Video Article

Quantitation of γH2AX Foci in Tissue Samples

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Abstract

DNA double-strand breaks (DSBs) are particularly lethal and genotoxic lesions, that can arise either by endogenous (physiological or pathological) processes or by exogenous factors, particularly ionizing radiation and radiomimetic compounds. Phosphorylation of the H2A histone variant, H2AX, at the serine-139 residue, in the highly conserved C-terminal SQEY motif, forming γH2AX, is an early response to DNA double-strand breaks[1]. This phosphorylation event is mediated by the phosphatidyl-inosito 3-kinase (PI3K) family of proteins, ataxia telangiectasia mutated (ATM), DNA-protein kinase catalytic subunit and ATM and RAD3-related (ATR)[2]. Overall, DSB induction results in the formation of discrete nuclear γH2AX foci which can be easily detected and quantitated by immunofluorescence microscopy[2]. Given the unique specificity and sensitivity of this marker, analysis of γH2AX foci has led to a wide range of applications in biomedical research, particularly in radiation biology and nuclear medicine. The quantitation of γH2AX foci has been most widely investigated in cell culture systems in the context of ionizing radiation-induced DSBs. Apart from cellular radiosensitivity, immunofluorescence based assays have also been used to evaluate the efficacy of radiation-modifying compounds. In addition, γH2AX has been used as a molecular marker to examine the efficacy of various DSB-inducing compounds and is recently being heralded as important marker of ageing and disease, particularly cancer[3]. Further, immunofluorescence-based methods have been adapted to suit detection and quantitation of γH2AX foci *ex vivo* and *in vivo*[4,5]. Here, we demonstrate a typical immunofluorescence method for detection and quantitation of γH2AX foci in mouse tissues.

Protocol

Tissues

1. Lung tissue, embedded in moulds in optimal cutting temperature (OCT) compound 4583, was sectioned (5 μm) using a CM Leica 1950 cryostat and mounted onto poly-L-lysine slides.

The frozen samples were obtained from Dr Simon Royce at the Murdoch Children's Research Institute. Lung tissue was obtained from untreated Balb/c mice and from a mouse model of chronic allergic airways disease which shows many of the pathological features of human asthma[6]. Experiments on animals were performed in accordance with the guidelines and regulations set forth by the Animal Ethics Committee of the Murdoch Children's Research Institute which adheres to the Australian Code of Practice for the care and use of laboratory animals for scientific purposes.

2. Sections were allowed to air dry at room temperature for 1 hour.

Immunofluorescence staining

1. Sections were fixed with 2% (w/v) paraformaldehyde in phosphate buffered saline (PBS) for 20 minutes.
2. Sections were equilibrated with two washes in PBS for 15 minutes each.
3. Sections were treated with 70% ethanol (chilled to -20°C) for 20 minutes followed by three washes in PBS for 10 minutes each.

At this point slides may be stored in sealed containers at 4°C for up to 2 weeks.

4. Sections were washed three times with PBS for 10 minutes each and then blocked with 100μL freshly prepared in PBS containing 8% BSA in PBS containing 0.5% Tween-20 and 0.1% Triton X-100 (PBS-TT) for 1 hr at room temperature.
   All incubations were performed in a humidified staining trough.
5. Sections were washed with PBS-TT for 5 minutes and a hydrophobic perimeter was marked around the tissue with a Pap pen.
6. Excess buffer was tipped off and 100μl of primary rabbit polyclonal anti-γH2AX antibody (diluted 1:500, in 1% BSA in PBS; Millipore), was added to each section for a 2 hour incubation at room temperature.
   Incubation with primary antibody may be performed overnight at 4°C.
   For further evidence, that γH2AX foci represent DSBs, a primary antibody recognising a DSB repair protein may also be used. For example, co-localization of γH2AX and phospho-ATM is a commonly used combination.
7. Sections were washed two times in PBS-TT for 5 minutes each and incubated with 100μl of secondary antibody (Alexa Fluor 488 goat anti-rabbit IgG diluted 1:500, in 1% BSA; Invitrogen) for 1 hour at room temperature in the dark. (Diluted antibody was kept in the dark throughout the procedure).
8. Sections were washed three times with PBS-TT for 5 minutes each and incubated with 0.5mg/mL RNAse A for 30 mins at 37°C.
9. Nuclear counterstaining and mounting was performed with Vectashield medium containing propidium iodide.

10. The slides were sealed with nail polish and stored overnight at 4°C in the dark before analysis.

Microscopy / Analysis

1. A Zeiss LSM510 Meta Confocal Microscope was used to acquire images using the standard GFP (for γH2AX - Alexa Fluor 488 goat anti-rabbit IgG) and PI (543 nm) lasers.

Images were acquired in a Z-series pattern with a step size of 0.5 µm. During analysis, individual planes were deconvoluted and stacked to produce a maximum projected image to minimize the overlap of foci.

The method of creating a maximum projected image prior to a particle count should only be used in circumstances where a thin section (e.g. 5µm) is used and where the background staining is insignificant. Where thicker sections with a significant background must be analyzed, a more complex analysis such as 3D Object Counter as described by Bolte and Cordelieres (2006) should be used[7].

2. Metamorph (Molecular Devices, USA) was used to create merged (red and green) images to quantitate numbers of foci.

Although Metamorph can be used to quantitate foci numbers, typically when using tissue sections foci are counted manually (by eye) for greater accuracy.

Specific cell types of interest can be selected for quantitation, for instance lung epithelial cells. This may be done based on histology and with reference to haematoxylin and eosin stained serial sections.

Discussion

For the purpose of this demonstration we used mouse lung tissue from untreated Balb/c mice and from a mouse model of chronic allergic airways disease. We quantitated differences in the number of foci per cell with slightly higher averages observed in the damaged lung compared to untreated sections. Specifically, quantitation indicated 6.5 foci per/cell and 9.4 foci per/cell (manual counting of 20 cells per section), in the untreated tissue and chronic allergic airways disease tissue, respectively. However, the use of a DSB-inducing agent, for example naphthalene, which is known to induce DSBs in the mouse lung model, would yield higher foci numbers. Indeed, this protocol is most suited for the investigation of the effects of ionizing radiation in tissues; more striking results, in terms of γH2AX foci numbers and resolution are obtained when ionizing radiation is used. It is well known that following exposure to X-rays, γH2AX foci form rapidly and foci numbers reach a maximum between 30-60 minutes[2]. Therefore, 1 hour time points (post-irradiation) are ideal for evaluating initial DSB formation and longer times (typically up to 48 hours) can be used to monitor DNA repair.

Overall, quantitation of γH2AX in tissues is useful for monitoring DSB formation and repair. Apart from its utility in the context of ionizing radiation, the method can be applied to evaluation of the efficacy of DSB-inducing compounds in various tissues, for example the commonly used anticancer anthracyclines such as doxorubicin are known to cause DSBs, and potentially to monitor various pathologies that are characterized by reactive oxygen species-mediated DSBs. Importantly, this protocol is suitable for different mouse tissues.

Disclosures

No conflicts of interest declared.

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