Published in final edited form as:

Methods Mol Biol. 2009 ; 523: . doi:10.1007/978-1-59745-190-1_11.

Cytometric Analysis of DNA Damage: Phosphorylation of Histone H2AX as a Marker of DNA Double-Strand Breaks (DSBs)

Toshiki Tanaka, Dorota Halicka, Frank Traganos, and Zbigniew Darzynkiewicz

Abstract

Phosphorylation of histone H2AX on *Ser* 139 is a sensitive reporter of DNA damage, particularly if the damage involves induction of DNA double-strand breaks (DSBs). Phosphorylated H2AX has been named γ H2AX and its presence in the nucleus can be detected immunocytochemically. Multiparameter analysis of γ H2AX immunofluorescence by flow or laser-scanning cytometry allows one to measure extent of DNA damage in individual cells and to correlate it with their position in the cell cycle and induction of apoptosis. This chapter presents the protocols and outlines applications of multiparameter cytometry in analysis of H2AX phosphorylation as a reporter of the presence of DSBs.

Keywords

γ H2AX; H2AX phosphorylation; DNA double-strand breaks; Multiparameter flow cytometry; Laser-scanning cytometry; Immunocytochemistry; Apoptosis

1. Introduction

DNA damage that involves formation of DSBs induces phosphorylation of *Ser*-139 at the carboxy terminus of histone H2AX (1, 2) one of several variants of the nucleosome core histone H2A (3–5). The phosphorylation takes place on H2AX molecules in megabase chromatin domains flanking the DSBs and is mediated by the PI-3-like protein kinases, ATM- (1, 2, 6, 7) ATR- (8), and/or DNA-dependent protein kinase (DNA-PK) (9, 10). The *Ser*-139 phosphorylated H2AX has been named γ H2AX (11). Development of an Ab specific to γ H2AX made it possible to detect H2AX phosphorylation and thus to assay immunocytochemically DNA damage and repair in situ, in chromatin of individual cells (12). Compared with the alternative method of DNA damage assessment which is based on analysis of electrophoretic mobility of DNA released from individual cells (comet assay) the immunocytochemical approach is less cumbersome and offers much greater sensitivity (13). Shortly after induction of DSBs, e.g., by ionizing radiation or other genotoxic agents, the presence of γ H2AX in chromatin can be detected with this Ab in the form of discrete nuclear foci (2, 11). Because each focus represents a single DSB2 their frequency is considered to report the incidence of DSBs. Several checkpoint and DNA repair proteins such as Rad50, Rad51, and Brca1 co-localize with γ H2AX (14). It was recently proposed that phosphorylated H2AX may function as an anchor holding broken DNA ends in close proximity in chromatin, facilitating their repair (15). γ H2AX also mediates translocation of the p53-binding protein 1 (53BP1) to the radiation-induced foci (7).

Western blotting and detection of γ H2AX immunofluorescence (IF) provide two major approaches to analyze H2AX phosphorylation. Measurement of γ H2AX IF by

multiparameter flow or laser-scanning cytometry (16–20) is particularly advantageous. The major benefit of the cytometric approach stems from the fact that H2AX phosphorylation in situ, in chromatin of individual cells, can be measured with high sensitivity and accuracy and the expression of γ H2AX can be directly correlated, within the same cells, with their DNA content, induction of apoptosis or any other cell attribute of interest. Large cell numbers, thus, may be rapidly analyzed and the data provide information on the extent of H2AX phosphorylation with respect to their cell cycle phase, commitment to die in response to DNA damage, their surface immunophenotype, etc. Cytometry also allows one to analyze intercellular variability in H2AX phosphorylation within cell populations and to identify rare cell subpopulations, otherwise undetectable by Western blotting. The chapter which follows is focused on applications of multiparameter cytometry in analysis of H2AX phosphorylation as a reporter of the presence of DSBs.

2. Materials

1. Primary antibody: anti-phospho-histone H2A.X (*Ser*-139) mAb, clone JBW301 (Upstate)
2. Secondary antibodies: Alexa Fluor 488 F(ab')₂ fragment of goat anti-mouse IgG (H +L) (Invitrogen)
3. Buffer: 1 × phosphate-buffered saline (PBS) (Sigma)
4. Solutions (stored at 4°C)
 - a 1% methanol-free formaldehyde (Polysciences, Inc.) dissolved in PBS
 - b 1% (w/v) solution of bovine serum albumin (BSA; Sigma) dissolved in PBS
 - b 1 µg/ml 4,6-diamidino-2-phenylindole (DAPI, Sigma) dissolved in PBS
 - d 0.1% Triton X-100 (Sigma) dissolved in PBS (*see* Note 2)
 - e 5 µg/ml PI in the presence of 100 µg/ml of RNase A (Sigma) dissolved in PBS
 - f 80% ethanol in dH₂O

3. Methods

3.1. Staining of Cells to be Measured by Flow Cytometry

3.1.1. Fixation of Cells

1. Suspend ~ 10⁶ cells in 0.5 ml of PBS.
2. Transfer this cell suspension to 5 ml of 1% formaldehyde in tube for 15 min at 1–4°C (on ice), then centrifuge (200 g, 5 min).
3. Suspend the pellet in 80% ethanol for at least 2 h; the cells can be stored in 80% ethanol for up to several days at –20°C.

3.1.2. Staining

1. Centrifuge (200 g, 5 min) cells suspended in 80% ethanol then rinse (200 g, 5 min them twice) with PBS.

²The presence of nonionic detergent such as Triton X-100 is helpful since it increases permeability of the plasma membrane to primary and secondary Abs

2. Add 2–3 ml 1% BSA solution into the tubes and centrifuge (200 g, 5 min).
3. Discard the supernatant and transfer the cells suspended in 100 µl of 1% BSA to new tubes.
4. Add 1 µl (1:100) γH2AX primary antibody into the suspension, cover the tubes with parafilm to prevent drying and incubate for 2 h at room temperature (*see* Note 1).
5. Add 2–3 ml 1% BSA into the tubes and centrifuge (200 g, 5 min).
6. Discard the supernatant, suspend cells to 100 µl 1% BSA solution and transfer to new tubes.
7. Add 1 µl (1:100) Alexa Fluor 488 anti-mouse antibody, cover the tubes to prevent drying and incubate for 30 min at room temperature in the dark.
8. Add 2–3 ml 1% BSA into the tubes and centrifuge them for 5 min.
9. Add 0.5–1.0 ml of 10 µg/ml propidium iodide (PI) – RNase solution into the tubes and transfer to tubes used for FACS-can. Incubate cells for 30 min at room temperature in the dark.
10. Measure the samples by FACScan.

3.2. Staining of Cells to be Measured by Laser Scanning Cytometry (LSC)

The cells may be deposited on slides either by growing them in “Chamber”-slide vessels (the cells that grow attached) or by attaching them to slides by cytocentrifugation (the cells that grow in suspension or cells detached by trypsinization).

3.2.1. Fixation (For Cells Growing on Chamber Slides)

1. Discard the medium and wash each chamber by PBS once.
2. Add ice-cold 1% formaldehyde into each chamber and fix for 15 min on ice.
3. Remove formaldehyde, wash each chamber by PBS once. (*see* Note 3)
4. Remove the chamber sides and immerse a slide into cold (–20°C) 80% ethanol. The slides can be stored in 80% ethanol for up to several days at –20°C.

3.2.2. Fixation for Cells Deposited on Slides by Cytocentrifugation

1. Place the cells-bearing slides in Coplin jars containing 1% formaldehyde at 0–4°C for 15 min
2. Rinse the slides with PBS and transfer them to Coplin jars containing 80% ethanol at –20°C and store them under these conditions

3.2.3. Staining

1. Wash the slide twice with PBS, for 5 min each time.
2. Incubate with 0.1% Triton X-100 for 15 min at room temperature.
3. Incubate with 1% BSA for 30 min at room temperature.

¹The concentration of H2AX Ab can be between 1:100 and 1:400. Titrate the Ab initially and use it subsequently at the concentration that gives maximal signal-to-noise ratio, i.e., the maximal IF difference between the untreated cells and the cells subjected to treatment that generated DNA damage

³Be careful while washing the cells growing on chamber slides because the cells in mitotic phase or undergoing apoptosis may be detached from the slide during vigorous washing.

4. Incubate with 50–100 μ l 1% BSA containing 1:200 H2AX antibody in each chamber area (100–200 μ l to each slide) for overnight at 4°C. Rigorously prevent drying by covering the site containing cells and antibody solution with a coverslip made of parafilm or polyethylene foil, and keeping the slides in the moist chamber.
5. Wash slide by PBS for 5 min once.
6. Incubate with 50–100 μ l 1% BSA containing 1:100 Alexa Fluor 488 antibody in each chamber area (100–200 μ l to each slide) for 40 min at room temperature preventing drying.
7. Wash a slide by PBS for 5 min once.
8. Incubate with 100 μ l 1 μ g/ml DAPI in each chamber area for 10 min at room temperature.
9. Wash a slide by PBS and put one drop of mounting (anti-fade) medium on each chamber or cytospin area, then cover it with the cover glass.
10. Measure cell fluorescence by LSC.

3.3. Instrument Settings

1. Flow cytometer

The fluorescence of red (PI) and green (Alexa Fluor 488) of individual cells induced by excitation with a 488 nm argon ion laser is measured using a FACScan cytometer (Becton Dickinson) with the standard optics and CELLQuest software (Becton Dickinson). At least 10,000 cells should be measured per sample. All experiments should be repeated at least three times.

2. LSC

Cellular blue (DAPI) and green (Alexa Fluor 488) fluorescence emission is measured simultaneously in the same cells using iCys LSC (CompuCyte) utilizing standard filter settings; blue and green fluorescence is excited with violet diode and 488-nm argon ion lasers, respectively. The intensities of integrated fluorescence and maximal pixel are measured and recorded for each cell. At least 3000 cells were measured per sample. Each experiment should be run at least in triplicate.

3.4. Results and Data Interpretation

The protocols should be able to measure the induction of H2AX phosphorylation in HL-60 cells treated with topotecan (Tpt) measured by FACScan as well as induction of H2AX phosphorylation in A549 cells treated with Tpt measured by LSC. As mentioned earlier, H2AX phosphorylation (γ H2AX expression) measured by cytometry is often a reporter of DNA damage, particularly when the damage involves formation of DSBs. This is the case, for example, when the damage of DNA was induced by DNA topoisomerase I inhibitor Tpt (as shown in Figs. 11.1 and 11.2), DSBs are caused by ionizing radiation (1, 2) or generated during apoptotic DNA fragmentation (13). It should be stressed, however, that H2AX may be phosphorylated on *Ser*-139 also in the absence of induction of DSBs. This can be seen during replication stress induced by inhibition of DNA synthesis (21) or during chromatin condensation such as during mitosis in some cell types as well as upon induction of premature chromosome condensation (PCC) (22). A caution, therefore, has to be exercised in interpreting expression of γ H2AX as a marker of DSBs. (*see* Notes 4 and 5)

⁴The gating analysis can be carried out to obtain mean values of γ H2AX IF for cells in G₁, S and G₂M phases of the cell cycle by selecting the cells with DNA index (DI) 1.0 $\pm$ 0.2 (G₁); 1.2 > (S) < 1.8 and 2.0 $\pm$ 0.2 (G₂), respectively.

References

1. Rogakou EP, Pilch DR, Orr AH, Ivanova VS, Bonner WM. DNA double-stranded breaks induce histone H2AX phosphorylation on serine 139. *J Biol Chem.* 1998; 273:5858–5868. [PubMed: 9488723]
2. Sedelnikova OA, Rogakou EP, Panuytin IG, Bonner W. Quantitative detection of 125IUdr-induced DNA double-strand breaks with γ -H2AX antibody. *Radiation Res.* 2002; 158:486–492. [PubMed: 12236816]
3. West MH, Bonner WM. Histone 2A, a heteromorphous family of eight protein species. *Biochemistry.* 1980; 19:3238–3245. [PubMed: 7407044]
4. Thatcher TH, Gorovsky MA. Phylogenetic analysis of the core histones H2A, H2B, H3 and H4. *Nucleic Acids Res.* 1994; 22:174–179. [PubMed: 8121801]
5. Pehrson JR, Fuji RN. Evolutionary conservation of histone macro-H2A subtypes and domains. *Nucleic Acids Res.* 1998; 26:2837–2842.
6. Burma S, Chen BP, Murphy M, Kurimasa A, Chen DJ. ATM phosphorylates histone H2AX in response to DNA double-strand breaks. *J Biol Chem.* 2001; 276:42462–42467. [PubMed: 11571274]
7. Anderson L, Henderson C, Adachi Y. Phosphorylation and rapid relocalization of 53BP1 to nuclear foci upon DNA damage. *Mol Cell Biol.* 2001; 21:1719–1729. [PubMed: 11238909]
8. Furuta T, Takemura H, Liao Z-Y, Aune GJ, Redon C, Sedelnikova OA, Pilch DR, Rogakou EP, Celeste A, Chen HT, Nussenzweig A, Aladjem MI, Bonner WM, Pommier Y. Phosphorylation of histone H2AX and activation of Mre11, Rad50, and Nbs1 in response to replication-dependent DNA double-strand breaks induced by mammalian topoisomerase I cleavage complexes. *J Biol Chem.* 2003; 278:20303–20312. [PubMed: 12660252]
9. Park EJ, Chan DW, Park JH, Oettinger MA, Kwon J. DNA-PK is activated by nucleosomes and phosphorylated H2AX within the nucleosomes in an acetylation-dependent manner. *Nucleic Acids Res.* 2003; 31:6819–6827.
10. Wang H, Wang M, Wang H, Bocker W, Iliakis G. Complex H2AX phosphorylation patterns by multiple kinases including ATM and DNA-PK in human cells exposed to ionizing radiation and treated with kinase inhibitors. *J Cell Physiol.* 2005; 202:492–502. [PubMed: 15389585]
11. Rogakou EP, Boon C, Redon C, Bonner WM. Megabase chromatin domains involved in DNA double-strand breaks in vivo. *J Cell Biol.* 1999; 146:905–916. [PubMed: 10477747]
12. Banath JP, Olive PL. Expression of phosphorylated histone H2AX as a surrogate of cell killing by drugs that create DNA double-strand breaks. *Cancer Res.* 2003; 63:4347–4350. [PubMed: 12907603]
13. Huang X, Okafuji M, Traganos F, Luther E, Holden E, Darzynkiewicz Z. Assessment of histone H2AX phosphorylation induced by DNA topoisomerase I and II inhibitors Topotecan and Mitoxantrone and by the DNA cross-linking agent Cisplatin. *Cytometry.* 2004; 58A:99–110. [PubMed: 15057963]
14. Paull TT, Rogakou EP, Yamazaki V, Kirchgesser CU, Gellert M, Bonner WM. A critical role for histone H2AX in recruitment of repair factors to nuclear foci after DNA damage. *Curr Biol.* 2000; 10:886–895. [PubMed: 10959836]
15. Bassing CH, Alt FW. H2AX may function as an anchor to hold broken chromosomal DNA ends in close proximity. *Cell Cycle.* 2004; 3:149–153. [PubMed: 14712078]
16. Tanaka T, Kurose A, Halicka HD, Traganos F, Darzynkiewicz Z. 2-Deoxy-D-glucose reduces the level of constitutive activation of ATM and phosphorylation of histone H2AX. *Cell Cycle.* 2006; 5:878–882. [PubMed: 16628006]
17. Tanaka T, Kurose A, Halicka HD, Huang X, Traganos F, Darzynkiewicz Z. Nitrogen oxide-releasing aspirin induces histone H2AX phosphorylation, ATM activation and apoptosis preferentially in S-phase cells. *Cell Cycle.* 2006; 5:1669–1674. [PubMed: 16861926]

⁵The data can also be expressed as a *change in mean* γ H2AX IF (Δ ; delta) either of all cells or after gating analysis cells in G₁, S or G₂M phase of the cycle, due to the treatment, by subtracting the mean IF value of the untreated cells from the mean IF value of the treated ones in respective phases of the cell cycle.

18. Tanaka T, Halicka HD, Huang X, Traganos F, Darzynkiewicz Z. Constitutive histone H2AX phosphorylation and ATM activation, the reporters of DNA damage by endogenous oxidants. *Cell Cycle*. 2006; 5:1940–1945. [PubMed: 16940754]
19. Tanaka T, Huang X, Jorgensen E, Gietl E, Traganos F, Darzynkiewicz Z, Albino AP. ATM activation accompanies histone H2AX phosphorylation in A549 cells upon exposure to tobacco smoke. *BMC Cell Biol*. 2007; 8:26. [PubMed: 17594478]
20. Tanaka T, Huang X, Halicka HD, Zhao H, Traganos F, Albino AP, Dai W, Darzynkiewicz Z. Cytometry of ATM activation and histone H2AX phosphorylation to estimate extent of DNA damage induced by exogenous agents. *Cytometry*. 2007; 71A:648–661. [PubMed: 17622968]
21. Kurose A, Tanaka T, Huang X, Traganos F, Dai W, Darzynkiewicz Z. Effects of hydroxyurea and aphidicolin on phosphorylation of ATM on *Ser* 1981 and histone H2AX on *Ser* 139 in relation to cell cycle phase and induction of apoptosis. *Cytometry A*. 2006; 69A:212–221. [PubMed: 16528719]
22. Huang X, Kurose A, Tanaka T, Traganos F, Dai W, Darzynkiewicz Z. Sequential phosphorylation of Ser-10 on histone H3 and Ser-139 on histone H2AX and ATM activation during premature chromosome condensation: Relationship to cell-cycle and apoptosis. *Cytometry A*. 2006; 69A: 222–229. [PubMed: 16528736]

Fig. 11.1.

This figure demonstrates selective response of S-phase to Tpt-induced DNA damage. The data was obtained using FACScan flow cytometer (BD Biologicals, San Jose, CA). FL-1 shows the level of γ H2AX immunofluorescence (*green fluorescence*) and FL-3 shows DNA content (*red fluorescence* of DNA-bound PI).

Fig. 11.2.

This figure also demonstrates selective response of S-phase to Tpt-induced DNA damage. The data was obtained using iCys laser scanning cytometer (CompuCyte, Westwood, MA). X axis shows the level of γ H2AX immunofluorescence and Y axis shows DNA content.