Published in final edited form as:

Methods Mol Biol. 2016 ; 1473: 71–76. doi:10.1007/978-1-4939-6346-1_8.

Determination of histone H2AX phosphorylation in DT40 cells

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Abstract

Visualization of DNA damage response protein recruitment to DNA damage sites enables measurement of the DNA damage. DNA double-strand breaks (DSBs) and blocked replication forks induce the phosphorylation of H2AX at serine 139 (γ H2AX), and accumulate γ H2AX which can then be detected as foci. The detection of γ H2AX foci by immunostaining with antibodies that recognize γ H2AX is an indicator of DSBs presence. This chapter describes the measurement of γ H2AX immunostaining using a high-content imaging platform in chicken DT40 B-lymphocyte cell lines.

Keywords

γ H2AX; High-contents imaging; DSB; immunostaining

1. Introduction

DNA damage response is crucial to maintain the homeostasis of cells. Damage that remains unrepaired or incorrectly repaired may lead to genetic mutations, instability, and increased risk of carcinogenesis. One of the most serious sources of damage in cells, DNA double-strand breaks (DSBs) are often induced by a various sources, including ionizing radiation and exposure to DNA-damaging chemical or environmental stress [1]. On the occurrence of DSB, cells initiate DNA response signaling and recruit DNA damage repair proteins to affected DNA sites to repair the altered DNA.

After the formation of DSBs, H2AX is rapidly phosphorylated on a serine residue to create γ H2AX [2]. H2AX is a variant form of histone H2A and is ubiquitously distributed throughout the genome. Its sequence is conserved well among species [3]. In the initial response to DSBs, H2AX is phosphorylated on serine 139 by three kinases: ataxia telangiectasia mutated (ATM), ataxia telangiectasia and Rad3-related (ATR), and DNA-dependent protein kinase (DNA-PK). The γ H2AX triggers the recruitment of various proteins involved in DNA repair [3]. H2AX is phosphorylated in megabase regions of surrounding the DNA break site [4]. Large numbers of γ H2AX molecules can be visualized as foci in nuclear region by immunostaining with antibodies that recognize γ H2AX. Monitoring of γ H2AX foci formation is useful for detecting the incidence of DSBs.

DT40 Chicken B-lymphocyte cells are widely used to make and analyze the DNA-repair gene knockout clones because of their high efficacy in targeted integration [5,6]. DT40 cells have a short cell doubling time (~ 8 h), a long S phase (about 70% of the cell cycle), and a lack of a G1/S checkpoint. DT40 cells are sensitive to chemicals that produce DSBs by disrupting replication forks because of their long S phase and their lack of a G1/S checkpoint [7].

Here, we describe the methods of γ H2AX immunostaining for high-content imaging analysis in 384-well plate format using the chicken DT40 B-lymphocyte cell line [8].

2. Materials

Prepare all solutions using ultrapure water (prepared by purifying deionized water to attain a sensitivity of 18 M Ω cm at 25 °C) and analytical grade reagents. Prepare all solutions at room temperature unless indicated otherwise.

1. DT40 cells (provided by S. Takeda, Kyoto University, Japan).
2. Culture medium: Roswell Park Memorial Institute (RPMI) 1640 medium supplemented with 10% heat-inactivated Fetal Bovine Serum (FBS), 1% chicken serum, 50 μ M β -mercaptoethanol, 100 U/mL penicillin and 100 μ g/mL streptomycin. Store at 4 °C.
3. Collagen I coated 384-well black wall/clear bottom plate (Corning Incorporated, Tewksbury, MA, USA).
4. Positive control compounds, adriamycin (CASRN (Chemical Abstract Services Registry Number) = 25316-40-9) and melphalan (CASRN = 148-82-3).
Chemicals are dissolved in dimethyl sulfoxide (DMSO) and prepared as 20 mM stock solutions prior to use.
5. Hank's balanced salt solution (HBSS)
6. 10 mg/mL Hoechst 33342 solution in water
7. Fixing solution: 12% Paraformaldehyde and 0.3% Hoechst 33342 in HBSS, 10 mg/mL of Hoechst 33342 solution in water is used. 32% Paraformaldehyde stock solution is used. Add 13 mL of 32% paraformaldehyde solution and 105 μ L of 10 mg/mL Hoechst solution to 22 mL of HBSS.
8. Permeabilization solution: 0.1% IGEPAL® (Sigma-Aldrich) in HBSS. Add 100 μ L of IGEPAL to 100 mL of HBSS and mix well using Vortex.
9. Blocking solution: 3% Bovine serum albumin (BSA) in HBSS. Dissolve 3 g BSA in 100 mL HBSS.
10. Anti-phospho-Histone H2AX antibody (EMD Millipore). Add 60 μ L of anti-phospho-Histone H2AX antibody to 60mL of blocking buffer. Keep on ice before use.

11. Alexa Fluor 594 goat-anti mouse IgG secondary antibody (Life Technologies). Add 60 μ L of Alexa Fluor 594 goat-anti mouse IgG secondary antibody to 60 mL of blocking buffer. Keep on ice before use.
12. 384-well plate sealing film.
13. Pipettes
14. 8-channel aspirator
15. ImageXpress Micro Widefield High-Content Screening System (Molecular Devices)

3. Methods

Carry out all procedures at room temperature unless otherwise specified.

3.1. Compound treatment

1. Plate the cells (6000–8000 cells/well/25 μ L) into collagen I coated 384-well black wall/clear bottom plate and incubate overnight at 37 °C under a humidified atmosphere and 5% CO₂. To allow the cell attachment on the bottom of the wells, incubate the plates overnight (See Note 1).
2. Next day, add 25 μ L/well of the compound on top at desired concentration into the wells and incubate at 37 °C for 24 h or time optimized for cell line of interest. Prepare 2 $\times$ final concentrations of compounds and add into assay plate, to get 1 $\times$ final concentration in assay plate.

3.2. Fixation and antibody staining

1. Add 25 μ L/well of fixing solution on top, and incubate for 10 min. Final concentration of paraformaldehyde is 4% and Hoechst is 0.1%.
2. Remove liquid gently using aspirator.
3. Wash with HBSS (50 μ L/well).
4. Add 25 μ L/well of permeabilization solution for 15 min.
5. Remove liquid gently using aspirator.
6. Wash twice with HBSS (50 μ L/well).
7. Add 50 μ L/well of blocking solution and incubate for 30 min at 37 °C.
8. Remove blocking buffer and add 25 μ L/well of anti-phospho-Histone H2AX antibody (1:1000 dilution, diluted in blocking buffer) and incubate for 1 h at 37 °C.
9. Wash three times with HBSS (50 μ L/well).

¹Maintaining DT40 cells in culture under 1 $\times$ 10⁶ cells/ml is critical for cell health. Due to the nature of the suspension cells, DT40 cells need to adhere to collagen coated surface as a crucial step in this assay in order to reduce the assay variability.

10. Add 25 μ L/well of Alexa Fluor 594 goat-anti mouse IgG secondary antibody (1:1000 dilution, diluted in blocking buffer) and incubate for 45 min at 37 °C.
11. Wash three times with HBSS (50 μ L/well).
12. Add 50 μ L/well of HBSS and seal the plate

3.3. Imaging readout

1. Take images using a 40 $\times$ objective in ImageXpress Micro Widefield High-Content Screening System using DAPI (acquire nuclear images), Texas Red (acquire γ H2AX foci) filter sets. Analyze at least 100 cells per compound treatment using the Transfluo module, a proprietary analysis protocol with MetaXpress software (Figs. 1 and 2).

3.4. Image Analysis

The Molecular Devices Systems proprietary MetaExpress Transfluo analysis module works in a way that it identifies Nuclei based on the size and intensity of DAPI channel as nuclei are stained with Hoechst. Total number of nuclei in a well will represent number of cells in the well. Foci are identified based on the size and intensity of Texas Red channel as foci are stained with red γ H2AX antibody. Thus the program quantifies the number of foci per nucleus. In the results, the average number of foci per cell (Fig. 3) was used to calculate the activity.

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Fig. 1. Induction of γ H2AX foci in wild-type DT40 cells. (A–C) Representative images of nuclei (blue) with γ H2AX (red) foci. (A) Without compound treatment (DMSO, control). (B) Treatment with 0.13 μ M adriamycin for 24 h. (C) Treatment with 30.7 μ M melphalan for 24 h.

Fig. 2. Induction of γ H2AX foci by Adriamycin and Melphalan in a concentration-dependent manner. The average number of γ H2AX foci per cell was measured after the compound treatment. Error bars represent SD from three independent experiments.

Fig. 3.

Segmentation: Transfluor analysis module correctly identifies the nucleus and H2AX foci