

# Chapter 2

## Ionizing Radiation-Induced DNA Strand Breaks and $\gamma$ -H2AX Foci in Cells Exposed to Nitric Oxide

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### Abstract

A number of studies have demonstrated that nitric oxide enhances radiosensitivity of anoxic and hypoxic cells in vitro and in vivo, and some evidence points to a role for DNA damage and repair in this phenomenon. We have recently observed that nitric oxide enhances the formation of DNA single- and double-strand breaks following ionising irradiation, measured by the alkaline comet assay and immunofluorescence microscopy for  $\gamma$ -H2AX.

**Key words:** Nitric oxide, DNA strand break,  $\gamma$ -H2AX, single cell gel electrophoresis, ionising radiation.

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### 1. Introduction

Whilst numerous studies have clearly demonstrated that nitric oxide (NO) enhances radiosensitivity of anoxic and hypoxic cells in vitro and in vivo (1, 2), the exact mechanisms underlying the observed radiosensitisation remain elusive.

NO has been reported to upregulate p53, PARP and the catalytic subunit of DNA-dependent protein kinase (DNA-PKcs), an enzyme involved in repairing DNA double-strand breaks via the non-homologous end-joining pathway (3–5). On the other hand, it has been reported to inhibit nucleotide excision repair (6). However, it is possible that these data were confounded by the presence of nitrogen dioxide (NO<sub>2</sub>). Furthermore, there is some evidence for the involvement of NO in ‘bystander’ responses (7) which may occur via the radiation-induced activation of nitric oxide synthase (NOS) (8).

Some recent evidence suggests a modulation of radiation-induced DNA damage and/or its repair by NO which appears to correlate with potent low-dose radiosensitisation of cell cultures at low concentrations of NO (9). Ionising radiation induces a wide spectrum of different DNA lesions, ranging from damaged bases and adducts to cross-links and strand breaks. However, few assays are available to measure the formation and repair of DNA damage following low-dose irradiation. We have observed the yield of DNA double-strand breaks in V79 Chinese hamster lung fibroblasts and MCF-7 human breast cancer cells, as detected by immunofluorescence microscopy for the phosphorylated histone variant  $\gamma$ -H2AX. Double-strand breaks increased twofold following X-ray-irradiation in the presence vs. absence of 1% v/v NO in nitrogen ( $N_2$ ), and repair time was longer in cells irradiated in NO than in air or  $N_2$  alone. Also, single-strand breaks, detected by alkaline single cell gel electrophoresis ('comet' assay), appeared to be enhanced in the presence of NO. Furthermore, loss of X-ray-induced  $\gamma$ -H2AX foci appeared to be slower in cells exposed to NO (9). These methods are described below.

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## 2. Materials

### 2.1. Cell Culture, NO Exposure and X-Irradiation

1. Eagle's MEM with Earle's salts supplemented with 10% (V79-379A) or 15% (MCF-7) foetal calf serum, penicillin, streptomycin, glutamine, sodium bicarbonate, sodium pyruvate and non-essential amino acids.
2. Solution of 0.25% trypsin and 1 mM ethylenediamine tetraacetic acid (EDTA).
3. NO (100% or 1% v/v in  $N_2$ ; 400 ppm v/v in  $N_2$  'INOmax' from INO Therapeutics, Sittingbourne, Kent, UK) and  $N_2$ .
4. Tubing, valves and fittings made of stainless steel, glass or PEEK polymer; glass syringes for irradiation of pre-gassed cell samples; glass scintillation vials with SubaSeal stoppers and gas entry and exit needles for irradiation of continuously gassed cell samples (*see Note 1*).
5. X-ray generator (Pantak, East Haven, CT, USA) with 4.3 mm aluminium filtration at 240 kV, 13 mA and a dose rate of approximately 0.5 Gy/min.

### 2.2. Immunofluorescence for $\gamma$ -H2AX

1. Lab-Tek II chamber slides and coverslips (22 mm  $\times$  50 mm  $\times$  0.13 mm).
2. Phosphate-buffered saline (prepared from PBS tablets).
3. 100% methanol (stored at  $-20^\circ\text{C}$ ).

4. Mouse monoclonal antibody (clone JBW301) for  $\gamma$ -H2AX from Millipore, Watford, Hertfordshire, UK (*see Note 2*). Stable at  $-20^{\circ}\text{C}$  for several years.
5. AlexaFluor 488-conjugated goat anti-mouse IgG secondary antibody. Stable at  $4^{\circ}\text{C}$  for 1 year. Protect from light.
6. 4,6-diamidino-2-phenylindole (DAPI) at a final concentration of  $0.5\ \mu\text{g}/\text{mL}$ . Stable at  $-20^{\circ}\text{C}$  for several years.
7. Mounting medium containing antifade, e.g. VECTASHIELD. Store at  $4^{\circ}\text{C}$ .
8. Clear, non-fluorescent nail varnish.

**2.3. Alkaline Single  
Cell Gel  
Electrophoresis  
for DNA Strand  
Breaks**

1. Glass slides and coverslips.
2. Lysis buffer (1 l): 1.2 M NaCl, 0.1% *N*-lauryl sarcosine, 0.26 M NaOH, 100 mM  $\text{Na}_2\text{EDTA}$ ,  $\text{pH}>12.5$ .
3. Electrophoresis buffer (2 L): 0.03 M NaOH, 2 mM  $\text{Na}_2\text{EDTA}$ ,  $\text{pH}>12.5$ . (The buffers can be made the day before and kept at  $4^{\circ}\text{C}$  but check pH before use.)
4. Rinse buffer: previously used electrophoresis buffer.
5. 1% pulsed-field certified agarose and 1% Type VII (low melting point) agarose.
6. Metal plate.
7. Horizontal electrophoresis chamber and power supply.
8. 70, 90 and 100% ethanol.
9. Sybr-Gold DNA staining solution. Store at  $-20^{\circ}\text{C}$ , protected from light.
10. DABCO antifade mounting medium. Store at  $-20^{\circ}\text{C}$ .
11. Clear nail varnish.

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### 3. Methods

Following the induction of a DNA double-strand break, hundreds to thousands of copies of the histone variant H2AX, which forms part of the nucleosome, are phosphorylated by the DNA damage-activated kinases, Ataxia Telangiectasia Mutated (ATM) and DNA-dependent Protein Kinase (DNA-PK), covering megabases of chromatin surrounding the site of the break. Immunofluorescence microscopy using commercially available antibodies for the phosphorylated form,  $\gamma$ -H2AX, can be employed to visualise and quantify these radiation-induced ‘foci’ which are used

as a surrogate marker for double-strand breaks and their repair (10, 11).

The comet assay detects DNA damage in single cells following gel electrophoresis of low concentrations of lysed cells embedded in agarose (12). The type of DNA damage detected in the comet assay depends primarily on the pH of buffers used for cell lysis and electrophoresis. Neutral conditions are used to measure double-stranded DNA breaks, whereas alkaline conditions allow the detection of both single- and double-stranded DNA breaks, as the alkaline conditions lead to denaturation of DNA. Given that ionising radiation induces 20–50 times more single- than double-strand breaks, most of the damage measured in the alkaline comet assay reflects single-strand breaks.

DNA damage is induced immediately during irradiation, and for determining initial yields of DNA damage, cells should be put on ice immediately after irradiation to suppress repair. This is especially important for DNA strand breaks measured with the alkaline comet assay because of the rapid repair of DNA single-strand breaks. However, for  $\gamma$ -H2AX-based measurements of DNA double-strand breaks, cells require post-irradiation incubation at 37°C to enable efficient phosphorylation of H2AX at sites of double-strand breaks. Depending on the quality of the immunofluorescence staining and on the background levels present in different cell lines, minimum incubation times of 3–30 min are commonly used, and, accordingly,  $\gamma$ -H2AX levels never reflect the full initial yield of damage (13). In the experiments described below, cells are irradiated in suspension and transferred to chamber slides. In this case the minimum incubation time is 30 min at 37°C to allow sufficient numbers of cells to attach to the slide surface.

### **3.1. Cell Culture, Nitric Oxide Exposure and X-Ray-Irradiation**

1. V79 and MCF-7 cells are split with trypsin/EDTA when approaching confluence and passaged in T25 tissue culture flasks to maintain cultures. Doubling times are 10–12 h for V79 and approximately 20 h for MCF-7 cells.
2. To prevent NO autoxidation, suspensions of exponentially growing cells ( $\sim 7 \times 10^5$  in PBS) are first pre-gassed by gently bubbling with argon (Ar) or N<sub>2</sub> for 30 min before gassing for 30 min with the appropriate gas (*see Note 3*).
3. All irradiations are performed at room temperature. Either the suspensions are irradiated in capped glass syringes and 1 mL samples are collected after each X-ray dose and placed on ice or 2 mL suspensions are irradiated in glass scintillation vials held nearly horizontal (5–10° angle with  $\sim 22$  mL headspace) whilst being continuously bubbled with the appropriate gas. The vials are placed on ice after exposure (*see Note 4*).

### 3.2. Immunofluorescence for $\gamma$ -H2AX

1. The cell suspensions are transferred into Lab-Tek II chamber slides and incubated at 37°C in a humid atmosphere containing 5% CO<sub>2</sub>.
2. Cells are fixed with 100% methanol at -20°C for 10 min (*see Note 5*).
3. Cells are incubated in PBS with 2% foetal calf serum for 3 × 5–10 min incubations at room temperature to block non-specific antibody binding sites (*see Note 6*).
4. Samples are incubated with anti  $\gamma$ -H2AX antibody (1:300 in PBS, 2% foetal calf serum) for 1 h at room temperature.
5. Samples are washed in PBS with 2% foetal calf serum for 3 × 5–10 min washes at room temperature.
6. Samples are incubated with secondary antibody (1:400 in PBS, 2% foetal calf serum) for 1 h at room temperature in the dark.
7. Slides are washed in PBS for 5–10 min at room temperature in the dark.
8. Cells are counterstained with DAPI for 3–5 min and washed in PBS for 5–10 min at room temperature in the dark.
9. Chambers are removed from slides and slides completely dried in the dark before applying mounting medium and mounting with a coverslip. Nail varnish is applied to seal the samples. Samples can be viewed when the varnish is dry and can be stored in the dark at 4°C for several weeks.
10. The slides are viewed using a fluorescence microscope. An example is shown in **Fig. 2.1**. Scoring of nuclear  $\gamma$ -H2AX foci typically requires at least an ×40 objective and can be

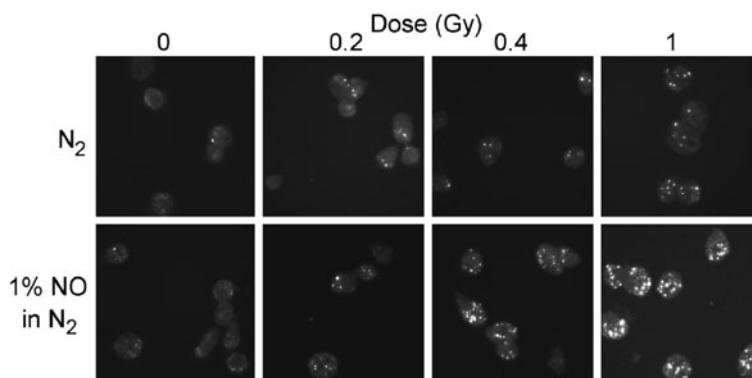


Fig. 2.1. Foci of  $\gamma$ -H2AX after irradiation of anoxic V79 cells in sealed syringes following incubation for 1 h at 37°C in 90/5/5% v/v N<sub>2</sub>/O<sub>2</sub>/CO<sub>2</sub>. *Upper panel*: irradiated with 0, 0.2, 0.4 and 1 Gy in N<sub>2</sub>; *lower panel*: irradiated with 0, 0.2, 0.4 and 1 Gy in 1% v/v NO in N<sub>2</sub>.

performed by eye either through the microscope eye pieces or on images obtained with a camera. Alternatively, a range of software packages have been used to facilitate automated scoring of  $\gamma$ -H2AX foci (14–17) (*see* Note 7).

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#### 4. Alkaline Single Cell Gel Electrophoresis for DNA Strand Breaks

1. Furnace BDH semi-frosted slides.
2. Coat non-frosted part of slides with 100  $\mu$ L of 1% PFGE agarose, air-dry slides and store in 50°C oven.
3. Label slides coated earlier using a pencil.
4. Make up 1% Type VII agarose and place in water bath at 37°C until needed.
5. Place metal plate in freezer.
6. Count cells and dilute to 50,000/mL, using cold medium.
7. Remove metal plate from freezer and cover with four sheets of paper towel.
8. For each slide aliquot 125  $\mu$ L cell suspension in 5 mL tube and keep on ice. Add 375  $\mu$ L of 1% Type VII agarose to cell suspension, gently mix and spread quickly on a coated slide. Place on metal plate and allow to set. Place in lysis buffer in a container in fridge and lyse overnight.
9. Carefully remove slides from lysis buffer and place slides in 650 mL of rinse buffer for 20 min, repeat twice.
10. Fill the electrophoresis tank with 1.6 L electrophoresis buffer. Put slides on plastic tray in electrophoresis tank (ensure that the slides are straight and all facing in the same direction). Run at 0.6 V/cm for 30 min. When the run ends, immediately remove the slides from the tank and place in cold 70% EtOH for 10 min, followed by 90% EtOH for 10 min and 100% EtOH for 10 min. Air-dry. The slides are now stable and can be left for any period of time before proceeding with the staining.
11. Make up a 1:10,000 dilution of Sybr-Gold in 1  $\times$  TBS. Sybr-Gold is light sensitive and must be kept in the dark. Incubate with slides for 30 min in the dark. Rinse twice for 5 min with distilled H<sub>2</sub>O, remove the slides and drain, but do not allow to dry out.
12. Put 50  $\mu$ L DABCO antifade along the centre of a coverslip. Lower the slide onto it and gently push down, leave for a few minutes to settle, then remove any excess. Seal around the edges of the coverslip with nail varnish. Leave to dry.

13. Slides are analysed either by an automated microscope image acquisition and analysis system which allows scoring of thousands of cells per hour or by manual analysis using a fluorescence microscope (*see* **Note 8**).

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## 5. Notes

1. Standard tissue culture plasticware and plastic tubing should be avoided as it contains oxygen which can leak out from the surface and thus contaminate the sample.
2. We have found this antibody to work very reliably for immunofluorescence. Numerous polyclonal rabbit antibodies for  $\gamma$ -H2AX are available from other commercial suppliers but have suffered from excessive non-specific staining and high variability in our hands.
3. Removal of NO<sub>2</sub> can be confirmed by the failure of the purified NO to oxidise N<sub>2</sub>-purged aqueous solutions of the dye ABTS [2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonate)] to its stable radical (green colour).
4. Continuous bubbling during irradiation ensures that NO levels are not diminished during the irradiation process. This is especially important at low concentrations of NO and at high radiation doses.
5. Fixation may have to be adjusted, depending on the cell line used. If the DAPI signal shows that DNA seems to be leaking out of the nuclei, then this indicates that the coarse precipitation of proteins facilitated by methanol treatment has broken up cell nuclei quite severely. Such disruption of cellular morphology occurs frequently in some cell types, e.g. leukocytes, when using methanol for fixation. It tends also to 'smear out' gamma-H2AX foci which, as a result, are more difficult to discern and score. To improve the preservation of cell morphology, cells can be pre-fixed with 2–4% buffered formaldehyde (prepared fresh in PBS from a buffered stock of 37% formaldehyde solution or from paraformaldehyde) for about 10 min at room temperature before a 10 min methanol fixation step at –20°C. The protein and DNA cross-links resulting from this treatment stabilise cellular structures and thereby help obtain more distinct gamma-H2AX foci.
6. The blocking efficiency of different batches of foetal calf serum may vary. Bovine serum albumin (fraction V) or other sera may be used as an alternative. This step and/or the subsequent incubation with primary antibody can be extended to overnight incubation at 4°C, if required or convenient.

7. In order to obtain the total number of foci per cell, nuclei must be imaged across their depth, and not just a single image taken. For software-based scoring, either 3D image analysis software or 2D analysis of maximum projections of z-stacks of images should be used. The required step size for image stacks depends on the focus depth of the optical system and is typically in the range of 200–1,000 nm. With increasing foci levels, more and more overlap between adjacent foci occurs which may result in ‘underscoring’. The extent of this effect depends on the optical resolution of the system and, for automated scoring, on the software algorithms used to detect and distinguish individual foci. Optionally, 3D deconvolution or confocal microscopy can be used to enhance the optical resolution along the depth axis. In practice, however, these rather time-consuming and expensive efforts rarely improve the accuracy of foci quantification.
8. A comet consists of a ‘head’ of DNA which cannot migrate out of the nucleus due to its size, and a ‘tail’ of fragmented DNA leaving the nucleus during electrophoresis and migrating according to the molecule size. The ‘tail length’ (distance between the centres of mass of the tail and head region), % of DNA in the tail and ‘tail moment’ (the product of tail length and % DNA in the tail) can be measured and calculated using dedicated image analysis software, and these terms are used to describe and quantify the amount of DNA damage measured. Alternatively, if analysing comets by eye, they can be classified into different categories of damage, depending on their appearance. *See* (18) for a more detailed discussion of analysis techniques and statistical issues in the use of the comet assay.

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