Creating Localized DNA Double-Strand Breaks Using Micro-Irradiation

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SUMMARY

We describe a protocol for creating localized DNA double-strand breaks (DSBs) with minimal requirements that can be applied in cell biology and molecular biology. This protocol is based on the combination of 5-bromo-2'-deoxyuridine (BrdU)-labelling and ultraviolet C (UVC)-irradiation through porous membranes. Cells are labelled with 10 µM BrdU for 48-72 hours, washed with Ca^{2+} and Mg^{2+}-free phosphate buffered saline (PBS (-)), covered by polycarbonate membranes with micro-pores, and exposed to UVC-light. Using this protocol, localized DSBs are created within subnuclear areas, irrespective of the cell cycle phase. Recruitment of proteins involved in DNA repair, DNA damage response, chromatin remodelling and histone modifications can be visualized without any specific equipment. The quality is the same as that obtained by the laser micro-irradiation or any other focal irradiation. DSBs become visible within 30 minutes of UVC irradiation.

INTRODUCTION

Nuclear proteins accumulate at specific subnuclear areas to execute their function. For example, ionizing radiation-induced DNA double-strand breaks (DSBs) recruit a number of proteins to chromatin regions flanking DSBs (1-7). The localization of these factors to a single DSB can be visualized as discrete foci using fluorescence microscopy, but not all factors are detectable. Presumably, the number of such factors accumulated at the site of a single DSB is insufficient for detection by standard immunofluorescence. To avoid this

problem, generating multiple DSBs within a limited nuclear area is necessary. A number of techniques have been developed to fulfill this purpose, however, all these methods require laser sources or other specific devices. For example, a study using an ultraviolet A (UVA) laser in combination with BrdU-labelling stimulated experiments using UVA lasers of different wavelengths to examine the spaciotemporal dynamics of the factors involved in DNA damage response and DNA damage repair (8-14). More recently, green and near-infrared lasers, which do not require BrdU-labelling, have been applied (15-17). Various types of micro-irradiation systems, which enable partial nuclear irradiation, have also been developed (18). These include microfocal irradiation with soft X-rays, and microbeam irradiation with charged particles or heavy ions (19-25). However, while laser micro-irradiation or focal irradiation techniques are advantageous, the requirement for specific devices limits their application.

Recently, we have reported the recruitment of proteins involved in DNA damage checkpoint and DNA damage repair to DSBs locally created using UVC micro-irradiation (26), which makes studies of localized DNA damage accessible to more laboratories. While the use of micro-porous membranes and UVC-light had been previously described, these original studies were intended to create UVC-induced cyclobutane pyrimidine dimers (CPDs) and (6-4) photoproducts (6-4PPs) (refs 27-29). We combined micro-irradiation and BrdU-labelling to generate localized DSBs (26). This protocol is derived from the original description in that study. Micro-irradiation is achieved by UVC-irradiation through

micro-pore membranes with different pore sizes. While UVA has been used for creating DSBs in combination with halogenated thymidine analogues (30), UVC is a preferable light source, as it is significantly more effective to photosensitize BrdU in DNA. This photochemical reaction is required for creating the DSBs (31). We showed that UVC micro-irradiation efficiently induced DSBs, which were quantitatively and qualitatively equivalent to those created by laser micro-irradiation. Furthermore, we found that UVC micro-irradiation enabled visualization of recruitment of proteins involved in DNA damage repair and response as well as in chromatin architecture and remodelling.

In summary, this protocol provides a simple, reliable and undemanding method for creating localized DSBs. It obviates the need for laser sources or specialized devices, and thus, the protocol can be followed easily by researchers without any previous experience. This will facilitate investigation of the impact of DNA damage on higher-order chromatin structure, genome rearrangements, transcription and replication. The disadvantage is that UVC exposure alone creates UV-specific base damage, such as CPDs and 6-4PPs, however, the effects of UV damage can be eliminated by including simple control experiments, in which the cells are exposed to UVC without BrdU-labelling.

Experimental design

BrdU labelling. This technique is applicable to any exponentially growing cell or tissue. BrdU is a widely-used synthetic analogue of thymidine, which is incorporated into newly

synthesized DNA. Cells cultured in a medium containing BrdU utilize it as a nucleoside source, and consequently those in the S phase are labelled with BrdU. BrdU incorporated into DNA is photoactivated by UV-light and creates single strand breaks in close proximity, which results in DNA double strand breaks. As cell growth kinetics and the thymidine pool sizes are very different between cells, the optimal concentration of BrdU should be determined carefully. Begin with BrdU concentrations between 0.4 and 10 μM, as most literature reports these concentrations. The optimal incubation time must also be determined carefully. Prolonged BrdU treatment of cells may cause DNA damage. For example, using diploid human BJ1-hTERT cells, BrdU treatment for 72 hours slightly increased the frequency of cells with spontaneous foci (2.9 ± 1.1% versus 5.7 ± 1.9%). We usually use 72 hr-incubation, which results in approximately 90% of cells being labelled. During the 72 hr-incubation, some cells are expected to pass through the S-phase twice, however, this is not essential. For example, the doubling time of the cells used here is approximately 24 hours, and the labelling indices are about 51% and 86% at 48 hours and 72 hours, respectively. Under these conditions, DNA damage-positive cells are approximately 50% and 85%, respectively, indicating that BrdU labelled for one cell cycle is sufficient for inducing DSBs.

UVC-irradiation. The protocol uses UVC-light, so a UV irradiation box equipped with germicidal lamps is required. The dose of UVC-exposure needs to be determined, as insufficient UVC-exposure may result in indistinct DSB formation. If a UVC dosimeter and a

UVC-irradiation box are available, expose cells at a dose rate of 1.0 J/m^2/sec. If these are not available, a UVC-lamp (15 W) attached to the clean bench can be used. At 50 cm distance from the UVC-lamp center, the dose rate is approximately 1.0 J/m^2/sec. Localized DSBs formation becomes apparent after a dose of 10 J/m^2 or more. In most cases we use 30 J/m^2. The number of DSBs in a 5 μm diameter area after exposure to 30 J/m^2 is estimated to be equivalent to that induced by 2 Gy of X-rays, which is about 80 DSBs.

Since UVC alone can induce phosphorylation of histone H2AX coupled with nucleotide excision repair (NER), the appropriate UVC dose should be examined carefully. While we were unable to detect phosphorylation of histone H2AX with 30 J/m^2 of UVC in our cells without BrdU-labelling, NER-coupled phosphorylation has been reported in the G1 or G2 phase with 60 to 100 J/m^2 of UVC (32, 33). In quiescent cells, phosphorylation was detected after 10 J/m^2 or less (33, 34). Thus, the UVC dose that dose not elicit any significant confounding effects, needs to be determined.

Membrane pore size. The pore size is another factor to be determined before experimentation. There are several types of micropore membranes with different pore size. As the pore size increases, the pore density decreases. With 5 μm micropore membrane, one or two subnuclear areas are targeted. With 3 μm and 2 μm micropore membranes, 2-4 and 5-7 areas are targeted, respectively (**Figure 1A**). We recommend beginning with a 5 μm micropore membrane.

Detection of DSB formation. Although multiple DSBs are locally created by the protocol, current biochemical techniques are not sensitive enough to detect those DSBs. Therefore, indirect detection using antibodies recognizing the factors related to DNA damage response is an alternative way to identify DSBs. So far, anti-phosphorylated histone H2AX at serine 139 antibody has been widely used to locate DSBs. Thus, while DSBs are formed immediately after the exposure, 30 min incubation is necessary for immunological reaction of the antibody.

Controls. To confirm that the results obtained reflect DSB formation, cells without BrdU-labelling are exposed to UVC in the same way. Under certain conditions, BrdU alone may also be harmful to cells, thus cells without micro-irradiation should be processed at the same time in the same way as negative controls. DSB formation can be determined by immunofluorescence assay using anti-phosphorylated histone H2AX at serine 139 antibody, and antibodies against phosphorylated ATM at serine 1981 and 53BP1 can also be used for confirmation of the results.

MATERIALS

REAGENTS

- Eagle's MEM (NISSUI, cat. no. 05900)
- Sodium Pyruvate (WAKO, cat. no. 199-03062)
- $NaHCO_3$ (WAKO, cat. no. 191-01305)

- HEPES (DOJINDO, cat. no. 342-01375)
- Fetal bovine serum (FBS) (ThermoTrace, cat. no. 15-010-0500V)
- Trypsin (BD-DIFCO, cat. no. 215240)
- Phosphate-buffered saline (PBS)
- Any exponentially growing cells can be used. In this protocol BJ1-hTERT cells (Foreskin-derived normal human diploid fibroblasts immortalized by the hTERT gene) (Invitrogen) were used.
- 5-Bromo-2'-deoxy-uridine (BrdU) (BOEHRINGER MANNHEIM, cat. no. 280879)
- Mouse anti-phosphorylated histone H2AX at serine 139 monoclonal antibody (BioLegend, clone 2F3, cat. no. 613402)
- Mouse anti-phosphorylated ataxia-telangiectasia mutated (ATM) at serine 1981 monoclonal antibody (Rockland, clone 10H11.E12, code no. 200-301-400)
- Rabbit anti-phosphorylated histone H2AX at serine 139 antibody (Bethyl, cat. no. A300-081A)
- Rabbit anti-p53 binding protein 1 (53BP1) antibody (Bethyl, cat. no. A300-272A)
- Rabbit anti-ring finger protein 8 (RNF8) polyclonal antibody (Abcam, cat. no. ab4183)
- Alexa Fluor 488-conjugated goat anti-mouse IgG (Invitrogen, cat. no. A11001)
- Alexa Fluor 594-conjugated goat anti-rabbit IgG (Invitrogen, cat. no. A11008)
- Tris(hydroxymethyl)aminomethane (Tris) (WAKO, cat. no. 207-06275)
- NaCl (WAKO, cat. no. 191-01665)

- Tween-20 (WAKO, cat. no. 167-11515)
- Skim milk (BD-DIFCO, cat. no. 232100)
- Formalin (WAKO, cat. no. 064-00406) !CAUTION Toxic by inhalation; work in a fume hood.
- Triton X-100 (SIGMA, cat. no. P2287)
- DAPI (Molecular Probes, cat. no. D-21490)
- Glycerin (WAKO, cat. no. 075-00616)
- Eukitt (O. Kindler GmbH & Co.) !CAUTION Contains xylene, which is harmful if swallowed or inhalated; work in a fume hood.

EQUIPMENT

- Clean bench for cell culture with vertical laminar flow and equipped with UVC light (HITACHI, CCV).
- Water bath with temperature control
- CO_2 incubator with temperature control
- Surgical forceps
- Micropore membranes with 25-mm diameter (MILLIPORE, ISOPORE MEMBRANE FILTERS, 5 µm pore, cat. no. TMTP02500, 3 µm pore, cat. no. TSTP02500, 2 µm pore, cat. no. TTTP02500)
- Glass coverslips (22 mm x 22 mm) (MATSUNAMI GLASS, No.1)

- Glass slides (MATSUNAMI GLASS, cat. no. S2215)
- UV dosimeter (UVP, UVX Radiometer)
- Fluorescence microscope (Leica, DM3000B)
- FW4000 digital image capture and analysis software (Leica)
- 25 cm^2 culture flask (BD Falcon, cat. no. 353082)
- 10 ml culture pipette (IWAKI GLASS)
- Cell counter (Sysmex, cat. no. CDA-500)
- 15 ml tissue-culture tube (BD Falcon, cat. no. 352096)
- UVC-irradiation box (UVP, cat. no. XX-15)
- UVC lamp (HITACHI, Germicidal lamp, GL-15)

REAGENT SETUP

Culture medium Make MEM stock solution by dissolving 9.4 g MEM powder in 500 ml of Milli-Q water and by adding 55 mg $liter^{-1}$ Sodium Pyruvate, 1.1 g $liter^{-1}$ $NaHCO_3$ and 4.768 g $liter^{-1}$ HEPES. Adjust pH to 7.2 by 1 N HCl. Prepare culture medium by mixing 450 ml of MEM stock solution and 50 ml of FBS. Culture medium can be stored for 1 month at 4°C.

PBS(-) Make 10x stock solution by dissolving 80 g liter-1 NaCl, 28.8 g liter-1 Na_2HPO_4, 2 g liter-1 KCl, and 2 g liter-1 KH_2PO_4 to Milli-Q water. This stock solution can be stored at room temperature, which is about 22°C, for several months.

0.2% trypsin/PBS Dissolve 2 g of Trypsine powder in 1 liter of PBS. Make 100 ml

aliquots and they can be store at -20°C for several months.

BrdU 10 mM BrdU solution was prepared in Milli-Q water. The stock solution can be stored at 4°C in the dark for 3 months at most.

TBS Make 20x stock solution by dissolving 48.4 g liter-1 Tris and 160 g liter-1 NaCl. Adjust pH to 7.6 by 1 N HaOH. This stock solution can be stored at room temperature for several months.

TBS-DT Dissolve 50 mg ml^{-1} Skim milk and 0.1% v/v of Tween-20 to TBS. Prepare fresh.

Antibody Solutions Dilute mouse monoclonal anti-phosphorylated Histone H2AX at serine 139 antibody (1:500), rabbit anti-phosphorylated Histone H2AX at serine 139 antibody (1:500), mouse monoclonal anti-phosphorylated ATM at serine 1981 antibody (1:400), rabbit anti-53BP1 antibody (1:500), or mouse monoclonal anti-RNF-8 antibody (1:500) in TBS-DT.

PROCEDURE

Cell preparation TIMING 15 min

1| Maintain normal human diploid BJ1-hTERT cells in an exponentially growing state by subculturing cells every three days. Wash cells with PBS (-) once and incubate cells with 0.2% trypsin/PBS (use 1 ml/cm^2 of culture area) for 3 min at room temperature. Add 5 ml of culture medium and collect cells into 15 ml tissue culture tube by pipetting with 10 ml culture pipette.

2| Count the number of cells using a cell counter, resuspend cells in culture medium at $1x10^5$ cells/ml, and pipette 0.5 ml of cell suspension onto 22 mm x 22 mm coverslips in 35 mm culture dishes so that the immunological detection of DSBs becomes possible (**Figure 2**). Incubate cells in a CO_2 incubator for 6-12 hours incubation.

BrdU-labelling 48-72 h

3| Replace the culture medium that the cells are in on the coverslip As cells are attached to the coverslip at this point, aspirate the culture medium and add 2 ml of fresh culture medium containing 10 μM BrdU. Culture cells for further 48-72 hours.

CRITICAL STEP Cell growth kinetics and the thymidine pool sizes are very different between cells. While the cells used in this protocol show DNA damage with 10 μM of BrdU, different cells may respond differently. Thus, the optimal concentration of BrdU should be determined carefully (see Experimental Design).

?TROUBLESHOOTING

UVC-exposure TIMING 45 min ~ 1 h

4| Remove medium from each culture (from Step 3) and store in different 15 ml culture tubes. [Thanks for this comment!] The stored medium was used in step 5. Wash cells with 2 ml of PBS (-) once, remove PBS (-) and immediately cover the cells with micropore membrane (25-mm diameter) (**Figure 2**), and expose to 10-30 J/m^2 UVC-light in

UVC-irradiation box. Use surgical forceps to handle micropore membranes.

!CAUTION Adequate precautions should be taken in using ultraviolet light sources. Exposure of the unprotected skin and eyes should always be avoided. Excessive exposure to high intensity UVC-light can cause inflammation of the conjunctiva, effects in the cornea, iris, and lens of the eyes as well as reddening or burning of the skin.

CRITICAL STEP This is the most critical step. Because cells are sensitive to osomotic change, complete this step as quick as possible. Also, it is important to remove PBS(-) as much as possible, as residual PBS(-) may cause diffraction of UVC-light, which results in diffused formation of DSBs (**Figure 1B**). Care should also be taken not to trap bubbles between the membrane and cells. A part of cells may be left uncovered (**Fig. 2**) so that BrdU-labelling and UVC-dose can be checked (**Figure 1C**).

?TROUBLESHOOTING

5| After exposure, add stored culture medium (from step 4) so that the membrane readily comes off from the cells. Remove the membrane and incubate cells in CO_2 incubator for 30 minutes at 37°C.

Immunological detection of DSBs TIMING 4 h

6| Wash cells with PBS(-) and fix cells in 2 ml of 4% formalin/PBS(-) for 10 min at room temperature. Remove 4% formalin, wash with PBS(-) and add 2 ml of 0.5% triton

X-100/PBS(-) for 5 min on ice. Wash with PBS(-) extensively.

PAUSE POINT Can be left for up to 3 days at 4°C.

7| Pipette 100 µl of antibody solution onto the cells and incubate the samples in CO_2 incubator for 2 hrs at 37°C. Multiple antibodies can be added to the same antibody solution.

8| Wash with PBS(-) once and pipette 100 µl of Alexa Fluor 488-labelled anti-mouse IgG antibody and Alexa Fluor 594-labelled anti-rabbit IgG antibody diluted in TBS-DT (1:1000) onto the cells. [I have provided the following sentences here. But, if you feel they should go to the Materials section, please move.] They can be added simultaneously, and any fluorochromes can be used. Incubate the samples in CO_2 incubator for 1 hr at 37°C.

9| Wash with PBS(-) and place the coverslips cell-side down onto the glass slides which are submerged in PBS(-) place the cover slips cell-side down onto the glass slides which have a drop of PBS containing 10% glycerol and 0.1 µg/ml DAPI. Turn the slide over and seal the edges of the coverslip with Eukitt.

PAUSE POINT Slides can be stored for up to 1 month at 4°C.

10| Examine slides with a widefield microscope equipped with motorized stages. Acquire z-stack images every 0.5 µm at a x400 magnification and create deconvoluted images by

maximum intensity projections. Capture images in individual colors using different filter units equipped to DM3000B microscope Acquire images using a Leica HCX PL FLUOTAR 40X dry, with an NA of 0.75 and filter cube A4 (Excitation filter: BP 360/40, Dichromatic mirror: 400, Suppression filter: BP470/40) for blue fluorescence, L5 (Excitation filter: BP 480/40, Dichromatic mirror: 505, Suppression filter: BP527/30) for green fluorescence and N3 (Excitation filter: BP 546/12, Dichromatic mirror: 565, Suppression filter: BP600/40) for red fluorescence. Create merged images, and analyze the merged digital images by FW4000 software according to the manufacturer's instruction [Unfortunately, there is no downloadable instruction]. Detect localized DSBs by visualizing phosphorylation of histone H2AX at serine 139. Or, detect protein localization and localized phosphorylation at DSB sites by visualizing various antibodies.

?TROUBLESHOOTING

TIMING

Steps 1-2, cell preparation, 15 mins

Step 3, BrdU Labelling, 48-72h

Steps 4-5, UVC exposure, 45 min-1h

Steps 6-10, Immunological detection of DSBs, 4h

?TROUBLESHOOTING

Troubleshooting advice can be found in **Table 1**.

Table 1 Troubleshooting table

Steps	Problem	Possible reasons	Solutions
3	Low BrdU incorporation	Insufficient cell growth	Increase BrdU concentration
		Large thymidine pool	Label cells for up to 72 hours
4	Cells are damaged	Osmotic shock	Make sure that cells are not dried up
			Complete the steps as quick as possible
10	Low DSB induction of diffused signal	Insufficient BrdU labelling	Determine optimal condition for BrdU-labelling
		Inappropriate UVC-dose	Increase UVC-dose up to 40 J/m^2

	PBS(-) aspiration is imperfect	Remove PBS(-) as much as possible, but do not to cause osmotic shock
Unexpected signal	UV-induced CPDs and 6-4PPs affects the results	Repeat the same experiment without BrdU-labelling

ANTICIPATED RESULTS

The protocol efficiently creates localized DSBs in a subnuclear area of a cell nucleus that are detectable by phosphorylation of histone H2AX at serine 139 (**Figure 3A**). DSB formation is confirmed by localized activation (phosphorylation) of ATM, which is the sensor protein for DSBs and the responsive kinase for phosphorylation of histone H2AX at serine 139 (**Figure 3B**). With this protocol, recruitment of proteins involved in DNA repair and DNA damage checkpoint are also detectable (**Figure 3B**). DSB signals induced by UVC-exposure alone in the absence of BrdU-labeling are very weak (**Figure 3A**), so that the effect of UV damage, such as CPDs and 6-4PPs, on DSB formation is negligible in G1 and G2 cells, although the possibility should always be taken into consideration. Inefficient DSB induction may result from insufficient BrdU-labelling and inappropriate UVC-exposure. This can be checked by anti-phosphorylated histone H2AX at serine 139, as UVC-exposure of BrdU-labelled cells

without micropore membrane generates strong whole-nuclei immunofluorescence signal (**Figure 1C**). The protocol provides simple and undemanding method for generating localized DSBs. It obviates the need for laser sources or specialized devises, and thus, the protocol can be followed easily by researchers without any previous experience.

Author contributions statements

K.S. conceived of the study, designed and performed the study, and drafted the manuscript. M.Y., Y.O. and M.S. provided reagents, carried out the immunofluorescence study, and edited the manuscript. S.Y. supervised the project.

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Competing financial interests

The authors declare that they have no competing financial interests.

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FIGURE LEGENDS

Figure 1. Use of micropore membrane. Exponentially growing normal human diploid cells were incubated for 72 hours with 10 μM BrdU. Cells were exposed to 30 J/m^2 of UVC through micropore membranes. They were incubated for 30 min, fixed with 4% formaldehyde, permeabilized with 0.5% Triton X-100, and incubated with anti-phosphorylated histone H2AX at serine 139. A, cells were exposed to UVC-light through 2 μm, 3 μm or 5 μm pore size membrane. With 5 μm micropore membrane, one or two subnuclear areas are targeted. With 3 μm and 2 μm micropore membranes, 2-4 and 5-7 areas are targeted, respectively. Bar indicates 3 μm. B, diffused fluorescence signals caused by diffraction of UVC-light as a result of inappropriate PBS(-) aspiration. The nucleus has four areas exposed (white arrow heads), but all areas show diffused signals. Bar indicates 3 μm. C, homogenous DSBs induction by UVC-irradiation without micropore membrane. Bar indicates 6 μm.

Figure 2. Cells covered by micropore membrane. Cells cultured on 22 mm x 22 mm coverslips (left) are covered by micropore membrane (right). It is critical to aspirate as much of PBS as much as possible (see **Fig. 1B**). Care should also be taken not to trap bubbles between the membrane and cells. A part of cells may be left uncovered so that BrdU-labelling and UVC-dose can be checked by the pan-nuclear signal of phosphorylated histone H2AX (**Figure 1C**).

Figure 3. Micro-irradiation creates localized DSBs and recruits DNA damage checkpoint factors. A; Exponentially growing normal human diploid cells were incubated for 72 hours with 10 μM BrdU (labeled BrdU/30 J/m^2) or without BrdU (labeled 30 J/m^2). The control sample is BrdU-labelled cells without micro-irradiation. Cells were exposed to 30 J/m^2 of UVC with 5 μm pore membrane. After UVC irradiation, cells were incubated for 30 min, fixed with 4% formaldehyde, permeabilized with 0.5% Triton X-100, and incubated with anti-phosphorylated histone H2AX at serine 139. DSB formation can be detected by localized signal of phosphorylated histone H2AX (middle), while UVC alone does not show localized DSBs. Bar indicates 3 μm. B; Exponentially growing cells were incubated for 72 hours with 10 μM BrdU. Cells were exposed to 30 J/m^2 of UVC through 5 μm pore size membrane. Then, incubated for 30 min, fixed with 4% formaldehyde, permeabilized with 0.5% Triton X-100, and incubated with antibodies against phosphorylated ATM at serine 1981 (ATM-P), phosphorylated histone H2AX at serine 139 (H2AX-P), p53 binding protein 1 (53BP1) and ring finger protein 8 (RNF-8). Colocalization of phosphorylated H2AX and phosphorylated ATM signals confirms DSB formation. Colocalization of 53BP1 and RNF-8 signals with that of phosphorylated H2AX indicates that these DNA repair and DNA checkpoint factors are recruited to the sites of DSBs. Bar indicates 3 μm.