Flow Cytometric Analysis of Phosphorylated Histone H2AX Following Exposure to Ionizing Radiation in Human Microvascular Endothelial Cells

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Flow cytometry/γH2AX/Ionizing radiation/DNA damage/N-acetyl-cysteine.

We applied a flow cytometric method to quantify IR-induced histone H2AX phosphorylation at serine 139 (γH2AX) and compared those values to those obtained using a standard microscopy based foci counting method. After PFA fixation, methanol permeabilization was suitable for both FITC- or Alexa647-γH2AX. In contrast, Alexa647-γH2AX was not suitable for ethanol permeabilization. Antibody concentrations at 1–2 µg/ml yielded the highest γH2AX positive percentage for both antibodies. Without DAPI staining, γH2AX formation can be measured as a relative fold increase. Values determined by bivariant flow cytometric analysis and those obtained using microscopic foci formation exhibited a good quantitative correlation. Values obtained by both methods could vary according to the gating or threshold setting used. γH2AX positive cells increased as a function of radiation dose (2–16 Gy) followed by a dose-dependent decay. The free radical scavenger N-acetyl-L-cysteine (NAC), if administered at a concentration of 4 mM 30 min before IR, was effective in reducing IR-induced γH2AX formation in all phases of the cell cycle. We have developed a simplified and quantitative flow cytometry based method to measure IR-induced γH2AX in cells and demonstrated strong correlation to values obtained by a standard automated digital microscopic foci analysis along with NIH ImageJ custom macro software (Appendix).

INTRODUCTION

DNA double-strand breaks (DSBs) are potentially damaging events in cells that are highly mutagenic when misrepaired and lethal if left unrepaird. Ionizing radiation (IR) induces a broad spectrum of different types of DNA lesions including a variety of base damages, single-strand breaks (SSBs) and DSBs in cells.1) Since DSBs are a major damaging lesion to the cells following IR, the ability to quantitatively measure them in a rapid manner would be extremely important for both biodosimetry and risk assessment applications. The measurement of γH2AX as a marker of DSB damage offers several advantages over the other analytical methods such as neutral elutriation, pulsed field gel electrophoresis and neutral comet assays that are described in detail elsewhere.2)

Recently developed γH2AX based analysis as a marker for DSBs is both relatively robust and practical to assess IR-induced damage in cells. Exposure to IR results in the rapid phosphorylation of a minor nucleosomal histone protein, H2AX, at the sites of DSB damage that precedes the involvement of repair enzymes involved in the processes of homologous recombination (HR) and non-homologous end-joining (NHEJ) DNA repair.3–5) An important modification that occurs specifically at the site of DSB damage is the rapid phosphorylation of H2AX5) covering about 2 Mb of DNA that surrounds the break.6) This can be detected using a specific antibody. The number of γH2AX foci in a nucleus is known to be directly proportional to the number of DSBs formed, and dephosphorylation coincides with DSB repair.7) Thus, γH2AX foci formation is a sensitive and specific marker for the detection of DSBs.7–9)

γH2AX can be detected visually as microscopic foci. Other effective techniques include: immunoprecipitation, Western blotting, flow cytometry and laser scanning cytometry. Thus, there are a number of methods utilizing γH2AX as a marker of DSBs available with each method having for its own merits and drawbacks. Although direct visualization of γH2AX is probably the most specific and efficient technique...
for spotting DSBs in cells it is very time consuming. The measurement of γH2AX within cells have here-to-fore largely relied on immunocytochemical approaches to detect microscopic foci formation to study the DNA repair mechanism(s), replication and recombination.\textsuperscript{10–12}

The intensity of γH2AX immunofluorescence (IF) measured by cytometry has been reported to correlate with the dose of IR.\textsuperscript{2,13,14} However, the frequency of γH2AX detected varies as a function of the phase of the cell cycle.\textsuperscript{15} This observation highlights the potential usefulness of flow cytometry in detecting γH2AX. When combined with DAPI nuclear staining, not only can the frequencies of γH2AX be integrated across the cell cycle, but specificity in quantification as a function of the specific phase of the cell cycle can be determined. Flow cytometric methods have been employed to assess drug- or UV-induced DNA damage related to apoptosis,\textsuperscript{16–20} At present, however, assessment of IR-induced γH2AX using flow cytometry is quite limited and usually expressed only in a relatively semi-quantitative manner.\textsuperscript{2,13,15,21} A further improvement and refinement is needed to develop a more rapid and quantitative technique to measure IR-induced DNA DSBs.

To assess DNA DSB damage following IR, we applied the flow cytometric analysis for the detection of γH2AX quantification and provided automated digital microscopic foci analysis using NIH ImageJ macro software (Appendix). We then compared these data with data obtained using flow cytometric analysis for IR-induced γH2AX measurement combined with DAPI staining. Because about two thirds of the biologic damage induced by low linear energy transfer (LET) IR, such as X-rays, is through the generation of highly reactive free radicals, i.e., the indirect effect of IR on DNA,\textsuperscript{22} we also investigated the role of a thiol containing free radical scavenger, N-acetyl-L-cysteine (NAC), on mediating the formation of IR-induced γH2AX across all phases of the cell cycle.

MATERIALS AND METHODS

Cells and culture conditions

Endothelial cells from human dermis immortalized with SV40 (HMECs)\textsuperscript{23} were obtained from Dr. T. J. Lawley, BioLogic Products Branch, Centers for Disease Control, Atlanta, GA. Cells were maintained in endothelial basal medium MCDB131 (Gibco/BRL, Grand Island, NY) supplemented with 15% fetal bovine serum (FBS) (Sigma, St. Louis, MO) 10 ng/mL epidermal growth factor (Collaborative Biomedical Products, Bedford, MA), 1 µg/mL hydrocortisone (Sigma), penicillin, and streptomycin (Gibco/BRL). HMECs were incubated in a humidified atmosphere of 5% CO\textsubscript{2} and 95% air at 37°C, subcultured weekly and new cell stocks were used every 3 months. HMECs were grown to confluence for all experiments and trypsinized with 0.25% trypsin and 1 mM EDTA (EDTA/Trypsin; Gibco/BRL).

Irradiation and a drug treatment conditions

Confluent HMECs were irradiated at room temperature by an X-ray generator (RT250, Phillips, Holland) operating at 250 kVp and 15 mA at a dose rate of 1.65 Gy/min. Radiation doses ranged from 0 to 16 Gy. NAC (Sigma) was dissolved in phosphate buffered saline (PBS; 8.1 mM Na\textsubscript{2}HPO\textsubscript{4}, 1.5 mM KH\textsubscript{2}PO\textsubscript{4}, 140 mM NaCl; Gibco/BRL) at a final concentration of 4 mM and was added to cells 30 minutes before irradiation and maintained throughout the procedure up to 1 hour after irradiation at which time the cells were harvested.

Cell preparation for γH2AX analysis

HMECs were grown to confluence in 60 mm dish. The preparation for spotting DSBs in cells it is very time consuming. The measurement of γH2AX within cells have here-to-fore largely relied on immunocytochemical approaches to detect microscopic foci formation to study the DNA repair mechanism(s), replication and recombination.\textsuperscript{10–12}

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plotted on the y-axis and an intensity level of 100. Contour or dot plots were used to set the gating strategy. At least three independent gating measurements were performed using contour patterns and the data were presented as a mean with standard deviation (SD).

**Quantification of microscopic γH2AX foci formation**

Microscopic γH2AX foci were immunolocalized and imaged using an automated multiparameter digital image microscope using the same samples that were prepared for flow cytometry. Cells were counterstained with 1 µg/ml DAPI and placed on a cover slip at the bottom of 35 mm dish. Cellular morphology and fluorescence were examined under an automated multiparameter digital image microscope (Olympus IX81), utilizing immersion oil with a 60x (N.A. 1.45) objective lens. The FITC and DAPI digital images were captured serially (MetaMorph, Universal Imaging Corp.), and more than 10 fields containing 100 cells were stored. Data were analyzed on a per nucleus basis using an NIH ImageJ software (v.2.34i) with a custom macro function designed to count particles. Nuclear boundaries and foci were automatically identified in images by a threshold algorithm on digitally processed images resulting in nuclear outlines along with the number of foci per cell and details of intensities and morphometry. The custom macro program for NIH ImageJ is available as an Appendix.

**RESULTS**

**γH2AX staining conditions for flow cytometric analysis**

We first examined the staining procedure to determine the conditions that might affect the relative intensity of γH2AX fluorescence. Confluent HMECs were irradiated with 8 Gy, harvested 1 hour after IR and fixed with 2% PFA. To assess the importance of the alcohol permeabilization step on fluorescence intensity, fixed cells were divided into two groups and separately permeabilized with either 70% ethanol or 90% methanol and then kept at 4°C or ~20°C, respectively. Cells were stained with either FITC- or A647-γH2AX antibodies, each at 4 µg/ml concentration.

Fig. 1a shows single stained FITC histograms derived under the various conditions. In general, histograms shifted to the right reflecting a higher γH2AX intensity in 8 Gy irradiated as compared to control groups with the exception of the ethanol permeabilized A647-γH2AX stained group. Under this experimental condition the histogram describing the irradiated cell population did not clearly separate from the untreated control background (Fig. 1a), making quantification of the γH2AX cell population induced by IR difficult. The resulting histograms are characterized as monophasic. Therefore, the mean, geometric mean and/or median fluorescence ratio can be calculated using relative γH2AX fluorescence intensities. This is done by taking the ratio of the histograms representing the irradiated to that of the control cells (Fig. 1b). Although use of these three parameters appears to yield similar ratios, the use of the median values represents the best choice due to the monophasic characteristic of the peaks.

From these results, the permeabilization step using methanol as compared to ethanol is superior for both FITC or A647-γH2AX staining. Both ethanol and methanol were equally effective in FITC-γH2AX staining. The reason for this discrepancy in the use of the two alcohols is not apparent at present. It may be due to differences related to quenching of Alexa647, the antibody concentration used, or differential effects on PFA in the fixation process.

**Bivariant flow cytometric analysis of γH2AX with DAPI-combined staining**

The use of single staining procedures to generate histograms reflecting γH2AX lacked the precision to effectively generate and analyze sufficiently quantitative data. The addition of DAPI staining offered the advantage of assessing γH2AX frequencies as a function of the phase of the cell cycle. This is important since it is known that background γH2AX intensity also varies as a function of the cell cycle phase.\(^\text{2,15}\)

To analyze γH2AX frequencies under conditions of mixed staining that included with DAPI, we used contour plotting to gate γH2AX positive cells from the remainder of the population expressing background γH2AX intensities. The intensity of FITC positive fluorescing cells was evaluated at 10% increments using contour plotting with the aid of FlowJo software. The resulting data are presented in both contour and the pseudo-color dot plots for comparison (Fig. 1c). A representative cell cycle distribution pattern is show in Fig. 1d. The results using this gating approach on the same samples are shown in Fig. 1e and 1f.

These data demonstrate the advantage of combining both γH2AX and DAPI staining on the same population of cells to obtain quantitative data on γH2AX formation as a function of the cell cycle as well as for better discrimination against variable cell cycle related background γH2AX intensities. It is important to note that in this experiment we used HMECs grown to full confluence and assessed γH2AX formation within 1 hour after IR exposure. Under these conditions no change in cell cycle distribution was observed.

**γH2AX antibody concentrations for bivariant flow cytometric analysis**

Although the antibody concentration of 4 µg/ml used in the above experiments was useful to discriminate the γH2AX positive population of cells from the negative-background population as shown in Fig. 1, we wanted to determine if there is a more optimal antibody concentration for γH2AX detection when DAPI is used in the combined staining method. First, we applied FITC- or A647-γH2AX antibody concentrations ranging from 4 to 100 µg/ml to confluent

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Fig. 1. Staining conditions for the flow cytometric detection of γH2AX formation induced in confluent HMECs by 8 Gy of IR. Cells were harvested 1 hour following IR, fixed with PFA, permeabilized with either ethanol or methanol and stained with either FITC- or A647-γH2AX antibodies and counter stained with DAPI. (a) Change in relative position of histograms describing γH2AX IF intensities as a function of radiation and permeabilization procedure. (b) Relative γH2AX IF values determined as the ratio of median values from histograms describing the different treatment conditions. (c) Demonstration of bivariant DAPI-combined γH2AX analysis. Cell cycle analysis is displayed using pseudo-color dot plots and contour plots with gating set at 10% increments for both control and IR groups. (d) Representative cell cycle distribution of confluent HMEC used in this study. There was no cell cycle phase difference between the control and irradiated cells analyzed 1 hour after 8 Gy. The combined percentages of control and irradiated groups are shown for each cell cycle phase. (e and f) Percent γH2AX positive cell frequencies derived from bivariant flow cytometric analysis as a function of antibody used and alcohol permeabilization method applied. Gating is set at 10% increments as shown in (d).

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ent HMECs irradiated with 8 Gy and assessed 1 hour after irradiation. The result was that a 4 μg/ml concentration of antibody was the most effective concentration with respect to measuring γH2AX frequencies for both of the fluorescent dye-conjugated antibodies used (data not shown). Next we evaluated both antibodies using confluent HMECs irradiated with 16 Gy. Antibody concentrations ranged from 1 to 8 μg/ml. As shown in Fig. 2a and 2b, both antibodies were most effective in the 1–2 μg/ml concentration range. These concentrations yielded the highest values of γH2AX frequencies detected. The relative efficiency of detection decreased as the antibody concentration was increased from 4 to 8 μg/ml. These results also reflect a higher frequency of γH2AX positive cells following 16 Gy as compared to 8 Gy exposures (see Fig. 1). From these results we chose to stain for γH2AX using antibody concentrations in the 1 to 2 μg/ml range for subsequent bivariant flow cytometric analysis.

**Automated digital microscopic foci analysis for γH2AX formation and its relationship to bivariant flow cytometric analysis**

To determine whether values of γH2AX frequency assessed using our flow cytometric method would correlate with those determined using a visual microscopy based method of detection, we utilized a standard cell/nucleus counting method. Cells were stained with FITC-γH2AX and DAPI. Identification and counting of positive cells containing γH2AX foci was made using the NIH ImageJ macro program which is provided as an Appendix. For this purpose we used the same cell samples scored by flow cytometry analysis to compare values obtained with the visual microscopy foci scoring method. Representative images obtained using an automated multiparameter digital image microscope are shown in Fig. 3a. The digitally captured images were further analyzed using the NIH ImageJ macro software. Unirradiated control HMEC images are shown at the top panel and images of cells irradiated with 8 Gy are shown in the lower panel. γH2AX foci and DAPI stained nuclei are shown in the left panel and the images used in automatic cell counting are shown in the right panel. The numbers reported represent positive cells for γH2AX as judged by numbers of foci counted representing values greater than the mean + 2SD as derived from the results presented in Fig. 3b. The distribution of background γH2AX foci/nucleus in untreated cells seems to fall below 10 with the average number being 3.6 with an SD of 3.9. If we take the mean + 2SD as a cutoff point, the upper limit of γH2AX in untreated cells as background number for future analysis is 11.4 foci per nucleus (Fig. 3b).

A comparison of the frequencies of γH2AX determined by the two methods for cells irradiated with 8 Gy is presented in Fig. 3c. γH2AX foci formation frequencies determined using the visual microscopy method with cutoffs set at two different SD (Mean + 2SD and +3SD) were compared with those obtained using flow cytometric analysis under two different gating conditions, i.e., 10% and 15% increments, respectively. The two methods yield substantially similar data with correlations of data sets being dependent on the respective threshold and gating values chosen.

**IR-induced γH2AX and phase of cell cycle**

A bivariant flow cytometric method was used to quantify the values of IR-induced γH2AX formation in confluent HMEC populations 1 hour following IR with doses ranging from 2 to 16 Gy (Fig. 4). Alterations in γH2AX frequency histograms (Fig. 4a), relative γH2AX frequencies (Fig. 4b), γH2AX single stained histograms (Fig. 4c), bivariant cell clustering distributions (Fig. 4d), radiation dose response of γH2AX formation (4e), and cell cycle distribution of γH2AX formation as a function of radiation dose (4f) are presented for comparative purposes and to describe the utility of this flow cytometric method in measuring γH2AX formation as a biomarker in irradiated cells. IR-induced γH2AX frequencies increased as a function of radiation dose up to 16 Gy with the response being linear up to 8 Gy. Between 8 and 16 Gy the dose response curve describing γH2AX formation

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**Fig. 2.** Determination of γH2AX formation detection as a function of γH2AX antibody concentrations used. Confluent HMECs were X-irradiated with 16 Gy and assessed 1 hour after IR by staining either FITC- or A647-γH2AX antibody.
Fig. 3. γH2AX foci formation using an automated multiparameter digital image microscope system. The images were captured using the same samples prepared for flow cytometric analysis. The captured digital images were further analyzed with NIH ImageJ custom macro (see Appendix). (a) Images of unirradiated control HMECs are shown in the top panel and the irradiated HMECs, 1 hr following 8 Gy, in the bottom panels. (b) γH2AX foci counted in unirradiated HMECs. Background or intrinsic average foci numbers and associated standard deviations were calculated and the mean + 2 SD was used as the threshold for the determination of positive cells shown in (a). (c) Correlation between flow cytometric analysis and microscopic foci analysis. γH2AX positive percentages using 10% and 15% contour increments are shown for flow cytometric analysis. Foci counting using digital microscopy utilized thresholds of mean + 2 SD and 3 SD to demonstrate the correlation between the two methods for the determination of γH2AX percentage.
Fig. 4. Radiation dose response of γH2AX using bivariant flow cytometric analysis. Confluent HMECs were irradiated with 2–16 Gy X-rays and γH2AX was assessed 1 hour after IR. All of the calculated results are shown for comparison purposes. (a) Changes in histogram position as a function of radiation dose. (b) Resulting γH2AX ratio as a function of geometric mean, mean, or median histogram values. (c) γH2AX percentages as a function of dose determined by gating of histograms derived from single stained cells. (d) γH2AX percentages as a function of dose determined by bivariant analysis combined with DAPI staining. The gating was set as shown in Fig. 1d. (e) γH2AX percentages ± SD as a function of radiation dose. (f) γH2AX percentages ± SD as a function of radiation dose and position in the cell cycle.

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flattened suggesting a saturation of the system in this very high dose range. IR induced $\gamma$H2AX formation in all phases of the cell cycle with the induction in G1 and G2 higher than that observed in S phase cells (Fig. 4f).

**Kinetics of IR-induced $\gamma$H2AX and its decay analyzed by bivariant flow cytometry**

A bivariant flow cytometric method was further utilized to analyze both IR-induced $\gamma$H2AX formation and its kinetics of decrease in confluent HMEC at 1, 4 and 24 hours following 2–16 Gy (Fig. 5). A radiation dose-dependent $\gamma$H2AX increase followed by a decrease as a function of time were evident as shown in Fig. 5. Induced $\gamma$H2AX returned to normal levels at 24 hours following IR exposure at every radiation dose used.

**Inhibition of IR-induced $\gamma$H2AX by a free radical scavenger NAC**

The bivariant flow cytometric method was applied to determine the effects of a free radical scavenging agent on the induction of $\gamma$H2AX in HMECs by IR. The free radical scavenger NAC was added to cultures of HMECs grown to confluence at a concentration of 4 mM for 30 min just prior to exposure to 8 Gy of IR. $\gamma$H2AX formation was assessed 1 hour after irradiation. As shown in Fig. 5, NAC reduced not only the $\gamma$H2AX formation in the population as a whole (Fig. 6a) but also in all phases of the cell cycle (Fig. 6b). Of considerable importance, the bivariant flow cytometric method also allowed for the determination that NAC exhibited a cell cycle phase-dependent radioprotective response (G1>G2>S) in an asynchronous population of cells.

**DISCUSSION**

Mammalian histone H2AX is a variant of H2A and accounts for approximately 2–10% of total histone H2A. The kinases that phosphorylate H2AX are nuclear PIKKs (phosphatidylinositol 3-OH serine/threonine protein kinase-like kinases) that include ATM (ataxia telangiectasia mutated), ATR (ATM and Rad3-related) and DNA-PK (DNA-dependent protein kinase). ATM and DNA-PKcs have been shown to phosphorylate histone H2AX in response to IR. During DNA synthesis the stalled replication forks as well as UV-induced lesions are regulated prominently by ATR. Under each of these conditions, the high concentration of $\gamma$H2AX protein localized in chromatin adjacent to DSBs can be detected by IF giving rise to easily identifiable distinct foci. It has been proposed that $\gamma$H2AX may function as an anchor to hold broken DNA ends in close proximity in chromatin, facilitating the DNA repair. As such, it has been reported that cells exhibiting enhanced radiosensitivity exhibit a relatively slower rate of $\gamma$H2AX loss and a higher retention of $\gamma$H2AX foci.

Because $\gamma$H2AX foci appear within minutes after IR exposure, $\gamma$H2AX focus formation is considered to be a sensitive and selective signal for identifying the existence of DSBs in cells. However, $\gamma$H2AX appears to be implicat-


ed in a number of a cellular responses that include mitotic DSB repair in yeast,\textsuperscript{11} V(D)J recombination in lymphoid cells\textsuperscript{10} and meiotic recombination in mice.\textsuperscript{12} During DNA replication arrest\textsuperscript{15} scheduled H2AX phosphorylation can occur as a result of intrinsic enzymatic processes. That can lead to a background frequency that is observed in untreated control cells.\textsuperscript{17,21} Thus, not all γH2AX foci that are detected are due solely to exogenously induced DNA damage but may also reflect the byproduct of normal cellular processes.

There are a number of methods now in use to detect γH2AX foci in cells. These include Western blotting, immunoprecipitation, flow cytometry, laser scanning cytometry and visual microscopy based methods. Of these methods, measurement of γH2AX IF by multiparameter flow cytometry\textsuperscript{2,20} is particularly advantageous. Large numbers of cells can be rapidly analyzed and the data can provide information relating to γH2AX incidence as a function of cell cycle distribution, the identification of rare cell subpopulations that might otherwise go undetectable by techniques such as Western blotting or microscopy. However, there are several important factors that must be addressed if cytometric techniques are to be used to quantitatively detect and analyze γH2AX incidence in IR exposed populations.

A potential confounding factor is the induction of the apoptotic process in radiation exposed cells. A number of papers have reported that the relative intensity of γH2AX IF measured by flow cytometer increases with the dose of IR or toxic chemicals used.\textsuperscript{2,13,20,21} Following exposure to these deleterious agents both damaged mitotic cells and cells undergoing apoptosis can express very high levels of γH2AX.\textsuperscript{4,17} The degree of H2AX phosphorylation in response to DSBs generated during apoptotic process has been reported to be many-fold higher compared with that representing initial or primary DSBs induced by damaging agent.\textsuperscript{19} The appearance of early apoptotic cells are easily identified in flow cytometric generated histograms and appear as a subpopulation cluster expressing 5 to 10 fold higher levels of γH2AX than are observed in non-apoptotic cells.\textsuperscript{15} This is due to the extensive DNA fragmentation that occurs during an apoptotic process. Thus, the challenge is to identify and dissociate the damage component occurring from the apoptotic process from that induced directly by the genotoxic agent such as IR.\textsuperscript{20}

As demonstrated in the histograms presented in Figs. 1a and 4a, cells exhibiting γH2AX formation following IR clearly overlap regions known to represent untreated control cells. Regardless, it is clear that the frequency of γH2AX foci increased with increasing doses of radiation.\textsuperscript{13,15} However, when we used the geometric mean, mean or median of these histograms we were able to calculate values representing a fraction of induced the γH2AX as the fold increase over the untreated population (Fig. 1a and Fig. 4a) resulting in a clear IR-dose response pattern (Fig. 4b). The relative level of γH2AX is defined as the ratio of the detectable γH2AX IF signal measured from the entire histogram from the irradiated populations to that of the unirradiated control. Ratios were obtained using comparisons of the geometric means, means or medians of the respective histograms. These parameters are easily calculated using FlowJo software as shown in Fig. 1b and Fig. 4b. Although each of these three parameters gave rise to qualitatively similar results, a comparison of median values was chosen as the most appropriate way to analyze these distribution histogram analyses. The median value approach has the advantage of not being heavily weighted by outliers as the mean value is. This is especially important when the frequency of positive γH2AX cells at a relatively low percentage as might be expected following exposure to low doses of IR.

In addition to the technical difficulty of gating due to the overlapping histograms, other biologically based considerations should be addressed. Cells in each phase of cell cycle will express fluorescence from γH2AX foci, with the intensity varying as a function of position within the cell cycle phase.\textsuperscript{15} Adjustment must be made to address this phenomenon to maximize the quantization of the flow cytometric method for γH2AX detection.\textsuperscript{13,15} Not only that, the level of the substrate H2AX is reported to be variable between different cell types and is dependent on total DNA content.\textsuperscript{5,15} Thus, not only the DNA content doubles during cell cycle progression, but histone content also doubles throughout the cell cycle. Therefore cells in S and G2/M have 1.5- and 2-fold higher levels of H2AX than G1 cells\textsuperscript{15} while the ratio of histone to DNA content remains invariable in cells.\textsuperscript{38}

In the case of stalled replication forks, unlike IR, H2AX phosphorylation is regulated prominently by ATR.\textsuperscript{32} This also occurs following exposure of cells to UV\textsuperscript{26,33} Stalled replication forks and UV-induced DNA lesions can result in DSBs and γH2AX formation predominantly in S phase cells.\textsuperscript{20} However, the intrinsic or background γH2AX are generally smaller in size and less distinct as compared with foci that are induced by genotoxic agents.\textsuperscript{2,17} UV-induced γH2AX in S phase cells are reported as resembling those induced by DNA topoisomerase inhibitors that can trigger apoptosis.\textsuperscript{19} These examples illustrate the importance of including cell cycle analysis to maximize the quantization of the flow cytometric assays in the detection of γH2AX formation frequencies following IR exposure of cells.

We quantitatively evaluated and compared the methods of automated digital microscopy and flow cytometry to detect γH2AX frequencies in cells treated under the same conditions. The automated digital microscopy method is generally well accepted for the detection of γH2AX foci because cells expressing this damage can be readily identified and thresholds for determining positive cells are easily set. As demonstrated in Fig. 3a, b, c both methods gave similar results. The automated digital imaging method we employed also included the use of NIH ImageJ macro software to assist in setting the appropriate thresholds. It is important to note that corre-
lation between the two methods is highly dependent upon the settings of the respective threshold or gating values used (Fig. 3c).

The frequency of $\gamma$H2AX formation as reflected by the intensity of IF increased linearly with the amount of damage induced\(^7\) but the accuracy of measuring discrete foci (size, number and intensity) in whole nuclei could be compromised somewhat when doses higher than 2 Gy (50 or so breaks per diploid cell) are used.\(^2,13\) This can be a limiting factor in the use of visual based microscopy based techniques to quantitatively measure $\gamma$H2AX frequencies at higher radiation doses.

While flow cytometry based approaches may lack some of the specificity in $\gamma$H2AX detection that can be attained with visual/digital based microscopy methods, they offer an advantage for rapid and mass screening of DNA damage in a quantitative manner. Not only can these approaches be used to routinely measure and evaluate in a rapid manner drug effects or radiation induction of $\gamma$H2AX formation, but cell cycle dependent effects can also be measured at the same time. It is known that DNA repair processes involving HR and NHEJ are dependent upon cell cycle phase.\(^39\) If these are involved in the removal of $\gamma$H2AX, flow cytometric approaches will be highly useful in assessing the kinetics of damage removal as a function of position in the cell cycle especially since most of these studies are performed using asynchronously growing populations of cells.

We have applied the present method routinely at radiation doses ranging from 1–8 Gy using other cell lines such as Chinese hamster AA8, several human head-and-neck cancer cell lines and human colorectal cancer cell lines, each exhibiting a strong radiation dose response. Fig. 5 is a representative plot of $\gamma$H2AX kinetics.

The background level of $\gamma$H2AX is both cell-type-dependent and cell-phase-dependent; levels of $\gamma$H2AX in G0/G1 phase cells are about three times lower than cells in S and G2 phases.\(^15\) Although $\gamma$H2AX foci formation occurs both in non-replicating\(^5,24\) and replicating DNA,\(^27\) histone H2AX phosphorylation has been reported to be more robust in cycling cells as compared to the cells arrested in G1.\(^15,40\) For these reasons we limited our current study to assessing $\gamma$H2AX under a confluent condition. $\gamma$H2AX formation induced by IR was observed as expected in all phases of the cell cycle (Fig. 4d and 4f). S phase cells exhibited a lower frequency of $\gamma$H2AX formation as compared to G1 or G2/M phase cells (Fig. 4f). Furthermore, the magnitude of $\gamma$H2AX formation induced by IR could be reduced with the use of a radioprotective drug. One such drug, NAC, if added to cells prior to IR, resulted in a reduced frequency of $\gamma$H2AX formation in all phases of the cell cycle (Fig. 6b). NAC is known to protect cells by scavenging ROS.\(^41\)

We have developed and contrasted to a well established digital microscopy based technique and a flow cytometric based method. Using bivariant flow cytometric method it was possible to detect and quantify in a rapid manner the frequency of $\gamma$H2AX formation induced by IR. Quantification of $\gamma$H2AX formation could be determined not only as a function of radiation dose but also as a function of cell cycle position.

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APPENDIX

A custom macro program for NIH ImageJ (file available on web)

// This macro adds particle analyzer outlines to the ROI Manager and uses those ROI
// to perform within-object counts on other color channels.
// This counts green particles per nucleus defined by DAPI outlines
// macro asks for input STK file
// ROI are numbered and overlaid on fitc image for documentation
// then save the RESULTS window data 'save as' EXCEL works nicely
// BE SURE TO CLOSE ALL IMAGE WINDOWS BEFORE RE-RUNNING
// modified 'RoiManagerAddParticles.txt' macro; balance by Vytas Bindokas, Univ. of Chicago, May 2005

// This is for 2-image stack, FITC then dapi order

requires("1.34k"); //this is the minimum ImageJ version needed to run macro

open();
t=getTitle();
t2= t + "ROI.tif";
setFont("SansSerif", 24);
run("Out"); //zoom out to make image appear smaller on screen
run("Convert Stack to Images");
run("Brightness/Contrast...");
run("Enhance Contrast", "saturated=0.5");
rename("dapi");
run("Out");
run("Duplicate...", "title=dapicopy");
selectWindow('0001');
run("Out");
rename("FITC");

selectWindow("dapi");
run("Subtract Background...", "rolling=60"); // flatten illumination
run("Gaussian Blur...", "radius=8"); // prep for watershedding
selectWindow("dapi");
setAutoThreshold();
// setThreshold(20, 255);
run("Threshold", "thresholded remaining black");
//run("Watershed"); //split apart touching nuclei

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// trace nuclear edges and build ROI list; edge particles are excluded here
run("Colors...", "foreground=black background=white selection=yellow");
selectWindow("dapi");
run("Analyze Particles...", "minimum=3000 maximum=15000 show=Nothing record exclude");
o = nResults;
for (i=0; i<o; i++) {
x = getResult(XStart, i);
y = getResult(YStart, i);
doWand(x,y); // this step does the tracing, next builds the ROI list
roiManager("add");
}

// switch to FITC staining for measurements within nuclear boundaries
selectWindow("FITC");
run("Set Scale...", "distance=1 known=.10729 pixel=1 unit=um "); // this calibrates for 60x oil objective
run("Set Measurements...", "area mean limit display redirect=None decimal=4");
n = roiManager("count");
run("Colors...", "foreground=white background=black selection=yellow");
roiManager("deselect");
for (i=0; i<n; i++) {
    write("foci for nucleus "+i);
    run("Analyze Particles", "minimum=3 maximum=999999 show=Nothing display");
    setThreshold(1180, 4095); // set FITC threshold as needed here
    roiManager("select", i);
write("FITC intensity for nucleus: "+i);
resetThreshold();
}
selectWindow("dapi");
run("Close");
run("RGB Merge", "red=*
None* green=FITC blue=dapicopy");
rename(t2);
selectWindow("Results");
