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Suitability of the γ-H2AX Assay for Human Radiation Biodosimetry

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1. Introduction

For biodosimetry purposes, there are only a few minimally invasive procedures. Two of them, fast and painless are particularly suited for large scale sample collection. One is to collect buccal cells, either by scraping the patient's inner cheek or using mouthwash. The other is to collect venous blood samples that are commonly used to obtain lymphocytes for chromosome analysis. Chromosome aberrations, such as dicentrics or translocations, have long been used as a dose indicator in biological dosimetry in cases of exposure or suspected overexposure to ionizing radiation (IAEA, 2001). The dicentric assay in peripheral blood lymphocytes is considered to be the reference method for biodosimetry: it is specific to ionizing radiation and stable enough to be used for dose estimation several months after exposure. However, it has several limitations; it is time consuming, it requires skilled personnel and needs a non-reducible period of 48-hour culture to obtain lymphocyte metaphases before chromosome scoring. One major application of biodosimetry is the identification, in the event of a large-scale radiation emergency, of the most severely exposed individuals. In this situation, a reliable bioassay is needed for population triage during the first few hours. Then, for faster dose estimation, current protocols for triage assessment call for the analysis of only 50 metaphases. This reduces the scoring delay somewhat but substantially augments the confidence interval and consequently decreases sensitivity, to 1 Gy (Miller, 2007; Voisin, 2004). The development of efficient new biodosimeters could overcome the limitations of dose sensitivity and avoid the lymphocyte culture step inherent to the dicentric assay.

Potential candidates include several proteins that are involved in the early steps of cellular response to ionizing radiation and specifically to DNA damage (Marchetti, 2006). One of the primary cellular effects of ionizing radiation is the induction of DSB (double-strand breaks). Following DSB induction, hundreds of histone H2AX molecules are phosphorylated in the chromatin flanking the DSB site and generates so-called γ-H2AX (Rogakou, 1998). The production of fluorescent antibodies specific for γ-H2AX coupled with fluorescence microscopy led to the development of sensitive assays that make it possible to visualize discrete nuclear foci at DSB sites (Rogakou, 1999). Foci induction and disappearance over time follows DSB rejoining in repair-competent cells, suggesting a correlation between initial as well as residual radiation-induced DSB and γ-H2AX foci (Rothkamm & Horn,

2009). The scoring of γ-H2AX foci is now widely used for quantitative evaluation of DSB formation and repair (Rothkamm, 2003) (Olive & Banath, 2004). Recent immunofluorescence studies show that the yield of these foci induced by ionizing radiation in humans increases linearly with the radiation dose after both *in vitro* and *in vivo* exposure (Leatherbarrow, 2006; Lobrich, 2005; Rothkamm, 2007; Sak, 2007). It has been shown that the scoring of γ-H2AX foci in human lymphocytes can be used to estimate very low doses (down to few cGy) at 30 minutes after *in vivo* radiation exposure (Lobrich, 2005; Rothkamm, 2007). However, since the level of γ-H2AX foci varies with time after irradiation, the sensitivity of its detection is necessarily time-dependent: very high sensitivity after few minutes but fair sensitivity at several hours post-exposure. Whilst microscopic imaging and scoring of γ-H2AX foci offers the highest sensitivity, intensity-based assays for γ-H2AX are widely used in experimental research and may offer some advantages in terms of throughput and automation (Rothkamm & Horn, 2009). In the contexts where the user is particularly interested in quickly quantifying the γ-H2AX signalling response, most attempts of developing a fast H2AX assay used flow cytometry (Ismail, 2007). This technique measures a relative intensity of the γ-H2AX staining instead of scoring the actual number of foci and is known to have a large level of inter-individual variation (Andrievski & Wilkins, 2009; Hamasaki, 2007; Ismail, 2007). Total γ-H2AX intensity levels are dose dependent and approximately linear up to a supralethal dose of 100 Gy (Ismail, 2007). Recently, a dual method has been developed to determine fluorescence yield using high-speed microscope imaging analysis. This workstation has been designed to fully automate the γ-H2AX immunocytochemical protocol, from the isolation of human blood lymphocytes to the image acquisition step (Turner, 2011).

The translation of γ-H2AX analysis into a reliable dosimetry device nonetheless requires both further validation and better automated methods in microscopy-based scoring. Despite the wide application of this appraoch, γ-H2AX counting is frequently carried out manually by eye and may be prone to investigator-related biases. Currently, manual counting of γ-H2AX foci on microscopy images is a time-consuming, tedious process, especially for doses higher than 0.1 Gy, where there are many foci throughout the nucleus. In the laboratories that manually score γ-H2AX foci today, there are a few numbers of trained investigators, to minimize scoring artefacts. Indeed, focus scoring uses measurement features like the focus size or brightness which are very difficult to evaluate objectively by eye. Therefore, automatic systems for γ-H2AX analysis may be used to allow a consistent and a fast quantification of γ-H2AX in a wide dose range that would be compatible with biodosimetry. Here, different methods that use either software already available or home-made developed for the automatic analysis of γ-H2AX are reviewed. A comparison of the results obtained for γ-H2AX responses in relation to dose, time since exposure, lower and higher limit of detection is made in this chapter.

2. γ-H2AX assay in peripheral lymphocytes

Peripheral blood mononuclear cells (PBMCs) are useful to evaluate the effects of ionizing radiation exposure, as they can be obtained with minimal invasiveness and under standard conditions. For the evaluation of γ-H2AX foci formation, PBMCs are very attractive because considerable amounts of cells can be easily obtained within a short time. Because monocytes and granulocytes can be excluded from the γ-H2AX analysis, the data obtained in PBMCs

mainly represent the result of a mixture of the different lymphocyte subpopulations. This cell selection can be done during the analysis either visually by the scorer according to morphological features or by a software using nucleus staining intensity criteria.

2.1 Microscopy-based γ-H2AX analysis

To count the foci present in the nuclei is currently the most sensitive method for γ-H2AX analysis. γ -H2AX foci become microscopically visible within minutes after irradiation, indicating the rapid phosphorylation of thousands γ-H2AX molecules. Thanks to this large scale formation of γ-H2AX, foci can be easily distinguished from a relatively homogeneous background signal so that one individual DSB can be detected (Fig. 1). In case of manual scoring by eye directly at the microscope, the speed can be relatively fast for a well-trained scorer, but this approach quickly becomes tiresome if many samples need to be analysed. This method of γ-H2AX-based visualization and quantification in PBMCs has been used to estimate the radiation dose received by adult patients who had undergone either multidetector computed tomography (CT) or radiotherapy treatment (Rothkamm, 2007; Sak, 2007). In these studies, blood samples obtained from patients before the exposure were

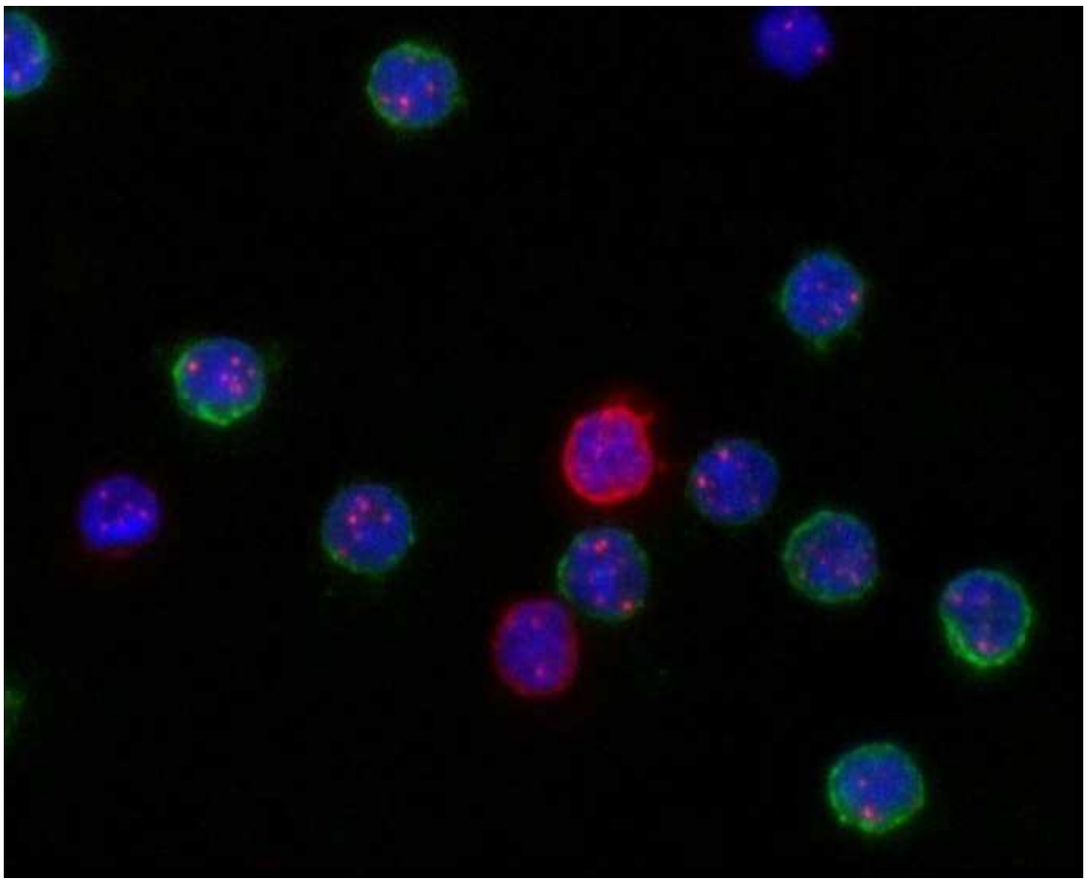

Fig. 1. Gamma-H2AX foci in human lymphocytes (CD4 and CD8 sub-types) after 0.5 Gy of γ-rays, 30 minutes post-exposure. The blue channel corresponds to DAPI staining; the magenta channel corresponds to γH2AX staining; the green and red channels correspond to CD4+ and CD8+ membrane staining, respectively.

irradiated *in vitro* at doses from 0 to 1 Gy. The results obtained *in vitro* from these studies show a good linear dose-effect relationship in PBMCs. However, although mean background of γ-H2AX were very similar in both studies (0.06 and 0.057 focus per cell, respectively) the induced foci yields 30 minutes after *in vitro* irradiation were quite different: 3 times higher in the CT study than in the radiotherapy study. This discrepancy in radiation-induced response of γ-H2AX could have been attributed to the difference in radiation quality types: X-rays 150 kV vs. γ-rays of Cobalt 60, respectively. Concerning the results obtained *in vivo*, the CT study shows that the doses obtained from the γ-H2AX focus yields after whole-body CT (16.4 mGy) or chest CT (6.3 mGy) were similar to phantom dosimetry–calculated doses (13.85 mGy and 5.16 mGy, respectively). In the radiotherapy study, it has been shown that the *in vivo* formation of γ -H2AX foci in dependence of the applied integral dose in cancer patients irradiated at different sites of the body follows a linear relationship. In another publication where γ-H2AX foci yields were also evaluated visually in PBMCs irradiated *in vitro* either with X-rays 100 kV or γ-rays of Cobalt 60; the slopes of the dose-effect relations were quite similar (about 10 foci per cell Gy^{-1}) in a dose range of 0.01 – 0.5 Gy (Beels, 2010). These results mean that the discrepancy between γ-H2AX foci yields observed in studies from different laboratories is rather due to the differences in scoring criteria than to true differences in radiation-induced responses. Furthermore, it is important to consider that these estimates are only true for blood samples that are processed within less than one hour after irradiation.

Although γ-H2AX foci can be detected just few minutes after irradiation, in a radiation emergency scene, it is however quite unrealistic to expect that blood samples can be obtained within an hour of exposure. Since the method will only be practical if it can be applied to samples taken at longer times post exposure, dose-response curves are needed for later time points. The response of lymphocytes to doses was examined visually in blood samples from individuals exposed *in vitro* to increasing doses (0.02–5 Gy) up to 2 days after irradiation (Redon, 2009). Thirty minutes post-exposure, doses as low as 0.02 Gy were detectable, and, for doses up to 2 Gy, the data followed a linear relationship between dose and γ-H2AX signal. For doses greater than 1 Gy, a substantial response was detected in the lymphocytes for up to 2 days after exposure (Redon, 2009). These results - obtained visually– have been confirmed *in vivo* in non-human PBMCs after irradiation of macaques receiving total body γ-ray doses ranging from 1 Gy to 8.5 Gy, for times up to 4 days after exposure (Redon, 2010).

Automatic systems may be used to avoid focus scoring bias that may prevent inter-laboratory comparisons. It allows fast scoring of γ-H2AX foci for biodosimetry purposes. Several groups have developed image analysis solutions for automated foci scoring in human cellular models (Bocker & Iliakis, 2006; Costes, 2006; Leatherbarrow, 2006; Mistrik, 2009). A semi-automatic approach, commercially available, has been used to score γ-H2AX foci in PBMCs for radiation doses assessment up to 16h after radiation exposure. Dose response curves were designed at different times post-exposure and the threshold of detection of the technique was evaluated at each time point (Roch-Lefèvre, 2010). The slopes of the dose-effect relation decreased with time post-exposure, from 10.7 γ-H2AX foci per cell Gy^{-1} at 30 minutes down to 0.50 foci per cell Gy^{-1} 16 hours post-exposure. The threshold of detection - defined as the lowest dose (D_{LD}) that could be detected was calculated for each time post-exposure. The calculated D_{LD} values were 0.05 Gy at 30 minutes, 0.3 Gy at 8 hours and 0.6 Gy, 16 hours post-exposure. It should be noticed that the disappearance of γ-H2AX can be inhibited by incubating the whole blood on ice at 0°C which is important in biological

dosimetry where blood samples are analyzed several hours after they have been withdrawn (for blood sample transport to the expert laboratory, for example) (Roch-Lefevre, 2010). It has been shown that blood samples could be stored on ice for extended periods of time without loss of the gamma-H2AX signal (Ismail, 2007).

2.1.1 Automation of image acquisition

Automation of image acquisition is a major issue for high throughout microscopy analysis. Resources commonly found in laboratories for automatic acquisition coupled with export of gray-scale TIFF files can be used to acquire images of lymphocyte nuclei and γ-H2AX foci. Actually, automated multi-colour image acquisition is becoming a standard function in many systems of computer-controlled fluorescent microscopes. Numerous possibilities exist, both commercial and open source, like micro-manager (http://micro-manager.org/) (Valente, 2011). Also in the interest of shortening both acquisition and analysis time, several technical parameters, like the lymphocyte density on slide and the minimum number of cells to score has to be optimized.

2.1.2 Automation of image analysis: Image cytometry

While fluorescence microscope in combination with a digital camera is standard equipment in many laboratories, the availability of specialized software, especially freeware, for the analysis of γ-H2AX foci is increasing (Carpenter, 2006) (Jucha, 2010) (Ivashkevich, 2011). Image analysis implies two distinct steps: selection of the cells and detection of γ-H2AX foci; therefore it needs to load two colour-types of grey scale images: one with counterstained nuclei for cell selection and the other one with fluorescent-coupled anti-γ-H2AX for focus detection. These two steps of analysis can be done manually, either fully automatically or semi-automatically, which all lead to advantages and limitations as described in Fig. 2. In this chapter, are compared two applications: Histolab™, a semi-automatic software and CellProfiler, a freeware that allows full-automated analysis (Roch-Lefevre, 2010) (Valente, 2011). Histolab™ is considered as semi-automatic since it still requires that the images be loaded separately by the user during analysis. It detects both nuclei and foci by applying a fixed threshold and a"top hat" threshold to the greyscale images of the nucleus and γ - H2AX staining, respectively. The major advantage of this software is that user interface is accessible, rendering parameters setting for foci detection very easy. Furthermore, in the current version of HistoLab™, the operator has the possibility of removing manually the cells with aberrant staining or morphology. But, with other software approaches where there is no operator intervention (as with CellProfiler for example); the cell type selection has to be done graphically using measurements of nucleus staining and morphology (Valente, 2011). For the nucleus analysis with CellProfiler an adaptive algorithm is applied on the counterstained-nucleus pictures to detect nuclei with a range of diameters. Inside these nuclei, another module can use a various choice of algorithms on the H2AX pictures (pre-subjected or not to a treatment) to detect foci with possibly minimum and maximum diameters. The last modules measure the area and intensity of both nuclei and foci. CellProfiler is a program that offers a great number of measurements and detection options. The main drawback of this freeware is a less user-friendly graphical interface that may render the vast number of detection algorithms and measurements overwhelming for a beginner. When CD-specific membrane staining takes place (as seen in Fig. 1.) CellProfiler is also able to measure the mean intensity of the associated colour channel inside each cell. The

optimization of this approach has been described for PBMCs isolated from blood exposed *ex vivo* to different doses of radiation (Valente, 2011).

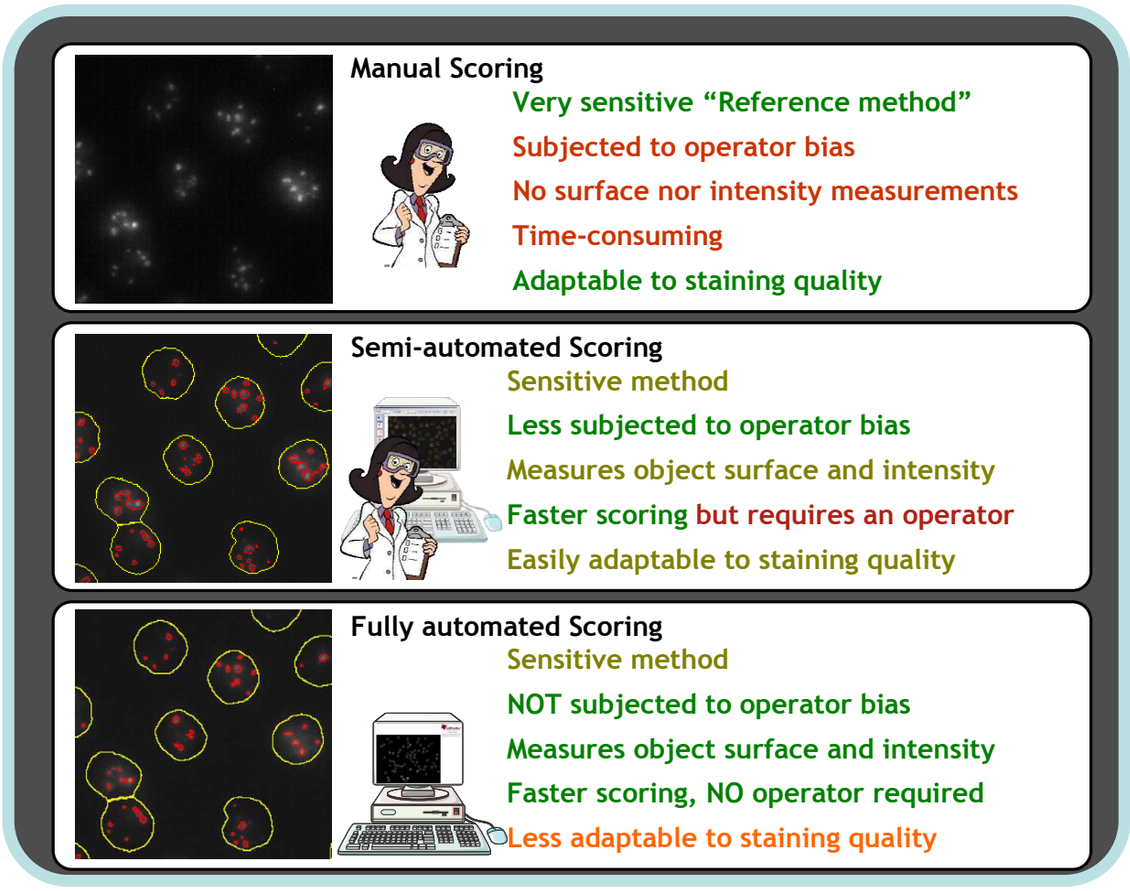

Fig. 2. Advantages and limitations for 3 methods of γ-H2AX focus scoring

All free (and paid) automated scoring applications seem to present good correlations to manual scoring once the right parameters are inputted. The free alternative that resembles most HistoLab™ is FociCounter (Jucha, 2010): it was developed specifically to score foci, with a simple interface and few parameters to change (faster to set up). These programs are ideal for simple scoring of a reduced number of cells/conditions, where the quality of the images/slides frequently requires operator intervention (to eliminate aberrant objects, for example). Since these programs require user intervention to select nuclei, the time of the analysis is their main disadvantage. Using CellProfiler, once the pipeline is set up, it requires very little operator intervention for analysis. A free equivalent is difficult to find. We do not compare it to imageJ as a CellProfiler module has been recently created to allow the user to run ImageJ macros and plugins as part of a CellProfiler image processing pipeline (http://cellprofiler.org/CPmanual/RunImageJ.html). In a recent publication, a new image cytometry program that will become freely available was presented (Ivashkevich, 2011). The details on the measurements that will be possible to obtain with this software are not yet disclosed, so we cannot fairly compare it with CellProfiler.

However, the computational approach they present is a good reference to help researchers select the parameters of other programs of this type (Ivashkevich, 2011).

2.2 Intensity-based γ-H2AX analysis

While fluorescence microscopy enables individual γ-H2AX foci and their characteristics to be imaged and analysed, flow cytometry provides a more rapid and straightforward method of γ-H2AX quantification, that is by measuring the total fluorescence intensity for each cell.

2.2.1 Flow cytometry γ-H2AX analysis

Using a rapid flow cytometry method, the dose response of γ-H2AX induction in lymphocytes and lymphocyte subsets was found to be linear in the dose range examined (0–10 Gy) (Andrievski & Wilkins, 2009). Although this linear dose relationship and speed of this assay are advantageous for using γ-H2AX as a biological dosimeter, it is limited by the high variability between experiments with standard errors of the mean often reaching more than 20% of mean values, indicating high intra-donor variability. This level of inter and intra-individual variation is consistent with previously reported data in human lymphocytes (Hamasaki, 2007). After an accidental exposure, the dosimetry will be complicated by the lack of control samples for each individual that could be used as a comparison to the post-exposure levels of expression.

2.2.2 Other intensity-based γ-H2AX analysis

γ-H2AX total fluorescence measurements were determined in lymphocyte nuclei from blood samples collected from four healthy donors, irradiated (*ex vivo*) with a range of γ-ray doses between 0 and 8 Gy (Turner, 2011). Dose response for γ-H2AX was analyzed 30 min after irradiation. Curve-fitting analysis showed that the induction of total γ-H2AX fluorescence was linear with increasing γ-ray doses up to 8 Gy. Statistical analysis of the individual data points at 2 Gy showed that there was a significant induction of total γ-H2AX fluorescence. However, with this method, the data for one of the four donors show that there was no significant difference in the γ-H2AX levels at 1 Gy, 30 min postirradiation, suggesting that the sensitivity of this method is above 1 Gy whereas sensitivity of γ-H2AX foci scoring methods are above 0.05 Gy at 30 minutes post-exposure (Roch-Lefèvre, 2010).

3. γ-H2AX assay in epithelial cells

3.1 γ-H2AX assay in exfoliated buccal cells

Buccal cells are expected to accumulate DNA damage after exposure to DNA strand-breaking agents such as ionizing radiation; making the system potentially useful to estimate a dose received by the very upper part of the body. To test the γ-H2AX assay for the detection of ionizing radiation-induced DNA damage in buccal exfoliated cells, an *in vitro* study was carried out with these cells from healthy donors exposed to γ-rays in a dose range of 0 – 4Gy (Gonzalez, 2010). The γ-H2AX foci rate of 0.08 foci per cell observed in non-irradiated buccal cells was similar to the one usually obtained in sham-irradiated lymphocytes. The number of γ-H2AX foci increased linearly with ionizing radiation dose in the interval from 0 to 4 Gy, and reached a rate of 0.82 foci per cell at 4 Gy which is much lower than in lymphocytes. Incubation experiments after *in vitro* irradiation revealed that

the number of γ -H2AX foci did not show a significant decrease 5 hours post-exposure (Gonzalez, 2010). It was concluded that it is possible to apply the γ -H2AX foci assay for the detection of ionising radiation-induced DNA damage in buccal exfoliated cells. The low removal of ionising radiation induced γ-H2AX foci in buccal cells may be a potential advantage for a biological dosimetry application.

3.2 γ-H2AX assay in plucked-hair cells

Plucking hairs is non-invasive and fairly painless compared to other types of sample collection. As buccal cells, it could be withdrawn by non-clinical personnel and then, it may be suited as well for large scale sample collection. It has been shown in macaques that the detection of γ-H2AX in plucked hairs can be used to monitor total body irradiation for several days after exposure (Redon, 2010). Moreover, the γ-H2AX signal decreased little or not at all between 1 and 2 days post-irradiation. It was concluded, as for buccal cells, that this slower rate of γ-H2AX foci loss compared to lymphocytes may be due to a lack of repair in hair differentiating hair cells and may be a potential advantage for biological dosimetry purposes (Redon, 2010) (Gonzalez, 2010).

4. Main issues to address for biodosimetry purposes

In contrast to chromosome-based dosimetry methods, nowadays well-established, a wide range of issues have to be addressed for the γ-H2AX assay before it will become completely relevant to its application in biological dosimetry. There are two main limitations that have to be addressed: inter- or intra-individual variability and partial-body exposure. A high individual variability leads to a decreased sensitivity of the γ-H2AX assay. This individual variability in radiation induced γ-H2AX focus yields was measured *in vitro* in the lymphocytes (from up to 27 healthy donors) before exposure and at different times after exposure (Roch-Lèfevre, 2010). In non-exposed lymphocytes, the inter-individual variation of the γ-H2AX yield was two to three times higher than the intra-individual variation, with a coefficient of variation of 31%. On the other hand, after irradiation, the inter-individual variation of the γ-H2AX yield was similar to the intra-individual one. Furthermore, radiation accidents are more commonly characterized by acute partial-body or inhomogeneous rather than homogeneous total-body radiation exposures. It has been shown that the yield of γ-H2AX foci formation can allow the estimation of the applied integral body dose after local radiotherapy to different sites of the body (Sak, 2007). However, further work is required to determine the effect of partial body exposure on γ-H2AX levels. In vivo exposures involving radiotherapy patients may help to determine the effects of lymphocyte circulation through the various body organs on γ-H2AX levels in blood samples taken at different time points after treatment. Also, changes in the distribution of γ-H2AX foci over time would have to be determined.

5. Conclusion

The gamma-H2AX assay will require additional *in vivo* experiments for validation. In particular, all usual limitations for human biodosimetry, particularly in regards to the different accident scenarios (delayed, fractioned, inhomogeneous exposures…) should be addressed. The γ-H2AX signal is also fairly short lived such that samples from potentially exposed individuals would need to be collected and processed quickly after exposure. These

issues make this assay have limited applications, for rapid screening of individuals when samples can be collected and processed within 24 hours.

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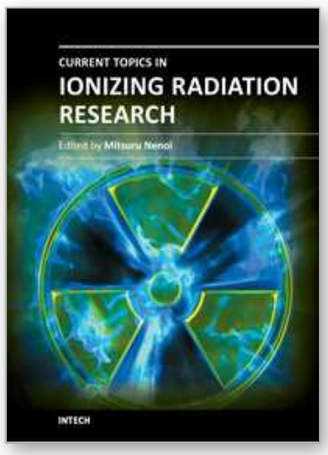

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Since the discovery of X rays by Roentgen in 1895, the ionizing radiation has been extensively utilized in a variety of medical and industrial applications. However people have shortly recognized its harmful aspects through inadvertent uses. Subsequently people experienced nuclear power plant accidents in Chernobyl and Fukushima, which taught us that the risk of ionizing radiation is closely and seriously involved in the modern society. In this circumstance, it becomes increasingly important that more scientists, engineers and students get familiar with ionizing radiation research regardless of the research field they are working. Based on this idea, the book "Current Topics in Ionizing Radiation Research" was designed to overview the recent achievements in ionizing radiation research including biological effects, medical uses and principles of radiation measurement.

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