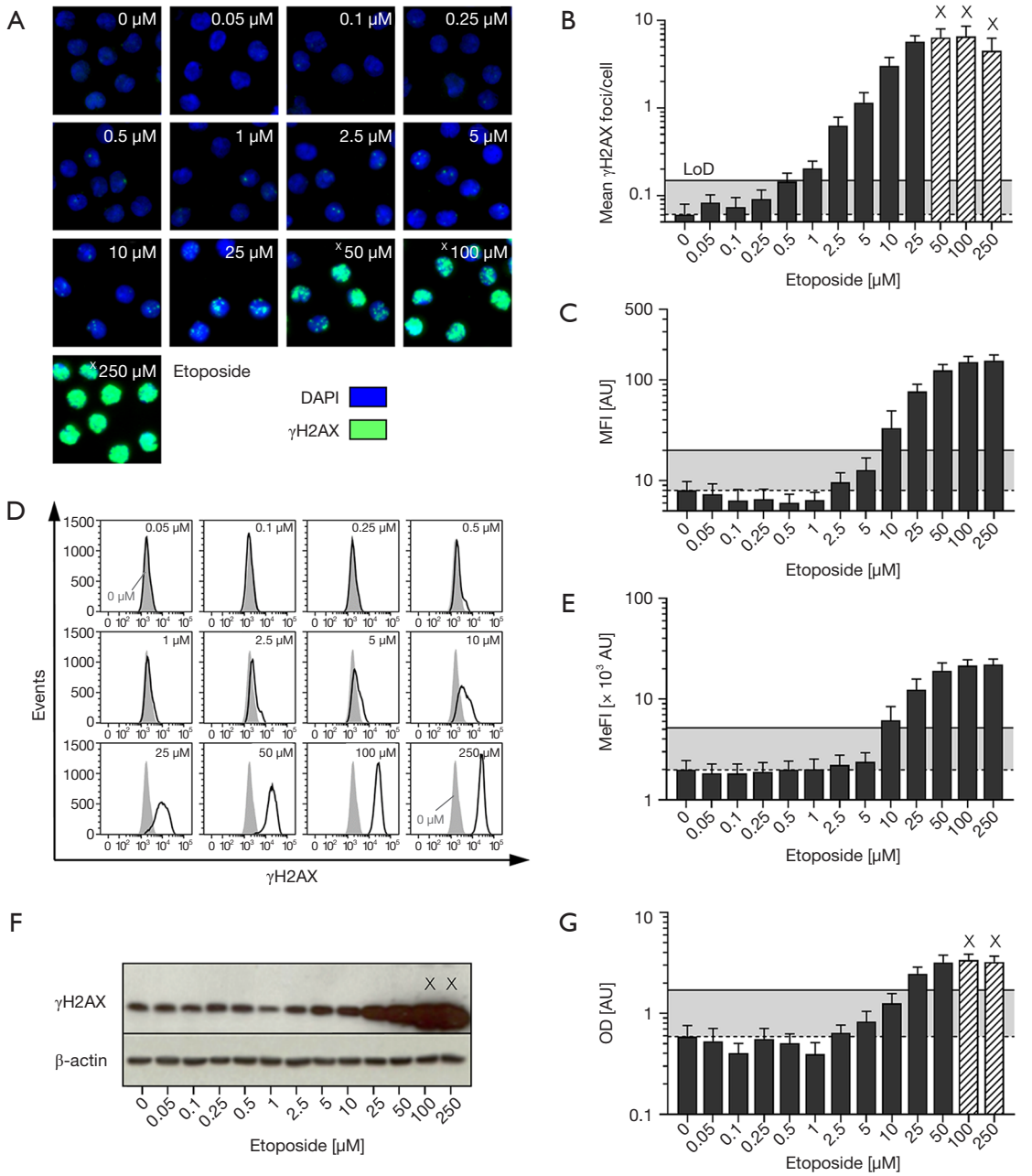

Figure 1 Dose-response curve of etoposide concentration and induced γH2AX level. PBMCs were exposed to indicate etoposide dose for 1 h and γH2cAX levels were quantified by automated fluorescent microscopic foci and intensity analysis, by flow cytometry or immunoblotting. (A) Representative fluorescence microscopy images of DAPI (blue) stained nuclei and γH2AX foci (green); (B) corresponding amount of γH2AX foci/cell and (C) mean fluorescence γH2AX intensity (MFI). Samples with >5% of cells showing a pan-nuclear γH2AX staining were excluded from quantification (indicated with X); (D) representative histograms obtained by γH2AX flow cytometry analysis (gray—untreated control) and (E) corresponding γH2AX intensity values; (F) representative immunoblot depicting γH2AX and β-actin levels obtained from cell lysates; (G) mean intensity values of bands converted into optical density (OD) units, normalized to individual β-actin loading control. Samples exceeding the quantification range are labeled with X. Gray rectangles in diagrams mark the mean γH2AX values of untreated samples (lower, dashed line) and calculated limit of detection (LoD; upper solid line). Bars represent mean ± SEM of five independent experiments. PBMC, peripheral blood mononuclear cell.

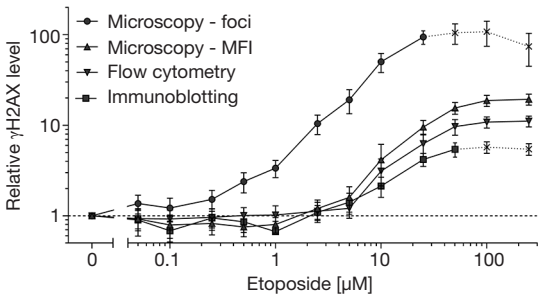

Figure 2 Relative γH2AX signal in relation to etoposide-exposure. Etoposide-induced γH2AX levels obtained by different immunoassays (*Figure 1*) were normalized to corresponding values of untreated cells (indicated with dashed line). Samples above the quantification range are marked with X. Data points represent mean ± SEM of five independent experiments. MFI, mean fluorescence intensity.

As shown, γH2AX foci quantification is the best approach when cells with low γH2AX levels are investigated. Besides its high sensitivity, γH2AX fluorescent microscopy furthermore allows assessment of additional parameters. This pertains to cell or foci intensity, size and shape, when combined with image acquisition and image processing. Although foci morphology is often not considered, Watters *et al.* observed different γH2AX patterns in mouse embryonic fibroblasts depending on the type of genotoxic treatment (20). Further, co-localization studies can be performed applying additional staining techniques, like different DNA damage proteins or chromatin markers (21-25). Besides analysis of single cells, fluorescent microscopy also enables histological tissue examinations of e.g., biopsies or hair follicles (26-29). Especially for research purposes, time-lapse microscopy of γH2AX formation and other repair foci can be performed in living cells by use of fluorescent-tagged proteins or fluorescent antibody fragments (nanobodies) (30-33).

In comparison to fluorescent microscopy, flow cytometry is more suitable for cells expressing more than one γH2AX focus (11) and depending on the intensity level signal strength and dynamic range can be further adjusted by settings of photomultiplier. As a high-throughput technology, flow cytometry enables measurement of several hundred to thousand cells per second and can be combined with additional labelling for DNA content or surface markers. Thereby, it facilitates cell cycle analysis as well as assessment of multiple and even small cell subpopulations. But in contrast to fluorescent microscopy, background intensity derived from autofluorescence or staining variations affect results more strongly and cannot be separated from weak γH2AX foci signals (2). To determine background levels, appropriate staining controls need to be included. Further, new approaches combining analytical characteristics of microscopy and flow cytometry, such as microscope-based laser scanning cytometry or imaging flow cytometry have also been used for γH2AX evaluation (34-37).

In contrast to single cell analysis, immunoblotting- or ELISA-based methods are less sensitive and results are not only dependent on the γH2AX level per cell but are also affected by total cell concentration. Further, apoptotic cells showing pan-nuclear γH2AX expression as well as different subpopulations cannot be distinguished. The ELISA can be performed as classical sandwich ELISA with immobilized capturing antibodies against whole H2A or H2AX molecules and γH2AX-specific detection antibodies. Additionally, modifications have been described utilizing direct plate coating with either cell lysate or whole cells, following fixation and permeabilization, and subsequent detection of γH2AX (38-40). A novel sandwich ELISA developed by Ji *et al.* does not only determine the level of γH2AX but also of total H2AX to allow quantification of relative γH2AX levels (41).

Immunoblotting experiments of our study were performed with chemiluminescence-based protein determination using ELC-substrate for detection of horseradish peroxidase. Cells treated with high concentrations of etoposide showed an overexposed γH2AX expression, which was above the quantification limit. But when the exposure time was reduced, bands of untreated samples could not be detected anymore. To improve this method, it needs to be investigated whether the substitution of enzyme-coupled secondary antibodies with fluorescence-labeled antibodies can increase the linear quantification range (42). For adjustment of cell concentration, lysates must be aligned according to the cell number or protein concentration. To confirm equal protein loading and to allow normalization of γH2AX levels across the gel, detection of constitutively expressed housekeeping gens, such as β-actin or lamin B1, and even better, total level of H2AX should be included (2,10). In contrast to assays requiring prompt staining of treated cells, lysate-based γH2AX detection by immunoblotting and ELISA has the advantage that a variety of samples can be prepared easily and stored over a certain period of time to allow subsequent and repeated analyses of γH2AX and additional protein levels.

In conclusion, we confirmed that microscopic fluorescent γH2AX foci quantification represents the most sensitive

immunoassay for γH2AX determination and is most suitable when γH2AX expression levels are low. Especially, the advancement in automated microscopy may facilitate translation of γH2AX foci analysis into clinical routine since the unsurpassed detection limit allows the assessment and follow-up of antitumor activity of chemotherapeutic substances such as etoposide. Further, improved assay protocols, such as novel ELISA tests as described by Ji *et al.* (41) should be considered due to their applicability for clinical diagnostics. In general, advantages and disadvantages of each technique should be taken into account to choose the most appropriate assay for the specific problem being investigated.

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Footnote

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