Chapter 1

Detection of DNA Double-Strand Breaks by γ-H2AX Immunodetection

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Abstract

DNA double-strand breaks (DSBs) are the most deleterious type of DNA damage and a cause of genetic instability as they can lead to mutations, genome rearrangements, or loss of genetic material when not properly repaired. Eukaryotes from budding yeast to mammalian cells respond to the formation of DSBs with the immediate phosphorylation of a histone H2A isoform. The modified histone, phosphorylated in serine 139 in mammals (S129 in yeast), is named γ-H2AX. Detection of DSBs is of high relevance in research on DNA repair, aging, tumorigenesis, and cancer drug development, given the tight association of DSBs with different diseases and its potential to kill cells. DSB levels can be obtained by measuring levels of γ-H2AX in extracts of cell populations or by counting foci in individual nuclei. In this chapter some techniques to detect γ-H2AX are described.

Key words DNA damage, Double-strand breaks, γ-H2AX, Immunofluorescence, Immunoblotting

1 Introduction

DNA can be damaged by endogenous and exogenous sources causing, among other lesions, double-strand breaks (DSBs). These can cause from cell death, if unrepaired, to loss of genetic material, if mis-repaired. Mammalian cells respond to agents that introduce DSBs with the phosphorylation of histone H2AX [1]. After the exposure to a damage-inducing agent, such as ionizing radiation, thousands of γ-H2AX molecules accumulate per DSB [2]. Given the high amplification of the signal, every DSB can be detected as a focus of γ-H2AX by immunological techniques using specific antibodies. These foci are crucial to the proper recruitment of repair factors to the site of damage [3]. The H2AX C-terminal tail that has the phosphorylated motif in mammals is also present in histone H2A from yeast, and it is also phosphorylated after DNA damage to facilitate DNA repair [4].

There are several detection techniques to evaluate DNA lesions such as DSBs: single-cell electrophoresis (Comet assay), terminal

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deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL), linear amplification-mediated high-throughput genome-wide translocation sequencing (LAM-HTGTS), DNA breakage detection-fluorescence in situ hybridization (DBD-FISH), and detection of DSB-associated molecular markers such as the Ku protein or γ-H2AX. These techniques have been thoroughly reviewed [5–11] and in this chapter we focus on the detection of phosphorylated H2AX as a measurement of DSBs because it is one of the earliest events after DSB formation and it is easily immunodetected. Given that DSBs can lead to genetic instability and eventually to cancer, and that paradoxically DSB induction is also used as cancer treatment, it is very useful to have a tool to monitor disease progression and/or treatment effectiveness based on the accumulation of γ-H2AX [12]. It is also useful for the understanding of the DNA damage response, repair, and all processes that control genomic integrity when factors of such pathways are missing or do not work properly [13, 14].

2 Materials

2.1 γ-H2AX Immunofluorescence in Mammalian Cells

1. DMEM-Dulbecco's Modified Eagle Medium.
2. 24-well plates.
3. Tweezers.
4. Round coverslips.
5. Bovine serum albumin (BSA).
6. PBS tablets.
7. Blocking solution: 3% BSA in phosphate-buffered saline (PBS).
8. anti-γ-H2AX antibody (Merck-Millipore).
9. Formaldehyde (methanol free) 10% ultra pure.
10. Alexa Fluor 594 goat anti-mouse.
11. Vacuum line.
12. DAPI solution (1 μg/ml in PBS).
13. Immu-Mount Mounting Medium.
14. Microscope slides.
15. Fluorescence microscope.

2.2 Detection of γ-H2AX in Mammalian Cells by Western Blotting

1. Lysis buffer (RIPA): 0.3 M NaCl, 1% (v/v) NP-40, 5% (w/v) sodium deoxycholate, 0.5% (w/v) SDS, 50 mM Tris–HCl pH 8.0.
2. Laemmli buffer 2×: 4% SDS, 20% glycerol (v/v), 2% 2-mercaptoethanol, 0.004% (w/v) bromophenol blue, 125 mM Tris–HCl pH 6.8.

3. Difco Skim Milk.
4. PBS tablets.
5. Protein ladder.
6. Running buffer: 25 mM Tris, 192 mM glycine.
7. Nitrocellulose blotting membrane with a pore size of 0.2 μm.
8. Transfer buffer: 25 mM Tris, 192 mM glycine, 20% (v/v) methanol.
9. TBS-T: 20 mM Tris–HCl pH 7.5, 150 mM NaCl, 0.1% Tween 20.
10. Blocking solution: 5% (w/v) Difco Skim Milk in TBS-T.
11. anti-γ-H2AX antibody (Merck-Millipore).
12. Goat anti-mouse HRP.
13. Supersignal West Pico PLUS Chemiluminescent Substrate.
14. High-sensitivity chemiluminescent films.
15. Phosphatase inhibitors.
16. Mini-PROTEAN Tetra Cell.

2.3 Detection of γ-H2AX in Yeast by Western Blotting

1. Trichloroacetic acid (TCA).
2. Difco Skim Milk.
3. Phosphatase inhibitors.
4. Laemmli buffer 2×: 4% SDS, 20% glycerol (v/v), 2% 2-mercaptoethanol, 0.004% (w/v) bromophenol blue, 125 mM Tris–HCl pH 6.8.
5. Glass beads acid-washed.
6. Multi-vortex mixer.
7. Running buffer: 25 mM Tris, 192 mM glycine.
8. Transfer buffer: 25 mM Tris, 192 mM glycine, 20% (v/v) methanol.
9. Nitrocellulose blotting membrane with a pore size of 0.2 μm.
10. Blocking solution: 5% (w/v) Difco Skim Milk in TBS-T.
11. TBS-T: 20 mM Tris–HCl pH 7.5, 150 mM NaCl, 0.1% Tween 20.
12. Anti-histone H2A (phospho S129) antibody.
13. Horseradish peroxidase conjugated goat anti-rabbit antibody.
14. Supersignal West Pico PLUS Chemiluminescent Substrate.
15. High-sensitivity chemiluminescent films.
16. Protein ladder.
17. Tris 1 M.
18. Mini-PROTEAN Tetra cell.

3 Methods

3.1 γ-H2AX Immunofluorescence in Mammalian Cells

1. HeLa cells are cultured on coverslips (*see* **Note 1**) at a concentration of 2×10^5 cells/well in a 6-well plate.
2. After 24 h, coverslips are transferred to a 24-well plate with cold PBS (*see* **Note 2**).
3. Aspirate the PBS with the vacuum system and add 1 ml of 2% formaldehyde in PBS (*see* **Note 3**). Incubate for 20 min at RT.
4. Remove formaldehyde solution (*see* **Note 3**).
5. Incubate the cells for 5 min at −20 °C in 70% ethanol.
6. Replace this solution with fresh 70% ethanol. Incubate 5 min at 4 °C (*see* **Note 4**).
7. Incubate the cells with blocking solution (1 ml/well) and incubate 1 h at room temperature.
8. Remove blocking solution and add anti-γ-H2AX (1:1000 in blocking solution) antibody in a total volume of 250 μl/well. Incubate 1 h at room temperature.
9. Wash twice in PBS (5 min each) and incubate with Alexa Fluor 594 goat anti-mouse secondary antibody (1:1000 in blocking solution) 1 h at room temperature.
10. Wash twice for 5 min each in PBS. Incubate with DAPI solution for 5 min at room temperature and wash twice more for 5 min each in PBS. Wash once in distilled H_2O.
11. Coverslips are removed using tweezers and placed on a microscope slide with Immu-Mount mounting medium (*see* **Note 5**).
12. The slides are placed flat at room temperature for 24 h and then stored at 4 °C (*see* Fig. 1).

3.2 Detection of γ-H2AX in Mammalian Cells by Western Blotting

1. Collect the cells and wash with ice-cold PBS.
2. Centrifuge at 13,000 × *g* 5 min. Discard the supernatant.
3. Add 100 μl of RIPA buffer with phosphatase inhibitors to 10^6 cells and incubate 30 min on ice.
4. Centrifuge 13,000 × *g* 10 min at 4 °C. Transfer the supernatant to a new tube.
5. Add an equal volume of Laemmli buffer 2× to a volume of cell extract. Boil samples at 95 °C for 5 min. Centrifuge at 13,000 × *g* 5 min (*see* **Note 6**).
6. Load 15–20 μl of each sample in a 12% acrylamide gel. Include a protein ladder. Run the electrophoresis at 200 V 45 min.
7. Transfer the acrylamide gel to a nitrocellulose membrane at 400 mA for 2 h (*see* **Note 7**).

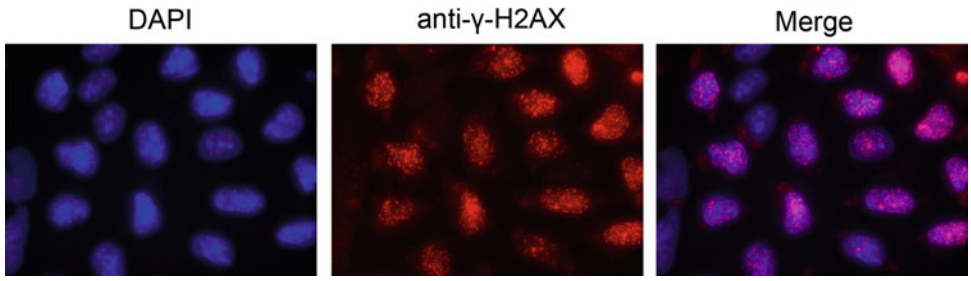

Fig. 1 Representative image of HeLa cells stained with DAPI (DNA) and γ-H2AX antibody. γ-H2AX foci can be observed in the nuclei

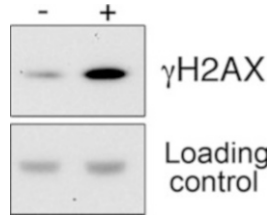

Fig. 2 Representative image of γ-H2AX immunoblotting. An increase in γ-H2AX is observed after DSB induction (+). The lower band corresponds to the loading control

8. Incubate the membrane in blocking solution.
9. Wash the membrane with TBS-T and add anti-γ-H2AX antibody (1:1000) in blocking solution. Incubate overnight at 4 °C with gentle shaking.
10. Wash twice with TBS-T. Add secondary antibody (1:10000) in blocking solution. Incubate 1 h at room temperature with gentle shaking.
11. Wash three times with TBS-T, 5 min each.
12. Prepare the detection reagent by mixing equal volumes of each solution.
13. Place the membrane face-up on a parafilm-coated plate and add the detection reagent. Incubate 5 min at room temperature in the dark.
14. Acquire the image using development techniques for chemiluminescence (*see* Fig. 2).

3.3 Detection of γ-H2AX in Yeast by Western Blotting

1. Grow 20 ml yeast cultures in the appropriate medium and temperature to an absorbance at 600 nm of about 0.8.
2. Harvest cells by centrifugation at 4000 × *g* 3 min. Discard supernatant.
3. Wash the cells with 1 ml of cold 20% TCA (w/v in H_2O) (*see* **Note 8**).

4. Spin down in a microcentrifuge tube for 75 s at 12,000 × *g*. Discard supernatant (*see* **Note 9**).
5. Resuspend the cell pellet in 200 μl of 10% TCA and add 200 μl of glass beads (*see* **Note 8**).
6. Vortex at high speed 10 min at 4 °C in a multi-vortex mixer. Spin down for 75 s at 12,000 × *g*.
7. Transfer supernatant to a new tube and wash the beads twice with 200 μl 10% TCA (*see* **Note 8**). Transfer the supernatant to the same tube (600 μl final volume).
8. Centrifuge 10 min at 1000 × *g* at room temperature. Discard supernatant.
9. Add 100 μl of Laemmli buffer 2× plus 50 μl of H_2O to the cell pellet (*see* **Note 6**). Resuspend by vortex and add 50 μl of Tris 1 M to neutralize (*see* **Note 10**). Mix by vortex.
10. Boil samples at 95 °C for 10 min. Mix by vortex.
11. Centrifuge at 1000 × *g* 10 min at room temperature.
12. Load 15–20 μl of each sample in a 12% acrylamide gel. Include a protein ladder. Run the electrophoresis at 200 V 45 min.
13. Transfer the acrylamide gel to a nitrocellulose membrane at 30 V overnight at 4 °C (*see* **Note 7**).
14. Incubate the membrane in blocking solution.
15. Wash the membrane with TBS-T and add anti-histone H2A (phospho S129) antibody (1:2000) in blocking solution. Incubate overnight at 4 °C with gentle shaking.
16. Wash twice with TBS-T. Add secondary antibody (1:2000) in blocking solution. Incubate 1 h at room temperature with gentle shaking.
17. Wash three times with TBS-T, 5 min each.
18. Prepare the detection reagent by mixing equal volumes of each solution.
19. Place the membrane face-up on a parafilm-coated plate and add the detection reagent. Incubate 5 min at room temperature in the dark.
20. Acquire the image using development techniques for chemiluminescence.

4 Notes

1. Use tweezers to place two to three round coverslips on a well before seeding the cells.
2. Fill the 24-well plate with 1 ml of PBS per well and place one coverslip per well with the cells on the upper side.

3. Formaldehyde is a sensitizing agent and a cancer hazard. Wear gloves and a lab coat and always work in a chemical fume hood.
4. In this step, coverslips can be kept in 70% ethanol at 4 °C for several weeks.
5. Place a drop of about 30 μl of mounting medium on a slide and place the coverslip with the cells facing the mounting medium.
6. 2-mercaptoethanol is toxic if inhaled and may cause damage to organs. Wear gloves and a lab coat and always work in a chemical fume hood.
7. Methanol is a hazardous chemical with significant toxic, flammable, and reactive properties that can produce deleterious impacts on human health and the environment when not properly handled. Wear a lab coat and gloves when working with methanol and always manipulate it in a chemical fume hood.
8. Trichloroacetic acid can cause skin burns and eye damage. Wear gloves and a lab coat when working with this reagent.
9. Pellets can be kept frozen at −20 °C.
10. Add Tris 1 M till the solution becomes blue.

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