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Review

Potential application of γ-H2AX as a biodosimetry tool for radiation triage

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ABSTRACT

Radiation triage and biological dosimetry are two initial steps in the medical management of exposed individuals following radiological accidents. Well established biodosimetry methods such as the dicentric (DC) assay, micronucleus (MN) assay, and fluorescence in-situ hybridization (FISH) translocation assay (for residual damage) have been used for this purpose for several decades. Recent advances in scoring methodology and networking among established laboratories have increased triage capacity; however, these methods still have limitations in analysing large sample numbers, particularly because of the ~ 48 h minimum culture time required prior to analysis. Hence, there is a need for simple, and high throughput markers to identify exposed individuals in case of radiological/nuclear emergencies. In recent years, a few markers were identified, one being phosphorylated histone 2AX (γ-H2AX), which measured a nuclear foci or nuclear staining intensity that was found to be suitable for triage. Measurement of γ-H2AX foci formed at and around the sites of DNA double-strand breaks is a rapid and sensitive biodosimetry method which does not require culturing and is thus promising for the analysis of a large number of samples. In this review, we have summarized the recent developments of γ-H2AX assay in radiation triage and biodosimetry, focusing chiefly on: i) the importance of baseline frequency and reported values among different laboratories, ii) the influence of known and unknown variables on dose estimation, iii) quality assurance such as inter-laboratory comparison between scorers and scoring methods, and iv) current limitations and potential for future development.

Contents

Abbreviations: DC, dicentric chromosomes; MN, micronucleus; CTA, computed tomography angiography; 3D-CRT, three-dimensional conformal radiotherapy; IMRT, intensity-modulated radiation therapy.

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1. Introduction

Since the discovery of X-rays in 1895 by Wilhelm Conrad Röntgen and radioactivity in 1896 by Henri Becquerel, industries making use of radiation and radioactive material such as medicine, agriculture, and the nuclear power industry have been expanding. While the benefits to society are obvious, there is growing concern regarding the associated occupational radiation exposure for those individuals working with the various technologies. Protection of workers, assessment of normal tissue toxicity in patients following radiotherapy, and estimates of occurrence of cancer or formation of cataract in patients following multiple computed tomographic (CT) scans are all challenging tasks related to radiation exposure [1–5]. In addition, the incidence of nuclear accidents, such as the Mayapuri incident, Chernobyl, and Fukushima, have raised public concern around the likelihood and magnitude of exposure during radiation accidents [6]. Knowledge regarding the absorbed dose of ionizing radiation received by an exposed person during these events is a vital step toward radiation triage and medical management, to support adequate and timely clinical intervention. Towards this goal, biological dosimetry methods such as the dicentric assay (DC), micronucleus assay (MN), fluorescence in-situ hybridization (FISH) translocation assay, premature chromosome condensation (PCC), and lymphocyte kinetic assay have been established and used in real-life exposure cases during the past several decades [7–10].

In recent years, several studies focused on the development of these methods for emergency response and have led to improved speed, quality, and capacity [8,11–14]. However, the established methods still have limitations associated with their usage during large scale accidents, most pressingly the time it takes to culture the blood samples and requirements of highly skilled cytogeneticists [15]. The culture time for analysis using the PCC fusion method has been reduced to 2 h, but this is technically difficult, expensive, and best for high doses [16]. A number of newly developed markers of radiation exposure including the gamma H2AX (γ-H2AX) assay, gene, and microRNA (miRNA) expression have shown promising results for radiation triage, particularly those which can exploit high throughput technologies for the analysis of a large number of samples [17–21].

Apart from radiation biodosimetry, the γ-H2AX assay has been employed in a variety of applications, such as basic research on DNA repair pathways, radiation biology, drug development, genotoxicity evaluation, cancer, diagnostic radiology, and radiotherapy protocol improvement. A number of recent review articles focus on the development and various applications of the γ-H2AX assay in these diverse areas [22–29]. However, in this review, we focus on recent developments regarding the γ-H2AX assay in radiation triage and biodosimetry, focusing chiefly on: i) the importance of baseline frequency and reported values among different laboratories, ii) the influence of known and unknown variables on dose estimation, iii) quality assurance, such as inter-laboratory comparison between scorers and scoring methods, and iv) current limitations and potential for future development.

2. Methodology

The literature search for this review was performed using the "PubMed" database (National Library of Medicine, National Institutes of Health, Bethesda, MD, USA). The search strategy was based on terms such as "γ-H2AX", "applications of γ-H2AX", "γ-H2AX and CT imaging", "γ-H2AX and interventional procedures", "γ-H2AX and radiotherapy", "γ-H2AX foci counting software", "baseline frequency of γ-H2AX foci", "γ-H2AX dose-response", "γ-H2AX inter-laboratory comparisons", and limited to studies using human blood samples. Based on the obtained results, the triage and routine biodosimetry applications of the assay and associated recent advancements have been classified in terms of their use and relevance to different radiation exposures scenarios.

3. Formation of γ-H2AX foci

In eukaryotes, deoxyribonucleic acid (DNA) is packed into nucleosomes that form the highly organized structure of chromatin. Each nucleosome contains 145 base pairs of nucleotides and an octamer of histone molecules (two molecules each of H2A, H2B, H3, and H4). Histone molecules in the nucleosomes undergo post-translational modification such as acetylation, methylation, ubiquitinylation, and phosphorylation. Consequently, these modifications of histones affect chromosome condensation, regulation of gene expression, and DNA repair mechanisms. Exposure to genotoxic agents, such as ionizing radiation, can cause a DNA double-stranded break (DSB).

A modification that occurs at this break site is phosphorylation of serine 139 (γ-phosphorylation) of H2AX (H2A histone family X), a highly conserved variant of the H2A [30]. The phosphorylated form of H2AX accumulates at the site of the DSB to initiate the repair process. At DNA DSB sites, the γ-H2AX protein is phosphorylated within 2–3 min of the initial lesion being formed by PI3 kinases such as ATM, DNA-PKc, and ATR [31]. Several molecules of γ-H2AX are phosphorylated and thus recruit the proteins that are involved in the DSB repair [MRN complex (MRE11/RAD50/NBS1), RNF8, BRCA1, 53BP1, *etc.*]. These proteins form a megabase chromatin domain and appear as repair foci that can be visualized using an antibody against the phosphorylated γ-H2AX [32–34]. Formation of γ-H2AX foci occurs due to the induction of strand breaks or a stalled replication fork and is evolutionarily conserved from lower (*Xenopus laevis*, *Drosophila melanogaster*, and *Saccharomyces cerevisiae*) to higher organisms (*Homo sapiens* and *Macaca mulatta*) and also in plants [35,36]. Measurement of γ-H2AX foci formation can be carried out in diverse cell types and tissue sections (cell lines, blood lymphocytes, splenocytes, bone marrow cells, buccal cells, plucked hairs, and cancerous tissue sections) by using microscopy and cell suspension flow cytometry [37]. The greatly reduced time for

analysis coupled with higher sensitivity when compared to DC and MN assays makes the γ-H2AX assay an attractive choice for various applications including radiation biodosimetry and related emergency triage [38].

4. Methods used to measure γ-H2AX levels

Four methods are commonly employed to measure the levels of phosphorylated γ-H2AX in cells exposed to ionizing radiation: quantitative measurement by microscopy, either automated or by eye, use of standard western blot techniques, qualitative analysis by fluorescence intensity using flow cytometry, and enzyme-linked immunosorbent assay (ELISA) (Fig. 1) [39–41].

4.1. Microscopic scoring of γ-H2AX foci

To observe the γ-H2AX foci using microscopy, the cells need to be placed on slides, fixed with paraformaldehyde/methanol, permeabilized using Triton X-100, blocked with BSA, and stained with both primary and secondary antibodies labeled with fluorescence molecules. Immunofluorescence stained phosphorylated H2AX foci can be visualized using either confocal- or epi-fluorescence microscopy at 60 to 100X magnification. Because of its accuracy and sensitivity, microscopic observation of γ-H2AX foci is the most commonly used method, as evidenced by the number of recent publications (Fig. 2) [42]. Confocal microscopy has an advantage over epi-fluorescence. Specifically, confocal microscopy has more accurate scoring of γ-H2AX foci thanks to improved discrimination of foci located above each other on different optical planes. In both cases, the sensitivity of microscopy methods is high and the minimum and maximum detectable doses are in the range of 0.02 and 4 Gy, respectively, for acute, low LET radiations [43,44]. However, the minimum and maximum detectable doses depend on many factors, such as the exact time since exposure, availability of pre-exposure samples from the same individual, and baseline levels determined in those samples. Furthermore, the specificity of microscopy method can be increased by measuring the colocalization of the γ-H2AX and 53BP1 foci [45]. Microscopic scoring might facilitate discrimination of the nature of exposure (>2 Gy); i.e whole-body exposure from that of partial body exposure, based on the distribution of γ-H2AX foci observed in the exposed cells [46]. Nonetheless, overlapping of the foci at higher doses (beyond 4 Gy), and variation between scorers are the key limitations of microscopy [43].

4.2. Measuring γ-H2AX protein using western blotting

Western blotting is a widely used molecular technique for the analysis of proteins. Phosphorylated γ-H2AX protein in cell lysates can be measured using western blotting and can then be related to the radiation absorbed dose. However, western blotting has a lower sensitivity than microscopic methods. Moreover, the time required for western blotting is longer compared to flow cytometric and microscopic analyses. These factors coupled with the fact that quantification comes with significant uncertainties, make this type of assay unattractive for radiation triage [39].

4.3. Measuring γ-H2AX fluorescence intensity using flow cytometry

Flow cytometry is a technique which is commonly used for the analysis of cells, molecules, and particles suspended in a fluid. Measurement of the γ-H2AX immunofluorescence with a flow cytometer is an alternative to microscopy; its key advantages are that greater numbers of samples/cells can be analyzed in a shorter time and that it is possible to identify cell cycle-dependent expression of the γ-H2AX (Fig. 3). Recent advances in terms of

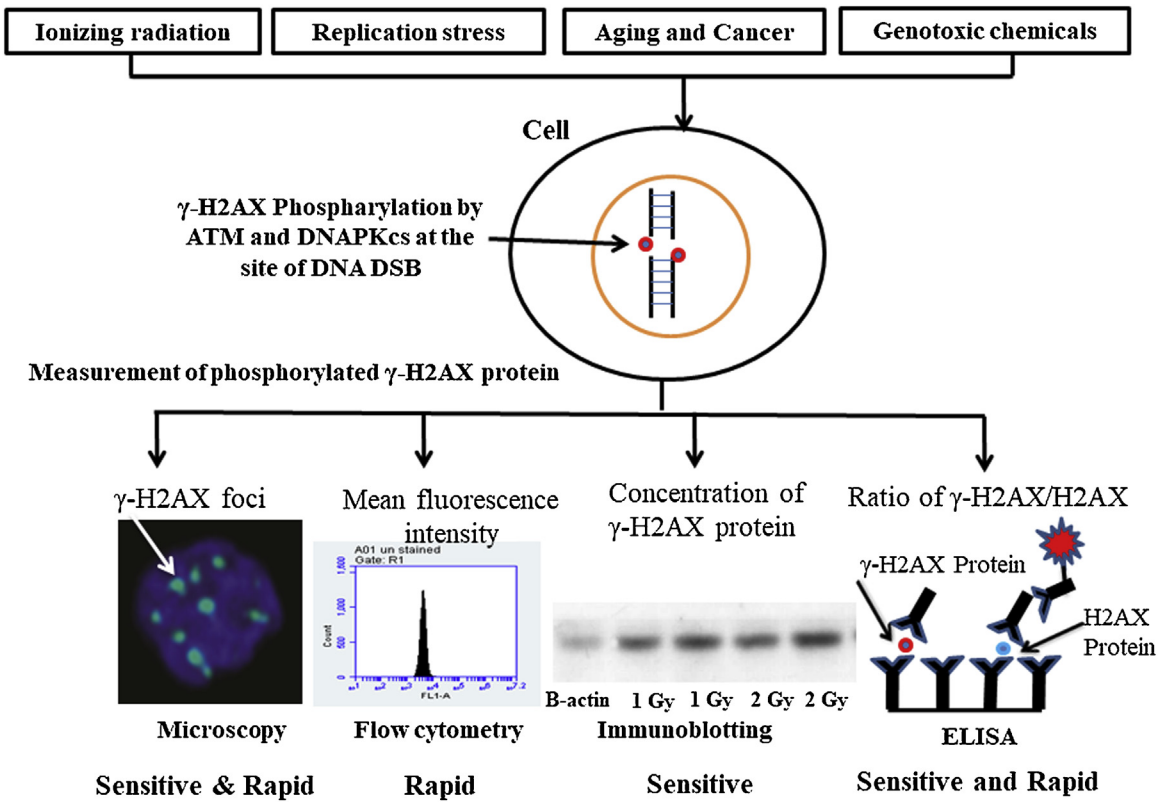

Fig. 1. Sources of damage to DNA, the formation of γ-H2AX foci, and methods to measure the phosphorylated γ-H2AX (microscopy, flow cytometry, western blotting, and ELISA) are depicted.

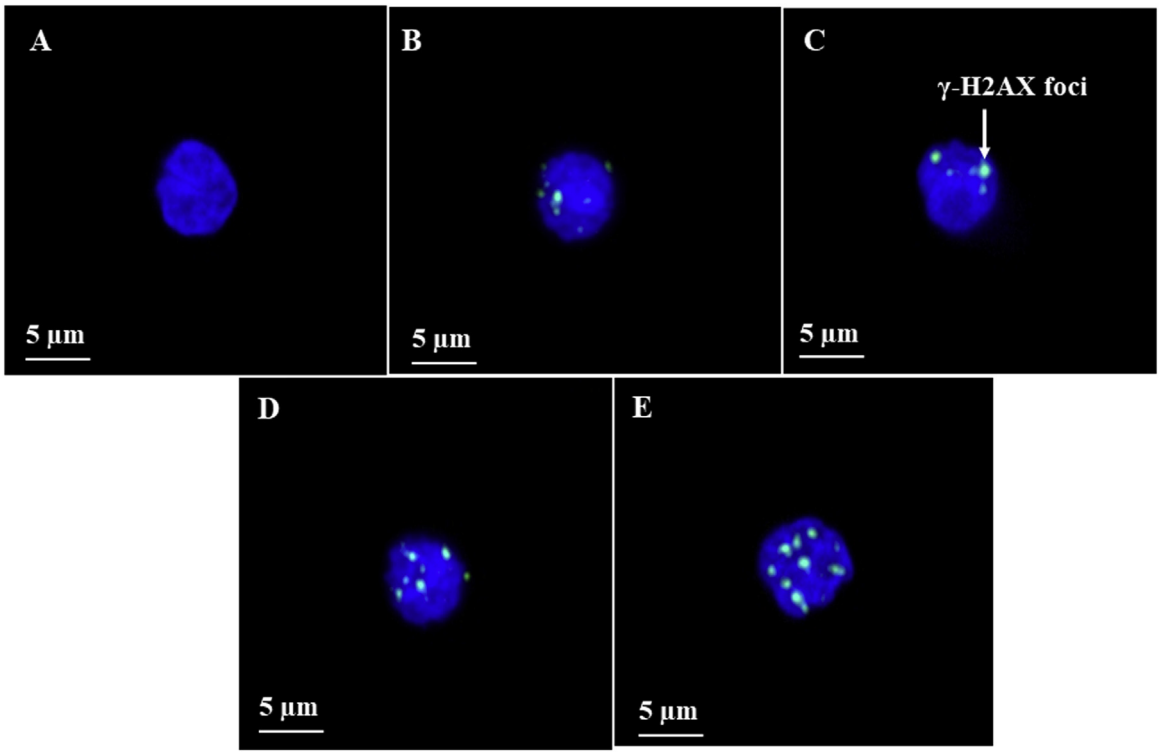

Fig. 2. Microscopic images of γ-H2AX foci obtained from lymphocytes exposed to different doses of 6 MV linear accelerator X-rays and incubated for 0.5 h at 37° C. **(A)** Control, **(B)** 0.1 Gy, **(C)** 0.5 Gy, **(D)** 1 Gy, and **(E)** 2 Gy. DAPI stained nucleus (blue color).

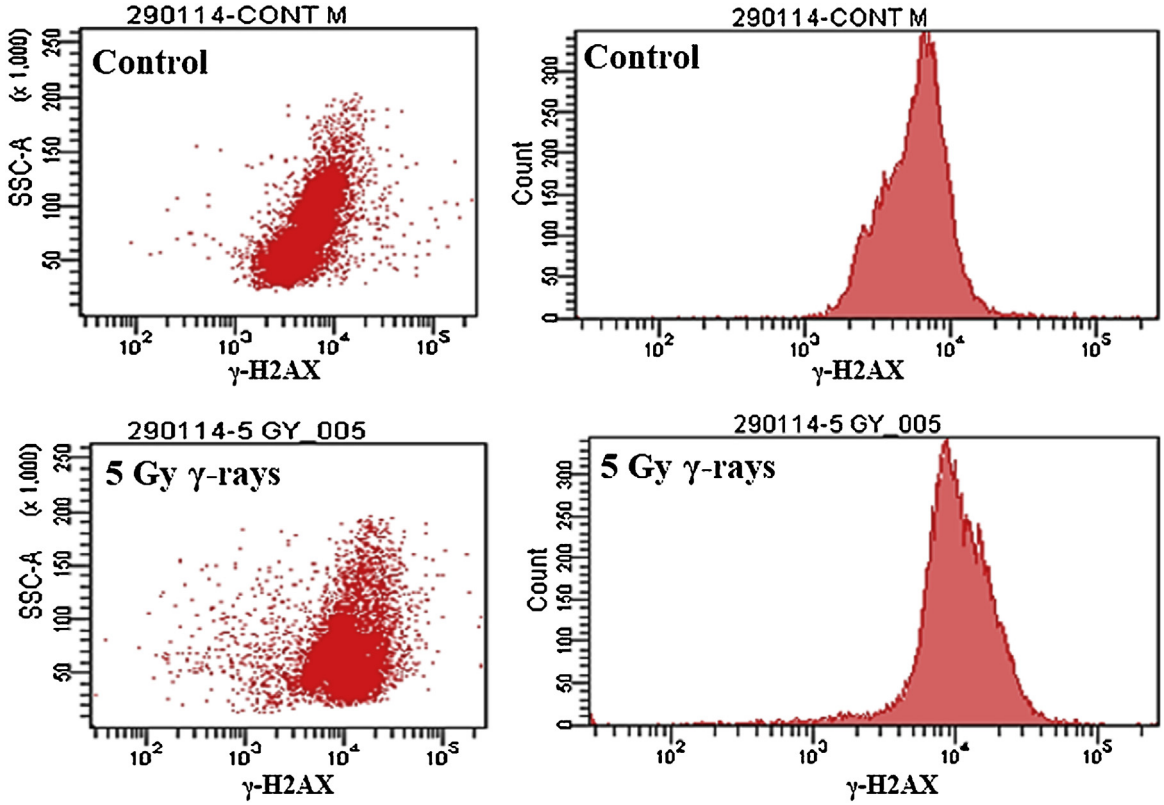

Fig. 3. Flow cytometric analyses of γ-H2AX fluorescence intensity in blood lymphocytes irradiated with 5 Gy ^{60}Co γ-rays and incubated for 0.5 h at 37° C. 10,000 events were recorded from each sample using flow cytometry. Dot plots (**x-axis** = γ-H2AX fluorescence, **y-axis** = Side Scatter-A) and histograms (**x-axis** = γ-H2AX fluorescence, **y-axis** = number of counts) show an increase in γ-H2AX fluorescence in 5 Gy γ-rays).

γ-H2AX immunofluorescence include the technique of imaging flow cytometry (the combination of microscopy and flow cytometry) and the development of immunofluorescence based portable devices [47]. Portable devices might eventually be suitable for deployment to the sites of radiation emergencies, something which is not currently possible with existing, well-validated, cytogenetic techniques [48,49]. However, the sensitivity of flow cytometry is lower than microscopy because flow cytometry looks at the total mean fluorescence instead of actual foci counts. This leads to an approximate minimum detectable dose of 0.25 Gy [50,51].

4.4. Measuring γ-H2AX using ELISA

Radiation-induced γ-H2AX can be measured using ELISA. ELISA measures the absolute amounts of both phosphorylated and total H2AX, which determines the percentage of γ-H2AX. The normalized value of γ-H2AX represents the amount of DNA damage caused by ionizing radiation. ELISA is a sensitive, rapid, and high throughput method. The major advantage of the ELISA-based estimation of γ-H2AX is the use of less instrumentation and less expertise required to analyse DNA damage [41,52]. Further studies are needed to evaluate the potential of ELISA for radiation triage in case of large radiological accidents.

5. γ-H2AX as a biomarker of radiation exposure

The emergence of the γ-H2AX assay as a potential marker of radiation exposure in recent years is due to its inherent advantages such as speed, sensitivity, ability to detect a range of doses, and the potential to perform the assay in lymphocytes, splenocytes, buccal cells, bone marrow cells, skin, and plucked hairs. Fig. 4 represents various sources of radiation exposure and samples that can be used for the assay, together with the potential application of the γ-H2AX assay for triage and biodosimetry.

5.1. γ-H2AX foci frequency in interventional radiology patients

The increasing usage of X-ray based imaging modalities in diagnosis and treatment has contributed significantly to public radiation exposures, which has, in turn, raised concern amongst the public and regulatory authorities regarding the increased risks of developing stochastic effects [53]. Given this, there is current interest in techniques to monitor the biological effects of low dose exposures for patients and health professionals working in imaging facilities. Among the radiation-based imaging modalities, interventional radiological procedures lead to the highest dose (depending on the procedure duration, dose varies between 0.4–8 Gy) for both interventional radiologists and patients [114]. Established biomarkers (DC and MN) are not well suited to measure the very low doses received during X-ray based imaging [7].

The level of γ-H2AX foci in pediatric patients (mean age 0.75 years) who underwent a cardiac catheterization procedure indicated an effective dose range of 0.5–53.4 mSv [54]. Patients with an age range from 23–88 years (n = 19), who underwent different angiographic procedures, showed increased frequencies of γ-H2AX foci, ranging between 0.3–1.5 foci/cell with foci frequency correlated with the dose area product (DAP) [55]. It has been reported that the γ-H2AX levels were higher in the blood samples of the patients who underwent interventional radiological procedures when compared to pre-exposure [56,57]. The levels of γ-H2AX foci in health workers (physicians and allied staff involved in radiological imaging procedures) were also found to be higher when compared to the baseline frequency obtained from the

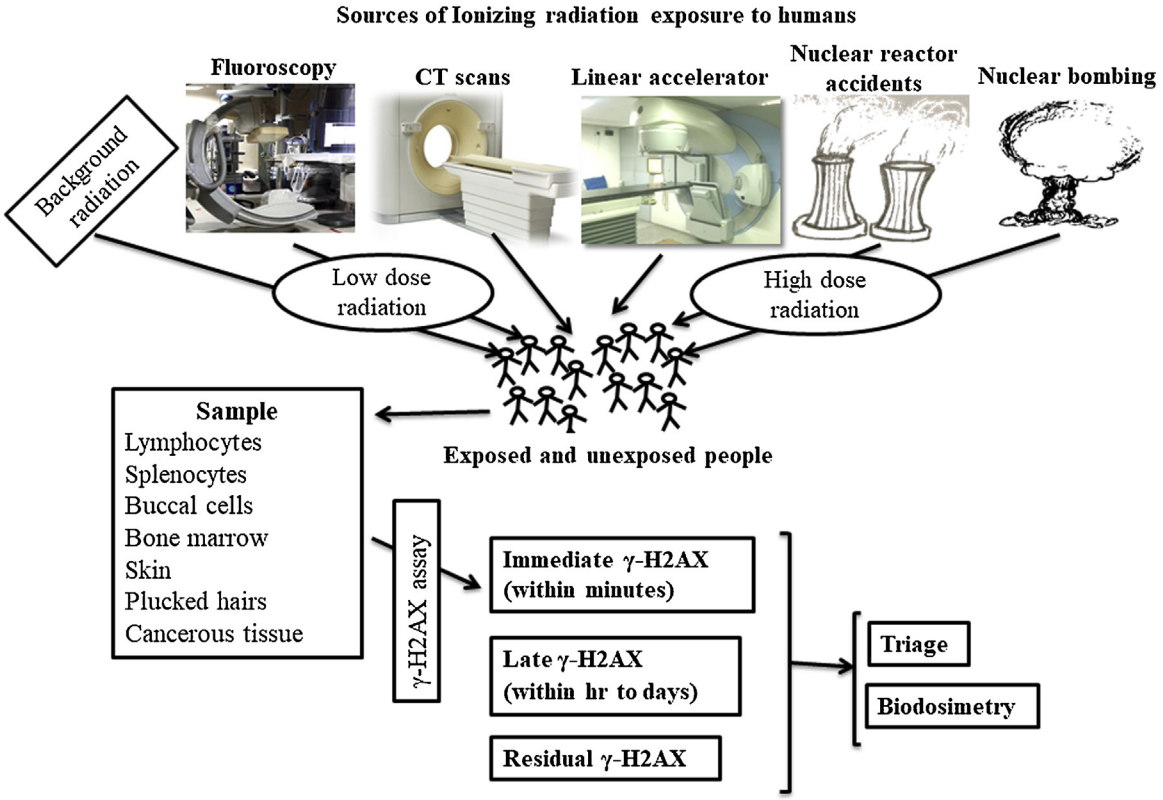

Fig. 4. Overview of the application of the γ-H2AX foci assay in radiation biodosimetry and triage.

healthy volunteers drawn from the same population [42]. Due to the sensitive nature of the γ-H2AX assay, it is an attractive choice for measuring DNA damage and can be related to the absorbed dose received during radiological procedures.

5.2. γ-H2AX foci frequency in CT imaging patients

CT is an imaging modality that allows the three-dimensional structure of organs or regions of interest in the body to be viewed. CT imaging delivers radiation doses in the range of 2–10 mGy to the patient. DNA damage caused by these low levels of ionizing radiation was detectable using the γ-H2AX foci assay [58,59].

The formation of γ-H2AX foci was observed in blood lymphocytes of patients who underwent CT examinations of the abdomen and/or thorax with and without contrast agents. The frequencies of γ-H2AX foci were found to increase linearly with the dose-length product (DLP Gy/cm) after CT procedure doses ranging from 500–1500 mGy/cm. A dose-dependent increase in γ-H2AX foci was also observed in the blood lymphocytes of young children (3–21 months) who underwent CT examinations (blood doses 0.22–1.22 mGy) [60].

The γ-H2AX assay was used to investigate the amount of DNA DSB in patient lymphocytes caused by X-ray contrast agents used in CT. The results indicate that the contrast agents combined with low diagnostic radiation doses did not show an increase in γ-H2AX foci, whereas higher doses caused significant increase in the γ-H2AX foci [61,62]. However, the consequences of using a contrast agent, in terms of overall radiation risk, are likely to be minor. The γ-H2AX assay yields after different CT procedures (helical and sequential coronary computed tomography angiography (CTA)) show good correlation with DLP and the yield of γ-H2AX foci was significantly lower after sequential CTA when compared to the original CTA [63]. It has also been reported that there was a significant increase in the levels of γ-H2AX intensity in patients who underwent CT and PET/CT [64,65]. The summary of these studies reveals that the γ-H2AX assay can be used as a potential marker to study the levels of DNA damage after CT procedures.

5.3. γ-H2AX foci frequency in radiotherapy patients

The γ-H2AX assay has been employed to predict the patient's response to therapy and monitor normal tissue toxicity [66]. The energy of radiation used for radiotherapy is in the mega electron volt (MeV) range, which helps to achieve sufficient tissue penetration to target deep-seated volumes. Nevertheless, not only the cancer cells but also surrounding normal tissue receives radiation, which leads to normal tissue toxicity and long-term effects. This issue is a major challenge in radiotherapy.

To monitor normal tissue toxicity and measure the radiosensitivity in patients who underwent radiotherapy, conventional methods such as clonogenic ability, accumulation of cells at G_0/G_2, and chromosomal aberration assays were used [67–69]. The potential of the γ-H2AX marker for predicting normal tissue toxicity has been demonstrated in breast cancer patients (n = 57) who underwent therapy. In this case, cells from the patients with acute skin reactions (grade 3) showed significantly increased γ-H2AX foci number compared to patients with normal skin reactions (grade 1) [70]. Another study in breast cancer patients treated with intensity-modulated radiation therapy (IMRT) or three-dimensional conformal radiotherapy (3D-CRT) found two-fold increases in γ-H2AX foci from IMRT as compared to 3D-CRT [71]. Sak et al., (2010) reviewed the importance of γ-H2AX foci measurement for a combination of drugs and radiation in monitoring patient responses to radiotherapy [72]. We have also measured the levels of γ-H2AX foci and quantified the absorbed dose in patients (n = 20) who underwent partial body radiotherapy. The results suggest that the assay can be used to estimate the radiation absorbed dose [21].

The kinetic profiles of γ-H2AX, 53BP1, and BRCA1 foci in various cell lines with known DNA repair disorders and mouse models show that the measurement of radiosensitivity is 87% in agreement with the standard colony survival assay [73,74]. Furthermore, the measurement of both γ-H2AX foci and chromosomal damages in patients who underwent partial and total body radiotherapy showed that the γ-H2AX measurement is a sensitive and rapid measure of the absorbed radiation dose [75]. For routine analysis, the protocols need to be standardized and should include analyses of known samples to assess variability and deviation in γ-H2AX foci scoring.

5.4. γ-H2AX foci frequency in occupational workers/accidental exposures to radiation

The potential of the γ-H2AX assay for the biomonitoring of individuals in radiological emergencies has been demonstrated extensively. Elevated levels of γ-H2AX foci were observed in two individuals who were exposed to γ-rays in an accidental exposure [76]. Flow cytometric measurement of γ-H2AX fluorescence intensity in Chernobyl clean-up workers (exposed 24–27 years ago) and shelter workers (who perform work activities at the zone of high radiation risk) revealed that the shelter workers had significantly higher γ-H2AX fluorescence intensity when compared to the clean-up workers (0.70 ± 0.93 *versus* 0.51 ± 0.27, respectively). Further subgroup analysis of the shelter workers based on their registered doses (stratified as 100–250 mSv or 250–1000 mSv), showed higher γ-H2AX fluorescence at higher doses. This study indicates that the γ-H2AX assay can be used as a diagnostic tool for low dose protracted exposures [77]. An increased level of γ-H2AX foci frequency was observed in health professionals (0.066 ± 0.005 foci/cell) working in a medical radiation facility when compared to the baseline frequency obtained from healthy volunteers (0.042 ± 0.001 foci/cell) [42].

5.5. Animal studies to evaluate the γ-H2AX assay

To evaluate the reliability of the *in vitro* γ-H2AX assay for accurate biodosimetry applications, animal studies have been performed in Rhesus Macaque and Swine models. Redon et al., (2010) evaluated the γ-H2AX assay in lymphocytes of total body irradiated non-human primate Rhesus Macaques exposed to 1–8.5 Gy of ^{60}Co γ-rays. The residual γ-H2AX foci were proportional to initial doses and a significant response was observed at 1 day (after 1 Gy), 4 days (after 3.5 and 6.5 Gy), and 14 days (after 8.5 Gy) in lymphocytes and at 1 day (after 1 Gy), 2 days (after 3.5 and 6.5 Gy), and 9 days (after 8.5 Gy) in plucked hairs [78]. Moroni et al., (2013) evaluated the γ-H2AX assay in a swine model after irradiation with both ^{137}Cs and ^{60}Co sources. Similar to human primates, a significant response in γ-H2AX foci was observed after 3 days (1.8 Gy ^{60}Co γ-rays) and 1 week (3.8 or 5 Gy of ^{60}Co γ-rays) in swine models. Both Rhesus Macaque and swine *in vivo* models can be used for validation of γ-H2AX dose-response in human lymphocytes after exposure to acute doses of γ-rays [79].

6. Recent developments in the use of the γ-H2AX assay for biodosimetry and triage

Radiation biodosimetry and the associated plans for using these techniques as secondary triage (following clinical signs and symptoms) are crucial during large scale nuclear accidents.

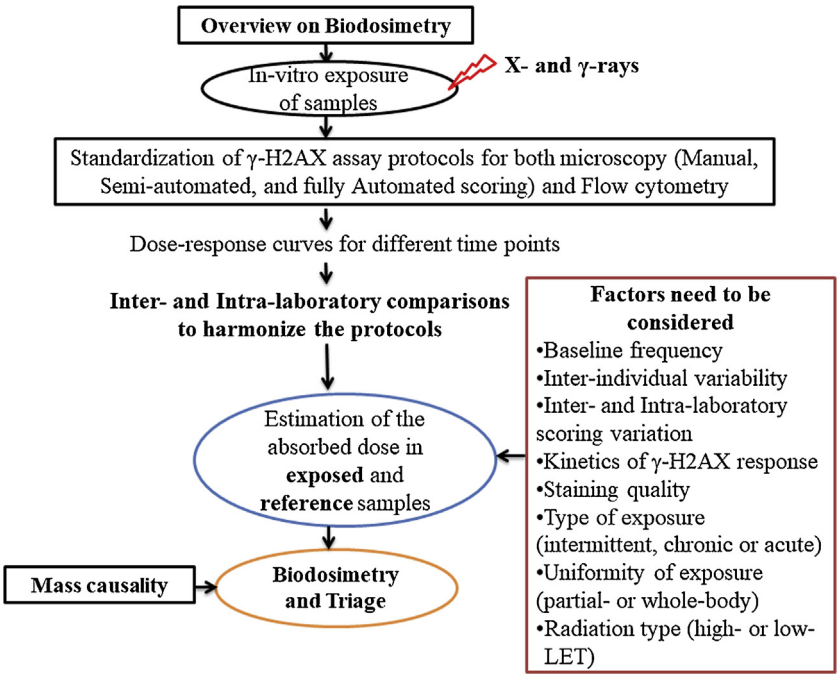

Fig. 5. Schematic representation of the steps involved in biodosimetry and triage using the γ-H2AX assay.

Extensive work on γ-H2AX suggests that it has the potential for use in triage when compared to existing markers. Here we have summarized recent developments such as the importance of adequate knowledge regarding population-specific baseline frequencies, dose-response relationships, automation in the scoring of γ-H2AX foci, rapid and field-level application devices, inter-laboratory comparisons, and limitations of the γ-H2AX assay as a potential biodosimeter and triage tool. Fig. 5 shows an overview of the methodology for biodosimetry using the γ-H2AX assay.

6.1. The basal level of γ-H2AX foci frequency

Knowledge regarding the population-specific expected baseline frequency of γ-H2AX foci and the variables that influence foci levels is extremely important for reliable dosimetry/triage applications. Inter-individual variability has been observed in the baseline frequency of γ-H2AX foci in blood lymphocytes of healthy individuals [80]. Basal levels of γ-H2AX foci have been reported to range from 0.001–0.3/cell, and the baseline levels are reported to be influenced by various factors (biological and experimental) (Table 1). It was reported that background radiation exposure, metabolic stress, various disease conditions, age, smoking, oxidative stress, inflammation, heat, genetic factors, race, gender, ethnicity, and alcohol consumption alter baseline frequencies of H2AX on an individual level [81–87]. Interestingly, a recent study of γ-H2AX foci levels in individuals living in a high background radiation (1.0–45.0 mGy/year) area in Kerala, India, reported no increase in DNA DSB [88]. A similar study from a different area of high background natural radiation (Mamuju, West, Sulawesi, Indonesia) showed higher γ-H2AX foci compared to control, but the increase was not statistically significant [89]. These studies highlight the importance of the baseline frequency of γ-H2AX foci. A summary of recent publications in which the baseline γ-H2AX foci has been measured is provided in Table 1.

6.2. Automation for the scoring of γ-H2AX foci using microscopy

Manual scoring of γ-H2AX foci is more accurate and preferred in many laboratories to find the exact number of foci and relate it to the dose accurately. However, during large scale nuclear accidents,

Table 1
Baseline frequency of γ-H2AX foci in the blood lymphocytes of healthy volunteers.

Study No	Mean γ-H2AX foci/cell	Number of samples	Age	Methods used to measure γ-H2AX foci	Publication
1	0.095 ± 0.009 (HLNRA) 0.084 ± 0.004 (NLNRA) (Mean ± SE)	Total (n = 91) (HLNRA) (n = 61) (NLNRA) (n = 30)	36 ± 7 y (HLNRA) 35 ± 6 y (NLNRA)	Epi-fluorescence microscopy (Carl Zeiss)	[88] Jain et al., (2016)
2	20.09 ± 2.01 24.9 ± 3.41 (Mean ± SEM)	Healthy volunteers (n = 94)	20–30 y 31–50 y	Rapid Automated Biodosimetry Tool (RABiT)	[87] Sharma et al., (2015) #
3	0.09 ± 0.05 0.07 ± 0.05 (Mean ± SD)	Healthy volunteers French (n = 21) Cuban (n = 6)	–	Epi-fluorescence microscope (Olympus)	[90] Roch-Lefevre et al., (2010)
4	0.13 (0.00–0.50) 0.31 (0.00–2.24) Mean (Range)	High Natural Background Area (n = 37) Control area (n = 8)	14–70 y	Fluorescence microscope (Nikon)	[89] Basri et al., (2017)
5	0.042 ± 0.001 (Mean ± SE)	Healthy volunteers (n = 130)	18–77 y	Epi-fluorescence microscopy (Olympus)	[42] Raavi et al., (2016)

HLNRA=High-Level Natural Radiation Area; **NLNRA**=Normal-Level Natural Radiation Area; **y**=Years; **SEM**=Standard Error of Mean; **SD**=Standard Deviation; **SE**=Standard Error; **#**=Radiation induced γ-H2AX total fluorescent yields (RIY).

this approach is impractical given the time needed to score thousands of samples. To increase the speed of scoring of γ-H2AX foci, different types of software programs have been developed which permit fully automated as well as semi-automated scoring. Many of these are available either free or on a commercial basis. FociCounter (Department of Radiobiology and Health Protection, Institute of Nuclear Chemistry and Technology, Poland) [91], Image-J (National Institute of Health, USA), Cell profiler, and the TRI-2 batch counting software (Health Protection Agency, UK) are freely available. Commercially available software programs include AutoQuantX (Meyer Instruments Inc, USA), HistoLab (Microvision Instruments, France), Image Pro (Media Cybernetics Inc., USA), MetaCyte (MetaSystems GmbH, Germany), and MetaMorph (Molecular Devices Inc., USA) [25,92]. The majority of these computer-assisted software programs identify and count the number of γ-H2AX foci based on predefined parameters, such as size and intensity, within the DAPI or PI stained nucleus. All these software programs will increase the scoring speed and facilitate making the assay suitable for use in triage. While these software programs reportedly have the potential to score γ-H2AX foci faster than the manual scoring, analyzing the γ-H2AX foci in complex tissue samples and overlapping of the foci remain unresolved [43,93]. A schematic representation of γ-H2AX foci counting using 'FociCounter' software is provided in Fig. 6 [91].

6.3. Flow cytometry, imaging flow cytometry, RABiT, portable devices, and rapid sample processing

Using flow cytometry to measure the γ-H2AX foci formation is a rapid tool, which can measure thousands of samples in a very short time [94,95]. In parallel, it can discern cell cycle-dependent occurrences of γ-H2AX, to increase the potential to discriminate and quantify higher doses, up to 25 Gy [50]. A recent development in γ-H2AX measurement is imaging flow cytometry (which has the advantages of both microscopy and flow cytometry) and permits quick and accurate measurement [47,96,97]. Furthermore, the rapid automated biodosimetry tool (RABiT) developed by Columbia University has increased the potential of the assay to analyze very large numbers of samples in a manner highly suitable for triage [98]. Even though flow cytometry, imaging flow cytometry, and RABiT have potential for application to triage, the main disadvantage is that they are not portable. To overcome this issue, portable fluidic fluorescence spectroscopy was developed for the analysis of samples near an accident, which increases the versatility of the assays. However, the sensitivity of these methods is lower compared to microscopy [48]. Dosikit, a new portable device based on the γ-H2AX assay, was developed for measuring radiation dose in the field [99]. DosiKit estimates doses from 0.5–10 Gy and subcategorizes individuals into three groups: below 2 Gy, 2–5 Gy, and above 5 Gy. The time required for the procedure is 45 min [100]. Dosikit can also measure γ-H2AX fluorescence in hair samples, which has the advantage of being a non-invasive method, but it still requires wider validation for use in the field [101]. In addition to the robustness of the instruments, sample processing with a minimal volume of blood and protocols requiring less time have been used to screen a large number of individuals during radiological emergencies [102,103]. The advantage of the assay, performed using these devices, is the ability to support fast triage, which could contribute to the effective management of radiation accidents.

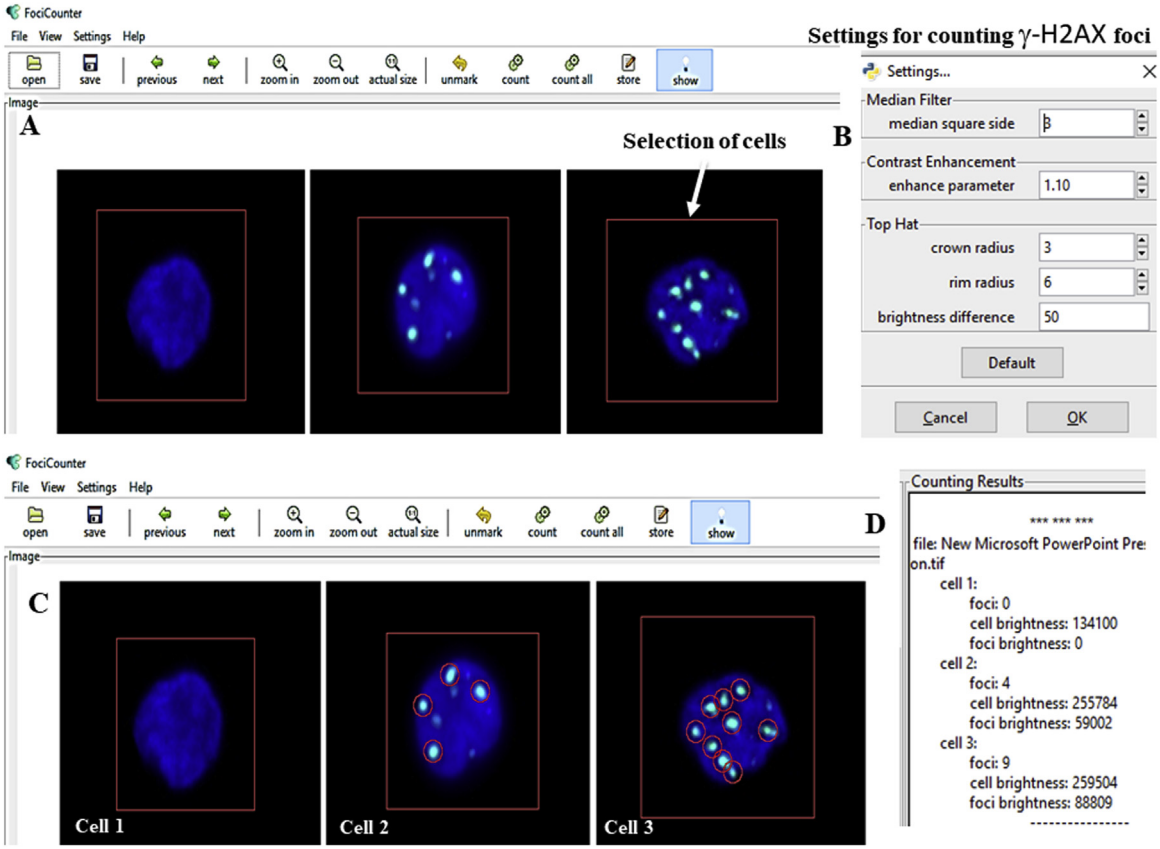

Fig. 6. Schematic representation of the counting of the γ-H2AX foci using 'FociCounter' software. (**A**) Loading the image into software, (**B**) Setting the parameters to count the γ-H2AX foci, (**C**) Counting the γ-H2AX foci, and (**D**) Result of the number of γ-H2AX foci in each cell.

6.4. γ-H2AX dose-response relationships in peripheral blood lymphocytes

To employ the γ-H2AX foci as a biodosimeter, it is necessary to define dose-response relationships for various types of radiation exposure in various scenarios (acute, protracted, and fractionated). Blood and lymphocytes exposed to ionizing radiation (*in vitro* and *in vivo*) showed a clear dose-dependent increase in γ-H2AX foci frequency in a variety of different studies [54,104–106]. Unlike well-established biomarkers, such as DC and MN for low LET radiations, radiation-induced γ-H2AX foci showed a linear dose-response [55]. As γ-H2AX foci formation and loss is a kinetic event, the dose-response kinetics shows bi-exponential decay, which can be used to measure the absorbed dose from samples exposed to ionizing radiation and sampled at various time points [46]. In contrast to DC and MN, multiple dose-response curves are required to estimate the dose from samples obtained at various time points. The yield of γ-H2AX foci is reported to vary across different labs, which will influence the dose-response curve coefficients. The coefficients are reported to vary based on the baseline γ-H2AX foci, type of radiation, dose rate, and time points post-exposure (Fig. 7). It is recommended that for accurate analysis, γ-H2AX foci yields should be measured from known exposed samples (as a positive control) along with the unknown samples [43]. A list of recently published articles on dose-response curve coefficients is provided in Table 2.

6.5. γ-H2AX assay inter-laboratory comparisons

To improve the quality and sharing of the workload in the case of an emergency, the methodology for sample transport, processing of samples, and scoring criteria were assessed as part of an inter-laboratory exercise for the conventional assays (CA and MN) used in biodosimetry [8,14]. Similar inter-laboratory comparison studies were performed among the five laboratories using different scoring methods for the γ-H2AX assay, which showed that the manual scoring provided better dose estimates than automated scoring [43]. The North Atlantic Treaty Organization (NATO) biodosimetry inter-laboratory comparison reveals the potential of γ-H2AX as a biodosimeter. Irrespective of the variation in γ-H2AX foci yields between the laboratories, the γ-H2AX foci assay could distinguish high and low dose samples with a satisfactory accuracy of 84% [109]. An inter-laboratory comparison with eight European laboratories was also carried out by Realising the European Network of Biodosimetry (RENEB). The results suggest consideration of variation in microscopes, spectral and brightness differences in fluorophores and light sources, wavelength ranges between different fluorescence filters, and the antibody from different suppliers for future inter-laboratory comparisons and/or use of the assay in a network response to an emergency [107]. The second γ-H2AX inter-laboratory comparison between RENEB laboratories demonstrated the potential of the assay for triage categorization of 46 samples (lymphocytes and whole blood) exposed to ^{60} Co γ-radiation [17]. A summary of the recent inter-laboratory comparisons on the γ-H2AX assay is given in Table 3.

7. Limitations associated with γ-H2AX foci assay in biodosimetry and triage

In spite of its advantages in triage, the γ-H2AX assay has several limitations in measuring doses during large scale radiological accidents. The advantages and the disadvantages of the γ-H2AX assay for triage and dosimetry are listed in Table 4. The formation of γ-H2AX foci starts within 5 min after irradiation and peaks at 45–60 min. This depends on the individual repair capacity [32]. Inter-individual variation in DNA repair capacity results in the variation of γ-H2AX foci in exposed samples at a particular time point after irradiation, which limits the sensitivity of the assay in estimating the accurate absorbed dose [87]. Unlike DC, the γ-H2AX foci assay is not specific to ionizing radiation and it can be formed by stalled replication forks, oxidative damage, and DNA damaging chemicals, which result in the formation of basal levels. Understanding baseline frequency of γ-H2AX foci and the factors affecting baseline frequency in a particular population might be helpful for segregating exposed from unexposed individuals and also for finding the variation in the dose-response curves used to

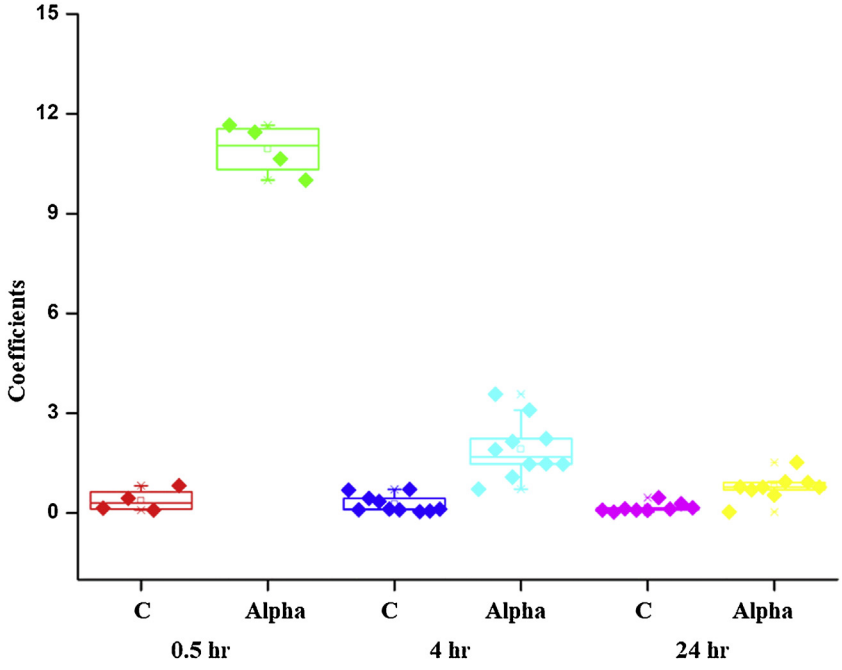

Fig. 7. Linear dose-response curves coefficients obtained from different post-irradiation time points (0.5, 4, and 24 h) of different laboratories. The linear curve coefficients (C and Alpha) reported in studies listed in Table 2 were used to draw the graph.

Table 2
γ-H2AX dose-response coefficients obtained from lymphocytes at different post-irradiation time points.

Study No	Dose-response coefficients	Time points	Type of radiation	Publication
1	Y = 0.09 + 10.65 D Y = 0.13 + 0.87 D Y = 0.15 + 0.50 D	0.5 h 8 h 16 h	^{60} Co γ-rays, 0.02–2 Gy, (0.5 Gy/min)	[90] Sandrine Roch-Lefèvre et al., (2010)
2	Y = 11.92*exp(20.3495*x) +3.552*exp(20.01843*x) Y = 8.593*exp(20.37*x) +1.136*exp(20.02*x)	1, 2, 4, 24, 48, 72, and 96 h (bi exponential function)	250 kVp X-rays, 0.5,1, 4 Gy, (1.7 Gy/min)	[46] Horn et al., (2011)
3	Y = 0.15 + 11.66 D Y = 0.15 + 2.44 D Y = 0.22 + 1.57 D	0.5 h 5 h 8 h	100 kVp X-rays, 0.02–1 Gy, (0.14 Gy/min)	[105] Mandina et al., (2011)
4	**(4 h)** Y = 0.10 ± 0.09 + 1.47 ± 0.22 D Y = 0.12 ± 0.07 + 3.10 ± 0.21 D Y = 0.71 ± 0.19 + 1.08 ± 0.17 D Y = 0.35 ± 0.26 + 1.48 ± 0.26 D **(24 h)** Y = 0.08 ± 0.02 + 0.54 ± 0.04 D Y = 0.09 ± 0.02 + 0.78 ± 0.03 D Y = 0.46 ± 0.09 + 0.94 ± 0.09 D Y = 0.13 ± 0.03 + 0.70 ± 0.05 D	4 h 24 h	^{60} Co γ-rays, 1, 2, 4 Gy, (1.3 Gy/min), (2.8 mGy/min)	[43] Rothkamm et al., (2013)
5	Y = 0.82 + 11.45 D Y = 0.12 + 1.52 D Y = 0.14 + 0.76 D	0.5 h 24 h 48 h	^{137} Cs γ-rays, 0.02−5 Gy	[44] Redon et al., (2009)
6	**(4 h)** Y = 0.04 ± 0.02 + 2.15 ± 0.09 D Y = 0.44 ± 0.16 + 1.90 ± 0.21 D Y = 0.06 ± 0.03 + 2.24 ± 0.09 D Y = 0.10 ± 0.09 + 1.47 ± 0.22 D Y = 0.12 ± 0.08 + 3.57 ± 0.23 D Y = 0.69 ± 0.06 + 0.72 ± 0.07 D **(24 h)** Y = 0.03 ± 0.04 + 0.79 ± 0.14 D Y = 0.28 ± 0.12 + 0.92 ± 0.14 D Y = 0.09 ± 0.03 + 1.35 ± 0.06 D	4 h 24 h	^{137} Cs γ-rays, 0.5, 1, 2, 4 Gy, (0.6 Gy/min)	[107] Barnard et al., (2015)
7	Y = 0.44 ± 0.20 + 2.01 ± 0.37 D Y = 0.38 ± 0.22 + 2.10 ± 0.56 D	0.5 h 2 h	^{60} Co γ-rays, 0.1–2 Gy, (1 Gy/min)	[108] Venkateswarlu et al., (2015)

Gy=Gray; **h**=Hours; **min**=Minutes; **kVp**=Peak Kilo Voltage.

measure the dose [42]. The influence of the inter-individual variability and the baseline frequency cannot be avoided in applications requiring accurate estimation of the dose from unknown exposures. However, in the case of radiation accidents, segregating exposed from unexposed individuals does not require accurate estimation of the dose and the influence of inter-individual variation, so the baseline can be ignored during large scale radiological emergencies [17].

Inter- and intra-laboratory scoring comparisons are critical for establishing consistent scoring and sharing workload. Inter-laboratory comparisons have been carried out for the γ-H2AX foci and the results showed a significant variation in γ-H2AX foci [17,107,109]. The variation between laboratories might be due to many factors, such as the selection criteria of cells, size of γ-H2AX foci considered for scoring (medium or large), differentiating the foci from the non-specific binding of the secondary antibody, and background noise. In addition to the scoring selection, cancer cells often showed higher γ-H2AX foci and have large γ-H2AX foci because of differential post-translational modifications of histones. In most cases, γ-H2AX foci in cancer cells may be unrelated to DSBs, which might affect the estimation of the dose from an unknown exposure. Therefore, to minimize the variability, there is a need for specific inclusion/exclusion criteria for consideration of γ-H2AX foci using manual scoring. However, the manual scoring variation can be avoided by using the automated scoring (pre-defined size) and semi-automated scoring during radiological emergencies [43]. Variation due to scoring of γ-H2AX foci can be avoided (with a compromise on the sensitivity) by measuring the fluorescence intensity using flow cytometry, imaging flow cytometry, and other portable devices to segregate the exposed individuals from unexposed during large scale radiological emergencies [97,100].

Variability in γ-H2AX may be due to the chemicals used, variation in the staining protocols, reproducibility of the spectral properties of the instruments used, and post-irradiation time. This variability limits the capacity of the assay for biodosimetry and triage [107,108]. The quality of staining depends on the type of slide, reagents, and incubation times. Irrespective of the instruments used (microscopy/flow cytometry), some variation attributable to chemicals and protocols cannot be avoided. Specificity in staining can be improved through co-localization of the DNA repair proteins. 53BP1 proteins colocalized with γ-H2AX might increase the specificity of staining and decrease the variability. Further, minimizing the complexity of the processing and the usage of ELISA-based methods might help to reduce variability in the γ-H2AX response, when used for triage in case of large scale radiological emergencies [52,73]. In view of the repair kinetics of γ-H2AX, It was suggested that the samples need to be analyzed <24 h of post-exposure to avoid loss of signal. However, immediately after collecting the blood samples, addition of paraformaldehyde (PFA)/phosphatase inhibitor (Calyculin) to the isolated lymphocytes has been reported to stabilize the γ-H2AX signal, which makes the assay more efficient in estimating the dose accurately, even several hours after irradiation [90]. Measurement of residual γ-H2AX foci might increase the time window for using the assay after large scale radiological accidents. When analyzing unknown samples, it is recommended that including reference samples will help identify sources of variability and deviation in staining protocols and scoring of γ-H2AX foci. To be prepared to conduct biodosimetry and triage during radiological accidents, laboratories must carry out standardization and validation of the protocols for γ-H2AX foci.

In addition to chemicals and protocols, the quality of the radiation (high or low-LET) and the nature of exposures also lead to

Table 3
Summary of the interlaboratory comparison studies on γ-H2AX foci in lymphocytes irradiated with ionizing radiation as a marker for measuring radiation absorbed dose during radiological accidents.

Study No	No. of laboratories	Scoring method	Time points	Type of radiation	Conclusion	Publication
1	7	Manual and automated	4, 24 h	^{60} Co γ-rays, 0.5, 2, 2.5 Gy, (0.67 Gy/min)	Second γ-H2AX foci inter-laboratory comparison was performed to strengthen (training and harmonization) the triage capacity of the laboratories. Despite using the standard protocol, the number of γ-H2AX foci varied among the laboratories, which suggests variation in sample processing, staining, and reagents used. However, the estimated dose correlated well with true doses. Manual scoring of γ-H2AX foci performed in blood and lymphocytes irradiated and incubated for 4 h resulted in accurate triage categorization. Out of 46 samples, only 3 samples were miscategorized, which might affect the clinical decision.	[17] Moquet et al., (2017)
2	8	Tele, manual and semi-automated	4, 24 h	^{137} Cs γ-rays, 0.5, 1, 2, 4 Gy, (0.6 Gy/min)	The first γ-H2AX foci inter-laboratory comparison exercise was performed among the European biodosimetry laboratories (RENEB). The results suggest that a significant difference in the γ-H2AX foci was reported between the laboratories. Despite the variation, the samples were correctly ranked from lowest to highest in the absence of calibration curves. No significant difference in the calibration curves was observed between manual and automated scoring. Manual scoring after 4 h of incubation showed more accurate triage categorization. Furthermore, the triage categorization was not influenced by scoring a lower number of cells.	[107] Barnard et al., (2015)
3	2	Manual and semi-automated	0.5 and 2 h	^{60} Co γ-rays, 0.1–2 Gy, (1 Gy/min)	Inter- and intra-laboratory comparisons were performed to harmonize the staining protocols and scoring of the γ-H2AX foci among two Indian biodosimetry laboratories. The scorers were trained to identify the γ-H2AX foci. Captured and blinded images were shared between the scorers and laboratories. The reported γ-H2AX foci showed consistency in scoring between the scorers and between the laboratories.	[108] Venkateswarlu et al., (2015)
4	7	Manual and automated	4 h	^{60} Co γ-rays, 0.5, 1.5, 1.82, 2.75 Gy, (0.3 and 1.183 Gy/min)	The potential of the γ-H2AX foci as a triage tool has been tested along with well-established assays such as DC and MN. γ-H2AX foci assay categorized 70% of samples (28/40) correctly and 85% (34/40) were categorized as correct or too high. There was no significant difference in the triage categorization among the laboratories.	[110] Ainsbury et al., (2014)
5	4	Manual	2, 24 h	240 kVp X-rays, 0.1–6.4 Gy, (1 Gy/min)	The performance of the γ-H2AX assay as a triage tool was evaluated in a NATO biodosimetry study. The γ-H2AX foci yields were significantly varied (four fold) and suggest that reproducibility is an important caveat for this assay. Irrespective of variations in γ-H2AX foci counts, high- and low-dose samples were distinguished. The participant laboratories reported the dose estimates 7 h after the receipt of the samples. The dose estimated using 2 and 24 h calibration curves showed an accurate dose estimation. The accuracy of dose estimation using uniformly irradiated samples was not affected by the number of cells scored (20, 30, 40, or 50 cells). However, in case of the nonuniform exposures, scoring more cells can give better dose estimates. Mean absolute difference values showed a 3.4-fold variation in the accuracy to estimate the dose.	[109] Rothkamm et al., (2013)
6	5	Manual and automated	4, 24 h	^{60} Co γ-rays, 1, 2, 4 Gy, (1.3 Gy/min), (2.8 mGy/min)	The effectiveness of the different scoring methods was evaluated by an inter-laboratory comparison among the five European laboratories. The obtained results suggest that the linear dose-response curves were fitted satisfactorily from all the laboratories. Manual scoring identified partial body exposures based on the γ-H2AX foci distributions, whereas the automated scoring showed more uncertainties in dose estimation. Even though the γ-H2AX foci assay was able to detect the protracted exposures, the dose estimation was not satisfactory. The scoring speed of the automated scoring compromises the accuracy and differentiation of partial body exposures.	[43] Rothkamm et al., (2013)
7	4	Manual and automated	4 and 24 h	240 kVp X-rays, 0.1–6.4 Gy, (1 Gy/min)	The dose assessment accuracy of the established and emerging markers for triage and radiation injury assessment were evaluated. The first laboratory reported the dose within 0.3–0.4 days (for γ-H2AX and gene expression), and 2.4 and 4 days (for DC and MN). Irrespective of the assay, a 2.5 to 4-fold difference in accuracy was observed among the laboratories. Doses $\geq$1.5 Gy showed a 10% decrease in the sensitivity of the γ-H2AX foci assay, however, it was comparable to MN assay.	[111] Rothkamm et al., (2013)
8	2	Flow cytometry	15 min, 1, 4 and 24 h	^{137} Cs γ-rays, 2 Gy, (0.3–0.6 Gy/min)	Two laboratories attempted to study the suitability of the γ-H2AX foci in segregating radiosensitive and normal patients. The results of this study suggest that the *in vitro* test is not suitable to differentiate normal patients and radiosensitive patients. Considerable inter-individual variation and the small effect of radiosensitivity was found to be responsible for the inability of the assay to categorize the patients.	[112] Greve et al., (2012)

Gy=Gray; **h**=Hours; **min**=Minutes; **kVp**=Peak Kilo Voltage.

variation in the formation of γ-H2AX foci, which in turn influences the estimation of dose [44]. The γ-H2AX foci size formed after exposure to high LET, low LET, and mixed beam radiations varies: i.e the γ-H2AX foci are generally bigger at low radiation doses (0.1–0.5 Gy) when compared to higher doses. The size of the 10 cGy radiation-induced γ-H2AX foci increased dramatically compared to endogenous foci. γ-H2AX foci more than doubled in size after nitrogen-ion exposure and almost doubled in size after X-ray exposure. This suggests that the mechanism by which the DNA is damaged after high and low LET radiations is different [77]. The

Table 4
List of the advantages and disadvantages of the γ-H2AX foci assay.

Advantages	Disadvantages
• γ-H2AX is a rapid assay (6–8 h) compared to DC and MN assays.	• Unlike DC and MN, the γ-H2AX assay is unstable for longer periods.
• The γ-H2AX assay did not require the culture of lymphocytes for 48 or 72 h.	• γ-H2AX foci overlapping at higher doses lead to counting error.
• High sensitivity (microscopy counting of γ-H2AX foci can be used to detect very low doses, 0.02 Gy).	• High cost compared to DC and MN assays (requires antibody) and high-end equipment.
• The γ-H2AX assay can be used to detect doses beyond 5 Gy (fluorescence measurement).	• Variation in γ-H2AX staining leads to misinterpretation of foci.
• High throughput methods such as flow cytometry, ELISA, and portable microfluidic devices can be used to measure the γ-H2AX.	• Not specific to ionizing radiation (many chemical/physical agents that lead to DNA-DSB can form γ-H2AX foci).
• γ-H2AX is can be performed in various cell types (lymphocytes, splenocytes, buccal cells, bone marrow, skin plucked hairs, and cancerous tissue).	• Significant Inter-individual variations in γ-H2AX foci can interfere with the estimation of the absorbed dose.
• Personal computer-based software programs can be used to quantify the number of γ-H2AX foci in the images captured using microscopy.	• It is not possible to have standard dose-response curves for the γ-H2AX assay; regular recalibration is required to estimate the dose accurately.

Gy=Gray; **h**=Hours.

large γ-H2AX foci formed after mixed beam irradiation was delayed and did not reach their maximum size until 1 h after irradiation [113]. The triage potential of the γ-H2AX assay in case of whole-body acute exposures was documented by a series of inter-laboratory comparisons, however, there is a lack of concrete evidence that the assay can be used to discriminate the nature of the exposure (i.e partial body and protracted exposures). Therefore, the quality of radiation and the nature of the exposure limits the utility of the γ-H2AX foci for dosimetry/triage during large scale radiological accidents [103,109].

The majority of the inter-laboratory comparison studies on γ-H2AX used blood lymphocytes, and it would be better if more information was generated using non-invasive samples, such as buccal cells or plucked hair. Because microscopy is more sensitive, the majority of the inter-laboratory comparisons were performed using microscopy, which clearly indicates that the assay can be used for triage. The potential of the flow cytometry/other portable devices in analyzing thousands of samples also needs to be tested using inter-laboratory comparisons. Co-localization-based methods need to be evaluated by inter-laboratory comparison, to reduce the staining variations. The ELISA-based method requires less instrumentation and has the advantage of measuring both H2AX and γ-H2AX, but inter-laboratory comparisons need to be performed to minimize the impact of methodological variations on dose estimation. The potential for the assay to differentiate the nature of exposure/type of radiation needs to be explored. The γ-H2AX assay has an advantage for triage over the cytogenic methods owing to the speed and potential for high-throughput analysis. When compared to gene expression assay, estimation of dose using γ-H2AX assay is variable. Both gene expression and γ-H2AX assays can be used for initial triage followed by the accurate dose estimation using gold standard “Dicentric Chromosomal” assay in case of large scale radiological accidents [111]. Overall, the γ-H2AX is a useful tool for triage, in the context of categorizing people who are exposed to below and above 2 Gy of low LET radiation.

8. Conclusion

The potential of the γ-H2AX assay for triage (high- *vs* low-levels) and/or to measure the radiation absorbed dose for exposed individuals has been reported in several *in vivo* and *in vitro* studies. For effective application of this assay for radiation triage and biodosimetry, one should consider the inter-individual variability, baseline frequency, dose-response curves, kinetics of γ-H2AX dose-response, scoring variation, inter-laboratory variation in scoring, staining quality, and reference sample analysis along with the unknown samples.

Declaration of Competing Interest

The authors report no declarations of interest.

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