Published in final edited form as:

Methods Mol Biol. 2015 ; 1292: 67–75. doi:10.1007/978-1-4939-2522-3_5.

Immunofluorescence-based methods to monitor DNA end resection

Bipasha Mukherjee, Nozomi Tomimatsu, and Sandeep Burma*

Division of Molecular Radiation Biology, Department of Radiation Oncology, University of Texas Southwestern Medical Center, 2201 Inwood Road, NC7.208, Dallas, TX 75390, USA

Summary

Double-strand breaks (DSBs) are the most deleterious amongst all types of DNA damage that can occur in the cell. These breaks arise from both endogenous (for example, DNA replication stress) as well as exogenous insults (for example, ionizing radiation). DSBs are principally repaired by one of two major pathways: non-homologous end joining (NHEJ) or homologous recombination (HR). NHEJ is an error-prone process that can occur in all phases of the cell cycle, while HR is limited to the S and G2 phases of the cell cycle when a sister chromatid is available as a template for error-free repair. The first step in HR is “DNA end resection”, a process during which the broken DNA end is converted into a long stretch of 3′-ended single-stranded DNA (ssDNA). In recent years, DNA end resection has been identified as a pivotal step that controls “repair pathway choice” i.e., the appropriate choice between NHEJ and HR for DSB repair. Therefore, methods to quantitatively or semi-quantitatively assess DNA end resection have gained importance in laboratories working on DNA repair. In this chapter, we describe two simple immunofluorescence-based techniques to monitor DNA end resection in mammalian cells. The first technique involves immuno-detection of Replication Protein A (RPA), a ssDNA-binding protein that binds to resected DNA. The second technique involves labeling of genomic DNA with 5-bromo-2′-deoxyuridine (BrdU) that can be detected by anti-BrdU antibody only after the DNA becomes single stranded due to resection. These methods are not complicated, do not involve sophisticated instrumentation or reporter constructs, and can be applied to most mammalian cell lines, and therefore, should be of broad utility as simple ways of monitoring DNA end resection *in vivo*.

Keywords

DNA Damage; DNA Double-Strand Break (DSB); DNA Repair; Homologous Recombination; DNA end Resection; Single Stranded DNA (ssDNA); RPA; BrdU

1. Introduction

Genomic insults like ionizing radiation (IR) or chemotherapeutic drugs cause double-strand breaks (DSBs) in our DNA. DSBs also arise from DNA replication stress due to the stalling and collapse of replication forks. Such breaks can trigger genomic instability and cause cell death or cancer if they are not repaired promptly and correctly. Two major pathways have

* Address correspondence to sandeep.burma@utsouthwestern.edu, Tel: 214-648-7440; Fax: 214-648-5995.

evolved to deal with these breaks in mammalian cells – non-homologous end joining (NHEJ) and homologous recombination (HR) [1]. NHEJ can occur throughout the cell cycle and rapidly rejoins the broken DNA ends following limited end processing and can thus be error-prone [2]. HR is restricted to post-replicative phases of the cell cycle and typically uses an undamaged sister chromatid as a template to restore genomic integrity and is thus potentially error-free [3]. A fine balance between the usage of NHEJ and HR is necessary for optimally maintaining genomic integrity. It would be important to minimize HR usage in G1 as recombination in the absence of a sister chromatid could lead to loss of heterozygosity (LOH) or chromosomal rearrangements due to recombination with the homologous chromosome or with homologous sequences elsewhere in the genome. However, use of HR in S and G2 phases would promote error-free repair and, indeed, HR would be the only means to repair one-ended replication fork-associated breaks. Hence mechanisms of “repair pathway choice” or the appropriate choice between NHEJ and HR are important for cell survival upon DNA damage, and these mechanisms control a critical step in HR termed “DNA end resection” [4–7].

DNA end resection is an early step in HR during which the broken DNA end is converted into a long stretch of 3'-ended single-stranded DNA (ssDNA). The ssDNA tail that is generated is rapidly coated by RPA (Replication Protein A), a heterotrimeric protein which prevents the formation of secondary structures and protects against degradation of the ssDNA [8]. Next, RPA is replaced with Rad51 to generate a recombinogenic nucleoprotein filament that searches for homologous sequences in the sister chromatid or elsewhere in the genome. As the generation of ssDNA promotes HR and thwarts NHEJ (by preventing the binding of NHEJ proteins), it is easy to understand why DNA end resection is a critical step at which repair pathway choice is exercised. DNA end resection occurs in a “two step” manner [6,7]. The first step, “initiation of resection”, involves the removal of ~50–100 bases of DNA from the 5' end by the MRX/MRN complex (Mre11-Rad50-Xrs2 in yeast and Mre11-Rad50-Nbs1 in mammals) in conjunction with Sae2/CtIP [9–13]. The second step, “long-range resection”, is carried out by two alternate pathways involving either the 5' to 3' exonuclease Exo1 alone or the helicase Sgs1/Blm in concert with Exo1 or the nuclease Dna2 [14–16]. Long-range resection proceeds at the rate of 4 kb per hour in yeast and the ssDNA tails generated can be several kilobases in length [6]. DNA end resection is regulated, at one level, by the mutually antagonistic relationship between Rif1-53BP1-PTIP and Brca1-CtIP wherein resection is blocked in G1 by 53BP1 which block is lifted by the action of Brca1 in S and G2 [17]. At another level, DNA end resection is regulated by CDKs that drive cells through S and G2 phases such that phosphorylation of resection nucleases CtIP, Exo1, and Dna2 by these CDKs promotes resection and restricts it to the post-replicative phases of the cell cycle [18–21]. DNA end resection also inactivates ATM kinase while activating ATR kinase at the same time thereby actuating a transition in the mode of cell cycle checkpoint signaling [22,16].

Given its pivotal role in repair pathway choice and cell cycle checkpoint signaling mechanisms, DNA end resection is currently an area of avid interest amongst researchers in the field of DNA repair and several assays are in use to quantitatively or semi-quantitatively monitor DNA end resection. Here, we describe two simple immunofluorescence-based techniques to monitor DNA end resection in mammalian cells. One technique is indirect and

visualizes the binding of RPA to resected DNA in the nucleus by immunofluorescence staining with anti-RPA antibody. The second method is more direct and involves labeling of genomic DNA with 5-bromo-2'-deoxyuridine (BrdU) that can be detected by anti-BrdU antibody only after the DNA becomes single stranded due to resection. The resected DNA can be visualized by fluorescence microscopy as punctate RPA or BrdU/ssDNA "foci" (Fig. 1) whose numbers can serve as a metric to quantify resection in cells responding to DNA damage or replication stress. These two protocols are a synthesis of methods published by other groups [23–27] that were further modified by us and described in previous publications from our group [28,16,29,21].

2. Materials

2.1. Tissue Culture Slides

1. Falcon™ Culture Slides (4 chamber) [Fisher Scientific; Catalog no: 08-774-209]
2. Fisherfinest™ Premium Cover Glasses (50 × 20 mm) [Fisher Scientific Catalog no: 12-548-5E]

2.2. Buffers and Solutions

1. Phosphate buffered saline (PBS), pH 7.4
2. Extraction Buffer 1: 10mM PIPES, pH 7.0; 100mM NaCl; 300mM Sucrose; 3mM MgCl₂; 1mM EGTA, 0.5% Triton X-100. Store at 4°C and use within 2 to 3 weeks.
3. Extraction Buffer 2: 10mM Tris-HCl, pH 7.5; 10mM NaCl; 3mM MgCl₂, 1% Tween 40, 0.5% sodium deoxycholate. Store at 4°C and use within 2 to 3 weeks.
4. 4% Paraformaldehyde solution (PFA). Dissolve 4 g of paraformaldehyde in 50 mL of water and 1 mL of 1 M NaOH (heat at 65°C in a water bath until powder is completely dissolved). Cool to room temperature. Add 10 mL of 10X PBS. Adjust the pH to 7.4 using HCl. Make up the volume to 100 mL with water. Filter through 0.2 µm filter. Store at –20°C in aliquots covered in aluminum foil and use within a month.
5. 0.5% Triton X-100 in PBS. Store at 4°C and use within 2 to 3 weeks.
6. 5% BSA (fraction V) in PBS. Store at 4°C and use within 2 to 3 weeks.
7. 1% BSA (fraction V) in PBS. Store at 4°C and use within 2 to 3 weeks.
8. 10 mg/mL BrdU in distilled water. Aliquot and store at –80°C and use within a year [Sigma; Catalog no: B5002]
9. Vectashield with DAPI [Vector Labs; Catalog no: H-1200].

2.3. Antibodies

1. Anti-RPA (Ab-3) mouse mAb [Calbiochem; Catalog no: NA191]
2. Anti-BrdU mouse mAb [BD Biosciences; Catalog no: 347580]

3. Alexa Fluor® 568 donkey anti-mouse IgG [Life Technologies, Catalog no: A10037]

3. Methods

3.1. Cell culture and Treatments

Both immunofluorescence protocols were standardized for U2OS cells and may need to be modified for other cell lines (*see* ^{Note 1}). DSBs can be induced by irradiating cells using a gamma- or x-ray irradiator. We use a Shepherd Mark 1–68 Cesium-137 irradiator. Alternatively, radiomimetic drugs (such as bleomycin or neocarzinostatin) or topoisomerase inhibitors (such as camptothecin or etoposide) may be added to the culture medium. Radiation doses or drug concentrations would have to be individually standardized depending upon the experiment and the level of background DNA damage in the cell line being used.

3.2. RPA Immunostaining Protocol

- 1 Seed cells in a tissue-culture treated 4-chamber slide (60,000 cells in 500 µL of cell culture medium per chamber) and allow them to attach and grow for about 16 h (*see* ^{Note 2}).
- 2 Irradiate cells with a total dose of 6Gy and incubate cells at 37°C in a CO₂ incubator for 3 h (*see* ^{Note 3}).
- 3 Wash cells with PBS. Aspirate off cell culture medium using low suction pressure, gently add 500 µL of PBS down the walls of the chamber, rock slide gently a couple of times and aspirate off PBS. Take care to not let slide dry between steps and minimize time between steps. Keep slide on ice as much as possible and all solutions cold (until Step 11).
- 4 Incubate cells in 500 µL of Extraction Buffer 1 for 10 min on ice (*see* ^{Notes 4 and 5}).
- 5 Wash cells with PBS.
- 6 Incubate cells in 500 µL of Extraction Buffer 2 for 10 min on ice.
- 5 Wash cells with PBS.
- 6 Fix cells in 500 µL of 4% PFA for 20 min on ice (*see* ^{Note 6}).

¹The protocol can be used for most human and murine cell lines. However “flatter” cells (for example, U2OS) provide better images.

²As a less expensive alternative to chamber slides, cells can be grown on coverslips in a 12-well plate and processed in the well.

³The incubation time after DNA damage induction may have to be varied depending upon the experiment and the cell line being used to obtain maximum number of foci. We recommend using a range of doses of the DNA damaging agent and varying incubation times while standardizing the protocol.

⁴The extraction steps with Buffers 1 and 2 are important for obtaining clear RPA or BrdU foci. The extraction time in each of the buffers is critical and may have to be standardized for other cell lines. We recommend doing a pilot experiment with different incubation times in the extraction buffers if using a cell line other than U2OS.

⁵Some cell lines may become more prone to detachment after the extraction steps. Handle slides carefully after extraction and check under microscope between steps to ensure that cells are not detaching.

⁶In our experience, PFA quality appears to be the most important factor affecting overall image quality. PFA should be made carefully and the pH balanced accurately. We prefer to not use PFA that is more than a month old. Freshly made PFA works best in our hands.

- 7 Wash cells with PBS
- 8 Permeabilize cells by incubating in 500 μ L of 0.5% Triton X-100 for 10 min on ice.
- 9 Wash cells with PBS.
- 10 Block slide by incubating in 500 μ L of 5% BSA in PBS on ice for 20 min.
- 11 Incubate with primary antibody (anti-RPA at 1:500 dilution in 1% BSA) for 16 h at 4°C in a humid chamber (a small plastic box with a tight lid and wet Kimwipes at the bottom works fine). At least 200 μ L of antibody solution should be used to prevent chamber from drying out.
- 12 Wash cells in PBS three times at room temperature; each time leave the PBS on for at least 10 min (*see*^{Note 7}).
- 13 Incubate with secondary antibody (Alexa Fluor® 568 anti-mouse at 1:1000 dilution in 1% BSA) in a dark box for 1 h at room temperature.
- 12 Wash cells in PBS three times at room temperature; each time leave the PBS on for at least 10 min keeping cells in a dark box.
- 13 Remove chambers and gently aspirate off excess PBS taking care to not scratch the cells. Air-dry the slide for a couple of minutes in a dark box (do not over-dry). Add a small drop of Vectashield containing DAPI to each chamber, cover with glass coverslip, tap gently to remove bubbles, and seal the coverslip with quick drying transparent nail polish. Store slides at –20°C in a dark box.

3.3. BrdU immunostaining protocol

- 1 Seed cells in a tissue-culture treated 4-chamber slide such that they will be about 50–60% confluent the next day (typically 40,000 cells in 500 μ L of cell culture medium per chamber) and allow them to attach and grow for about 24 h (*see* Note 2).
- 2 Add BrdU to cells at a final concentration of 10 μ g/mL and incubate for 16 h (approximately one cell cycle).
- 2 Irradiate cells with a total dose of 10 Gy and incubate cells at 37°C in a CO₂ incubator for 1 h (*see*^{Note 3}).
- 3 Wash cells with PBS: Aspirate off cell culture medium using low suction pressure, gently add 500 μ L of PBS down the walls of the chamber, rock slide gently a couple of times and aspirate off PBS. Take care to not let slide dry between steps and minimize time between steps. Keep slide on ice as much as possible and all solutions cold (until Step 12).
- 4 Incubate cells in 500 μ L of Extraction Buffer 1 for 10 min on ice (*see*^{Notes 4 and 5}).

⁷Longer washes after incubation with primary or secondary antibodies may help reduce non-specific signals.

- 5 Wash cells with PBS.
- 6 Incubate cells in 500 μ L of Extraction Buffer 2 for 10 min on ice.
- 5 Wash cells with PBS.
- 6 Fix cells in 500 μ L of 4% PFA for 20 min on ice (*see* ^{Note 6}).
- 7 Wash cells with PBS
- 8 Permeabilize cells by incubating in 500 μ L of 0.5% Triton X-100 for 10 min on ice.
- 9 Wash cells with PBS.
- 10 Block slide by incubating in 500 μ L of 5% BSA in PBS on ice for 20 min.
- 11 Incubate with primary antibody (anti-BrdU at 1:100 dilution in 1% BSA) for 16 h at 4°C in a humid chamber (a small plastic box with a tight lid and wet Kimwipes at the bottom works fine). At least 200 μ L of antibody solution should be used to prevent chamber from drying out.
- 12 Wash cells in PBS three times at room temperature; each time leave the PBS on for at least 10 min (*see* ^{Note 7}).
- 13 Incubate with secondary antibody (Alexa Fluor® 568 anti-mouse at 1:1000 dilution in 1% BSA) in a dark box for 1 h at room temperature.
- 12 Wash cells in PBS three times at room temperature; each time leave the PBS on for at least 10 min keeping cells in a dark box.
- 13 Remove chambers and gently aspirate off excess PBS without touching areas with cells. Air-dry the slide for a couple of minutes in a dark box (do not over-dry). Add a small drop of Vectashield containing DAPI to each chamber, cover with glass coverslip, tap gently to remove bubbles, and seal the coverslip with quick drying transparent nail polish. Store slides at –20°C in a dark box.

3.4. Quantification of Foci

Images of nuclei can be obtained using a fluorescence microscope at magnifications ranging from 40X to 100X. If a confocal microscope is used to capture *z*-sections, the sections should be merged so that all foci are visible on a single plane. The average numbers of foci per nucleus are determined after manually counting 50–100 nuclei using the Image J Cell Counter software (*see* ^{Notes 8 and 9}).

⁸Since extensive resection occurs only in cells in S and G2 phases of the cell cycle, all cells will not show the presence of RPA or BrdU/ssDNA foci.

⁹While both these methods allow quantification of the number of breaks undergoing extensive resection at any given time, these methods do not allow us to measure the extent of resection at a given break. A different protocol can be used to directly assess the length of resection at a restriction enzyme-generated DSB at a sequence-specific site [30]. In addition, one can indirectly measure the extent of resection at micro-laser-induced DSBs in living cells in real time by quantifying the accumulation of GFP-tagged RPA [16].

Acknowledgments

SB is supported by grants from the National Institutes of Health (RO1 CA149461) and the National Aeronautics and Space Administration (NNX13AI13G).

References

1. Wyman C, Kanaar R. DNA double-strand break repair: all's well that ends well. *Annual review of genetics*. 2006; 40:363–383.
2. Lieber MR. The mechanism of double-strand DNA break repair by the nonhomologous DNA end-joining pathway. *Annual review of biochemistry*. 2010; 79:181–211.
3. Heyer WD, Ehmsen KT, Liu J. Regulation of homologous recombination in eukaryotes. *Annual review of genetics*. 2010; 44:113–139.
4. Chapman JR, Taylor MR, Boulton SJ. Playing the end game: DNA double-strand break repair pathway choice. *Molecular cell*. 2012; 47:497–510. [PubMed: 22920291]
5. Shrivastav M, De Haro LP, Nickoloff JA. Regulation of DNA double-strand break repair pathway choice. *Cell research*. 2008; 18:134–147. [PubMed: 18157161]
6. Symington LS, Gautier J. Double-strand break end resection and repair pathway choice. *Annual review of genetics*. 2011; 45:247–271.
7. Huertas P. DNA resection in eukaryotes: deciding how to fix the break. *Nature structural & molecular biology*. 2010; 17:11–16.
8. Fanning E, Klimovich V, Nager AR. A dynamic model for replication protein A (RPA) function in DNA processing pathways. *Nucleic acids research*. 2006; 34:4126–4137. [PubMed: 16935876]
9. Gravel S, Chapman JR, Magill C, et al. DNA helicases Sgs1 and BLM promote DNA double-strand break resection. *Genes & development*. 2008; 22:2767–2772. [PubMed: 18923075]
10. Mimitou EP, Symington LS. Sae2, Exo1 and Sgs1 collaborate in DNA double-strand break processing. *Nature*. 2008; 455:770–774. [PubMed: 18806779]
11. Zhu Z, Chung WH, Shim EY, et al. Sgs1 helicase and two nucleases Dna2 and Exo1 resect DNA double-strand break ends. *Cell*. 2008; 134:981–994. [PubMed: 18805091]
12. Makharashvili N, Tubbs AT, Yang SH, et al. Catalytic and noncatalytic roles of the CtIP endonuclease in double-strand break end resection. *Molecular cell*. 2014; 54:1022–1033. [PubMed: 24837676]
13. Nicolette ML, Lee K, Guo Z, et al. Mre11-Rad50-Xrs2 and Sae2 promote 5' strand resection of DNA double-strand breaks. *Nature structural & molecular biology*. 2010; 17:1478–1485.
14. Nimonkar AV, Genschel J, Kinoshita E, et al. BLM-DNA2-RPA-MRN and EXO1-BLM-RPA-MRN constitute two DNA end resection machineries for human DNA break repair. *Genes & development*. 2011; 25:350–362. [PubMed: 21325134]
15. Bolderson E, Tomimatsu N, Richard DJ, et al. Phosphorylation of Exo1 modulates homologous recombination repair of DNA double-strand breaks. *Nucleic acids research*. 2010; 38:1821–1831. [PubMed: 20019063]
16. Tomimatsu N, Mukherjee B, Deland K, et al. Exo1 plays a major role in DNA end resection in humans and influences double-strand break repair and damage signaling decisions. *DNA repair*. 2012; 11:441–448. [PubMed: 22326273]
17. Daley JM, Sung P. 53BP1, BRCA1, and the choice between recombination and end joining at DNA double-strand breaks. *Molecular and cellular biology*. 2014; 34:1380–1388. [PubMed: 24469398]
18. Chen X, Niu H, Chung WH, et al. Cell cycle regulation of DNA double-strand break end resection by Cdk1-dependent Dna2 phosphorylation. *Nature structural & molecular biology*. 2011; 18:1015–1019.
19. Ferretti LP, Lafranchi L, Sartori AA. Controlling DNA-end resection: a new task for CDKs. *Frontiers in genetics*. 2013; 4:99. [PubMed: 23760669]
20. Huertas P, Cortes-Ledesma F, Sartori AA, et al. CDK targets Sae2 to control DNA-end resection and homologous recombination. *Nature*. 2008; 455:689–692. [PubMed: 18716619]

21. Tomimatsu N, Mukherjee B, Catherine Hardebeck M, et al. Phosphorylation of EXO1 by CDKs 1 and 2 regulates DNA end resection and repair pathway choice. *Nature communications*. 2014; 5:3561.
22. Shiotani B, Zou L. Single-stranded DNA orchestrates an ATM-to-ATR switch at DNA breaks. *Molecular cell*. 2009; 33:547–558. [PubMed: 19285939]
23. Mirzoeva OK, Petrini JH. DNA damage-dependent nuclear dynamics of the Mre11 complex. *Molecular and cellular biology*. 2001; 21:281–288. [PubMed: 11113202]
24. Kaidi A, Weinert BT, Choudhary C, et al. Human SIRT6 promotes DNA end resection through CtIP deacetylation. *Science (New York, NY)*. 2010; 329:1348–1353.
25. Hu Y, Scully R, Sobhian B, et al. RAP80-directed tuning of BRCA1 homologous recombination function at ionizing radiation-induced nuclear foci. *Genes & development*. 2011; 25:685–700. [PubMed: 21406551]
26. Cuadrado M, Martinez-Pastor B, Murga M, et al. ATM regulates ATR chromatin loading in response to DNA double-strand breaks. *The Journal of experimental medicine*. 2006; 203:297–303. [PubMed: 16461339]
27. Raderschall E, Golub EI, Haaf T. Nuclear foci of mammalian recombination proteins are located at single-stranded DNA regions formed after DNA damage. *Proceedings of the National Academy of Sciences of the United States of America*. 1999; 96:1921–1926. [PubMed: 10051570]
28. Tomimatsu N, Mukherjee B, Burma S. Distinct roles of ATR and DNA-PKcs in triggering DNA damage responses in ATM-deficient cells. *EMBO reports*. 2009; 10:629–635. [PubMed: 19444312]
29. Costelloe T, Louge R, Tomimatsu N, et al. The yeast Fun30 and human SMARCAD1 chromatin remodellers promote DNA end resection. *Nature*. 2012; 489:581–584. [PubMed: 22960744]
30. Zhou Y, Caron P, Legube G, et al. Quantitation of DNA double-strand break resection intermediates in human cells. *Nucleic acids research*. 2014; 42:e19. [PubMed: 24362840]

Fig. 1. Representative images of mock-irradiated or gamma-irradiated U2OS cells immunostained with anti-RPA antibody (red) or with anti-BrdU antibody (red). Nuclei are stained with DAPI (blue).