Video Article

Quantification of γH2AX Foci in Response to Ionising Radiation

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Abstract

DNA double-strand breaks (DSBs), which are induced by either endogenous metabolic processes or by exogenous sources, are one of the most critical DNA lesions with respect to survival and preservation of genomic integrity. An early response to the induction of DSBs is phosphorylation of the H2A histone variant, H2AX, at the serine-139 residue, in the highly conserved C-terminal SQEY motif, forming γH2AX[1]. Following induction of DSBs, H2AX is rapidly phosphorylated by the phosphatidyl-inosito 3-kinase (PIKK) family of proteins, ataxia telangiectasia mutated (ATM), DNA-protein kinase catalytic subunit and ATM and RAD3-related (ATR)[2]. Typically, only a few base-pairs (bp) are implicated in a DSB, however, there is significant signal amplification, given the importance of chromatin modifications in DNA damage signalling and repair. Phosphorylation of H2AX mediated predominantly by ATM spreads to adjacent areas of chromatin, affecting approximately 0.03% of total cellular H2AX per DSB[2,3]. This corresponds to phosphorylation of approximately 2000 H2AX molecules spanning ~2 Mbp regions of chromatin surrounding the site of the DSB and results in the formation of discrete γH2AX foci which can be easily visualized and quantitated by immunofluorescence microscopy[2]. The loss of γH2AX at DSB reflects repair, however, there is some controversy as to what defines complete repair of DSBs; it has been proposed that rejoining of both strands of DNA is adequate however, it has also been suggested that re-instatement of the original chromatin state of compaction is necessary[4-8]. The disappearence of γH2AX involves at least in part, dephosphorylation by phosphatases, phosphatase 2A and phosphatase 4C[5,6]. Further, removal of γH2AX by redistribution involving histone exchange with H2A.Z has been implicated[7,8]. Importantly, the quantitative analysis of γH2AX foci has led to a wide range of applications in medical and nuclear research. Here, we demonstrate the most commonly used immunofluorescence method for evaluation of initial DNA damage by detection and quantitation of γH2AX foci in γ-irradiated adherent human keratinocytes[9].

Video Link

The video component of this article can be found at https://www.jove.com/video/1957/

Protocol

Cell Preparation

1. Human keratinocytes (FEP-1811) were grown in Keratinocyte-Serum Free Medium (K-SFM; Invitrogen) supplemented with epidermal growth factor, bovine pituitary extract and 20 µg/ml gentamicin, at 37°C and 5% CO_2.
2. A single cell suspension was prepared by detaching with trypsin-EDTA (0.05% v/v)
3. Cells were seeded in 8-well Lab Tek II microchamber slides (10,000 cells/well) and slides were incubated for 3 days at 37°C and 5% CO_2.

Irradiation

1. Cells were irradiated on ice with 2 Gy using a ^{137}Cs source (Gammacell 1000 Elite irradiator; Nordion International, ON, Canada; 20.6 seconds/Gy)
2. Unirradiated control and 2 Gy irradiated cells were incubated for 1 hour at 37°C and 5% CO_2.

Immunofluorescence staining

1. Media was tipped off and cells were washed with 300µl of PBS (w/o Ca^{2+} or Mg^{2+}) per well and were rotated on an orbital mixer for 5 minutes.
2. The buffer was tipped off and 100µl of freshly prepared 4% (v/v) paraformaldehyde was added to each well and slides were incubated at room temperature for 10 minutes.
   All incubations were performed in a humidified staining trough

3. Cells were then washed with PBS (w/o Ca^{2+} or Mg^{2+}). Slides were placed in a Coplin jar and rotated on an orbital mixer for 5 minutes. This wash step was repeated a further two times.
4. The buffer was tipped off and excess PBS was gently blotted.
5. Cells were permeabilized using 100ml Triton X-100 (0.1% v/v) per well and a 10 minute incubation at room temperature.
6. Cells were washed with PBS (w/o Ca^{2+} or Mg^{2+}) as described in steps 3 and 4 above.
7. Non-specific protein binding was blocked with 100ml of BSA (1% v/v) per well and 20 minute incubation at room temperature.
8. Excess BSA was tipped off and 100µl of primary mouse monoclonal anti-phospho histone-H2AX antibody (diluted 1:500, in 1% BSA; Millipore), was added to each well for a 1 hour incubation at room temperature.
9. Cells were washed with PBS (w/o Ca^{2+} or Mg^{2+}) as described in steps 3 and 4 above and incubated with 100µl of secondary antibody (Alexa Fluor 488 goat anti-mouse IgG diluted 1:500, in 1% BSA; Invitrogen) per well for 45 minutes at room temperature in the dark. (Diluted antibody was kept in the dark throughout the procedure)
10. Cells were washed with PBS (w/o Ca^{2+} or Mg^{2+}) as described in steps 3 and 4 above. However, exposure to light was minimized using foil.
11. Nuclear counterstaining was performed with 100ml TOPRO3 (diluted 1:500; Invitrogen) per well and a 10 minute incubation at room temperature
12. Cells were washed with PBS (w/o Ca^{2+} or Mg^{2+}) as described in step 10 above.
13. The chambers were carefully removed from the slides, excess moisture was blotted and slides were allowed to air dry.
14. One drop of ProLong GOLD anti-fade solution (Invitrogen) was added per well and slides were mounted (22x50 mm coverslip) and any excess liquid around the edges of slide was blotted.
15. The slides were kept in the dark for a further 30 minutes at room temperature before sealing with nail polish.
16. The slides were stored overnight at 4°C in the dark before analysis.

Microscopy / Analysis

1. Zeiss LSM510 Meta Confocal Microscpe used to acquire images using the standard GFP (for γH2AX - Alexa Fluor 488 goat anti-mouse IgG) and far red lasers (for TOPRO-3). Typically, a 63 x oil immersion objective lens is used.
   Images are acquired in a Z-series pattern with a step size of 0.5 µm. A step size of 0.5 µm was chosen to minimize loss of foci present in different planes in the nuclei. During analysis, individual planes are deconvoluted and stacked to produce a maximum projected image to minimize the overlap of foci (Top-hat filter applied).
2. Metamorph (Molecular Devices, USA) was used to analyse number of foci.
   The program quantitates the number of foci in each cell after the threshold has been applied to exclude background. The information is logged in a Microsoft Excel spreadsheet for further analysis.

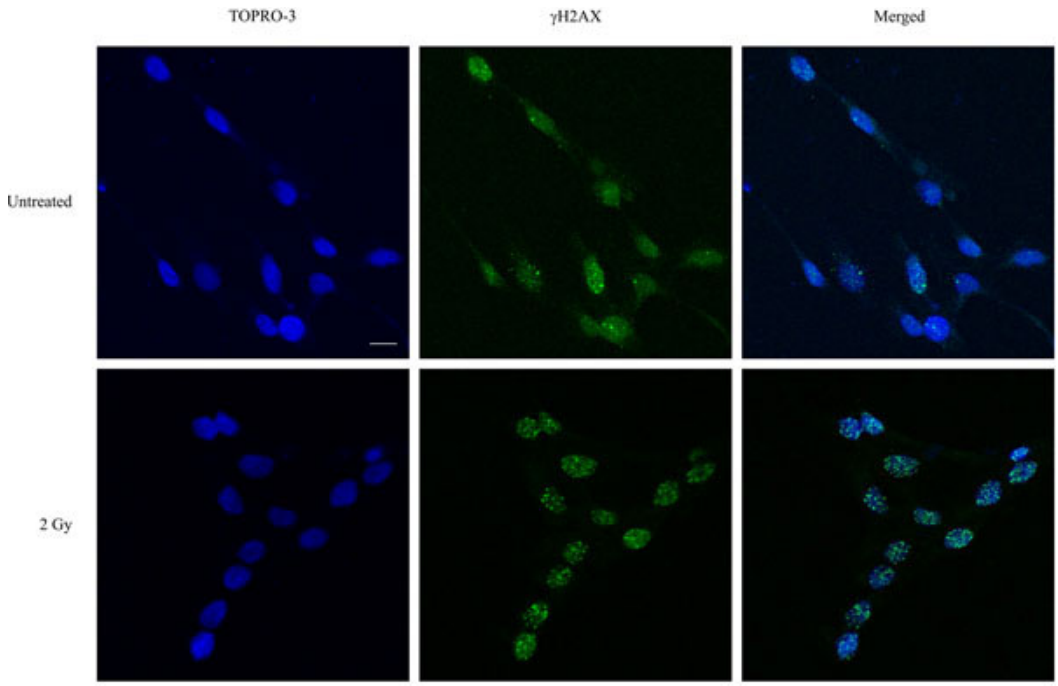

Figure 1. Immunofluorescence visualization of γH2AX foci (green) in untreated human keratinocytes and in cells irradiated with 2 Gy and incubated for a further 1 hour at 37°C, 5% CO_2. DNA was stained with TOPRO-3 (blue). Images were acquired using a Zeiss LSM 510 Meta Confocal microscope. Bar = 10 µm.

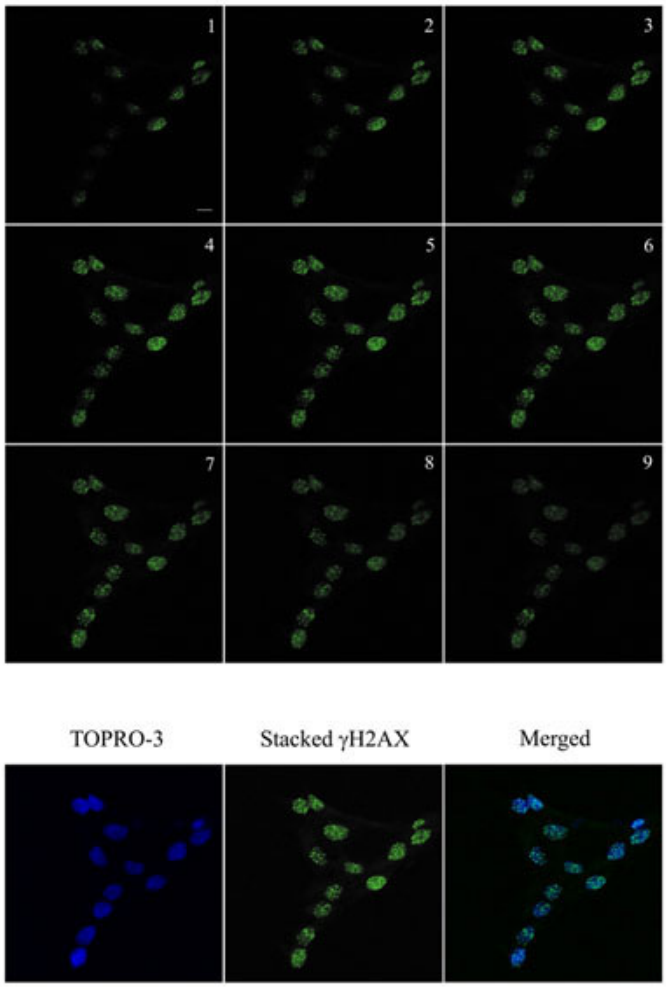

Figure 2. Immunofluorescence visualization of γH2AX foci (green) in human keratinocytes and in cells irradiated with 2 Gy and incubated for a further 1 hour at 37°C, 5% CO_2. Images were acquired using a Zeiss LSM 510 Meta Confocal microscope using 0.5 µm Z-sectioning (1-9) to ensure all foci were acquired. The images was then stacked for quantitation using Metamorph. DNA was stained with TOPRO-3 (blue). The stacked γH2AX and blue images were stacked for visualization. Bar = 10 µm.

Discussion

Following exposure to ionising radiation (γ-rays), γH2AX foci form rapidly and foci numbers reach a maximum between 30-60 minutes[2]. Therefore, our 1 hour post-irradiation time point reflects initial DSB formation. We have used the clinically relevant radiation dose of 2 Gy for our experiment. However, the method can be used for radiation doses up to 4 Gy for detection of initial DSB formation; significant overlap of foci precludes accurate quantitation at higher doses. Higher radiation doses may be used for longer post-irradiation incubation times, as γH2AX foci are lost due to repair, resulting in quantifiable numbers. Typically, 4 hour and up to 24 hour post-irradiation incubation times are used for monitoring DNA repair. We have used the DNA stain TOPRO-3 due to limitations in the excitation lasers of our confocal microscope. More commonly, 4,6-diamidino-2-phenylindole dihydrochloride (DAPI) is used for nuclear counterstaining. Although we used a confocal microscope, epifluorescent microscopes with z-sectioning capacity are adequate. The immunofluorescence method is suitable for other adherent cancer and normal cell lines; we have tested T98G human glioblastoma and normal human endothelial cells and rat H9c2 embryonic ventricular myocytes.

Finally, quantitation of γH2AX is useful in the context of ionising radiation-induced DNA damage, for monitoring DNA damage – as illustrated by our experiment – and repair (radiation sensitivity). The assay is also useful for evaluating the efficacy of compounds that modulate cellular responses to radiation (i.e. radiation protectors and sensitizers). Further, γH2AX is emerging as a potential molecular marker in aging and disease, predominantly in cancer[10].

Disclosures

No conflicts of interest declared.

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