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A Flow Cytometry-Based Method for Analyzing DNA End Resection in G_0- and G_1-Phase Mammalian Cells

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Abstract

DNA double strand breaks (DSBs) constantly arise in cells during normal cellular processes or upon exposure to genotoxic agents, and are repaired mostly by homologous recombination (HR) and non-homologous end joining (NHEJ). One key determinant of DNA DSB repair pathway choice is the processing of broken DNA ends to generate single strand DNA (ssDNA) overhangs, a process termed DNA resection. The generation of ssDNA overhangs commits DSB repair through HR and inhibits NHEJ. Therefore, DNA resection must be carefully regulated to avoid mis-repaired or persistent DSBs. Accordingly, many approaches have been developed to monitor ssDNA generation in cells to investigate genes and pathways that regulate DNA resection. Here we describe a flow cytometric approach measuring the levels of replication protein A (RPA) complex, a high affinity ssDNA binding complex composed of three subunits (RPA70, RPA32, and RPA14 in mammals), on chromatin after DNA DSB induction to assay DNA resection. This flow cytometric assay requires only conventional flow cytometers and can easily be scaled up to analyze a large number of samples or even for genetic screens of pooled mutants on a genome-wide scale. We adopt this assay in G_0- and G_1- phase synchronized cells where DNA resection needs to be kept in check to allow normal NHEJ.

Keywords: DNA DSB, Resection, ssDNA, NHEJ, HR, RPA, Flow cytometry

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Background

Genome stability relies in large part on a timely response to and repair of DNA damage that arises during normal physiological processes such as DNA replication and exposure to genotoxic agents such as ionizing irradiation. Disruptions in these activities will lead to death of the affected cells in response to unrepairable damage or genomic rearrangement that promote oncogenic transformation (Tubbs and Nussenzweig, 2017). One of the major DNA lesions that cells encounter is DNA double strand breaks (DSBs). While all DSBs are detrimental if not repaired properly, many of them are important intermediates of critical cellular activities. For example, DSBs can be created by topoisomerase II to relieve torsional strain and supercoiled or catenated DNA during DNA replication or transcription (Nitiss, 2009). During meiosis, DSBs are induced by the nuclease SPO11 and other accessory proteins to initiate meiotic recombination (Lam and Keeney, 2014). In developing lymphocytes, DNA DSBs are generated by the RAG endonuclease, composed of RAG1 and RAG2, during V(D)J recombination to assemble functional antigen receptor genes (Schatz and Swanson, 2011).

The two major repair pathways for DNA DSBs are homologous recombination (HR) and non-homologous end joining (NHEJ). HR is utilized during S and G_2 phases of the cell cycle when a sister chromatid is available as a template for accurate repair (Prakash *et al.*, 2015). NHEJ is the predominant DNA DSB repair pathway in G_0, also termed quiescence, and G_1 phases of the cell cycle due to the lack of sister chromatids, although it is also functional in other cell cycle phases (Chang *et al.*, 2017). The critical choice of HR or NHEJ for DNA DSB repair depends on whether the broken DNA ends are resected to generate extensive single strand DNA (ssDNA) overhangs, which inhibit NHEJ and are quickly bound by the trimeric ssDNA binding complex replication protein A (RPA) to initiate HR (Symington and Gautier, 2011; Ceccaldi *et al.*, 2016; Scully *et al.*, 2019). Given the importance of appropriately employing HR or NHEJ in different cell cycle phases and at distinct genomic territories, DNA end processing is sophisticatedly regulated by nucleases and accessory proteins that promote resection, and DNA protection proteins that counter nucleolytic activities at DSBs (Symington, 2016; Setiaputra and Durocher, 2019; Mirman and de Lange, 2020; Cejka and Symington, 2021). Proper DNA end protection is especially important for cells in G_1 and G_0 phases of the cell cycle as extensively resected DNA DSBs cannot be repaired by NHEJ. The most studied DNA end protection protein is 53BP1, and since the initial discovery of its DNA protection function, many downstream effectors have been identified, including RIF1, the Shieldin complex, and the CST complex (Setiaputra and Durocher, 2019; Mirman and de Lange, 2020).

To decipher the complex regulation of DNA end resection and protection, numerous approaches have been established to qualitatively and/or quantitatively monitor the levels of ssDNA in cells in response to endogenous or exogenous DNA damage. For example, a quantitative PCR approach on restriction enzyme-digested genomic DNA purified from cells with endonuclease-induced DSBs allows for low resolution detection of resected ssDNA near DSBs that are resistant to cleavage by restriction enzymes (Zhou *et al.*, 2014). HCoDES and End-seq utilize different DNA structure capturing designs coupling with next generation sequencing to determine resected DNA end structure at nucleotide resolution (Dorsett *et al.*, 2014; Canela *et al.*, 2016). BrdU, when incorporated in the genome during DNA replication, can be used to reveal the presence of BrdU-labeled ssDNA in cells by anti-BrdU antibody staining and immunofluorescence imaging or flow cytometry in the absence of DNase I treatment (Mukherjee *et al.*, 2015; Tkac *et al.*, 2016).

Given that RPA rapidly binds to ssDNA with high affinity *in vivo* and *in vitro*, immunofluorescence imaging- or flow cytometry-based approaches have also been widely used to visualize the chromatin-bound RPA, as a surrogate of ssDNA, by anti-RPA antibody staining following detergent extraction to remove soluble RPA in cells (Forment *et al.*, 2012; Mukherjee *et al.*, 2015). Here we describe a high-throughput flow cytometry-based method, originally developed by Josep V. Forment, Rachael V. Walker, and Stephen P. Jackson, to monitor DNA end resection (Forment *et al.*, 2012). The flow cytometric assay described here and in (Forment *et al.*, 2012) can easily provide quantitative measurement of the levels of chromatin-bound RPA in large number of cells (tens to hundreds of thousands of cells) on conventional flow cytometers that are readily available to most research communities. We include steps that allow optimizing this assay in multiple cell types. While this method can be used for cells in any phase of the cell cycle, we also include protocols for synchronizing cells in G_0 and for identifying G_1 cells in a proliferating culture, where DNA end integrity is critical for DNA DSB repair by NHEJ (Chen *et al.*, 2021).

Materials and Reagents

1. 6-well plates (Corning, catalog number: 3506)
2. 24-well plate (Corning, catalog number: 3524)
3. Round bottom polystyrene tubes for FACS (Thermo Fisher Scientific, catalog number: 149595)
4. Abelson leukemia viral kinase transformed pre-B cells (abl pre-B cells, custom made in lab)
5. MCF10A (ATCC, catalog number: CRL-10317)
6. Triton X-100 (Sigma, catalog number: T-8787)
7. Rat monoclonal anti-RPA32 (4E4) antibody (Rat monoclonal) (Cell Signaling Technology, catalog number: 2208S)
8. Mouse monoclonal anti-phospho-H2AX (S139) antibody (Millipore Sigma, catalog number: 05-636)
9. Alexa Fluor 488, goat anti-rat IgG (BioLegend, catalog number: 405418)
10. Alexa Fluor 647, mouse anti-rat IgG (BioLegend, catalog number: 405322)
11. Click-iT™ EdU Alexa Fluor™ 647 Flow Cytometry Assay Kit (Life Technologies, catalog number: C10419)
12. 7-AAD (BD Biosciences, catalog number: 559925)
13. Mouse FITC-anti-BrdU antibody (BD Biosciences, catalog number: 556028)
14. BD Pharmigen™ BrdU Flow Kit (BD Biosciences, catalog number: 559619)
15. DMEM, high glucose, no glutamine (Thermo Fisher Scientific, catalog number: 11960077)
16. Fetal bovine serum/FBS (GeminiBio, catalog number: 100-106)
17. MEM non-essential amino acids (Thermo Fisher Scientific, catalog number: 11140050)
18. L-glutamine (Thermo Fisher Scientific, catalog number: 25030081)
19. Sodium pyruvate (Thermo Fisher Scientific, catalog number: 11360070)
20. Penicillin Streptomycin (Thermo Fisher Scientific, catalog number: 15140122)
21. 2-mercaptoethanol (MP Biomedicals, catalog number: 194705)
22. DMEM/F-12 (Thermo Fisher Scientific, catalog number: 11330032)
23. Horse serum (Thermo Fisher Scientific, catalog number: 16050122)
24. EGF (Pepprotech, catalog number: AF-100-15)
25. Hydrocortisone (Sigma, catalog number: H-0888)
26. Cholera toxin (Sigma, catalog number: C-8052)
27. Insulin (Sigma, catalog number: I-1882)
28. Trypsin (Gibco, catalog number: 15090046)
29. Imatinib (SelleckChem, catalog number: S2475)
30. FACS tubes/Corning Falcon round bottom polystyrene tubes (Thermo Fisher Scientific, catalog number: 14-959-5)
31. DMEM media (for abl pre-B cells) (see Recipes)
32. MCF10A media (see Recipes)
33. FACS Wash (see Recipes)

Equipment

1. Allegra X-14R Centrifuge (Beckman Coulter)
2. XRAD 320 irradiator (Precision X-ray Inc.)
3. BD LSRFortessa X-20 cell analyzer (BD Biosciences)

Software

1. FlowJo (BD Life Sciences)

Procedure

A. Synchronization of cells in G_0 phase

1. Abl pre-B cells
   a. Dilute imatinib solution in DMEM media to 3 μM.
   b. Resuspend abl pre-B cells in DMEM + 3 μM imatinib at 2×10^6 cells/mL.
   Notes:
   1) All cells are cultured at 37°C with 5% CO_2.
   2) All cells are spun in an Allegra X-14 R centrifuge (Beckman Coulter) at 1,200 rpm for 5 min in procedures spinning is required for collecting cells.
   c. Culture abl pre-B cells in DMEM + 3 μM imatinib for at least 48 h.
2. MCF10A cells
   a. Plate 2.5×10^5 MCF10A cells in 5 mL of MCF10A media per well of 6-well tissue culture plates.
   b. Culture cells for 1 day.
   c. Aspirate media from each well and wash cells with pre-warmed 1× PBS or EGF-free MCF10A media.
   d. Add 5 mL of EGF-free MCF10A media to each well and culture cells for 2 days.
3. Verification of G_0 synchronization efficiency using BD Pharmigen™ BrdU Flow Kit with modification (for both abl pre-B cells and MCF10A cells)
   a. Add BrdU to cells (1mL of imatinib-treated abl pre-B cells or 1 well of EGF-deprived MCF10A cells in a 6-well plate) to a final concentration of 10 μM.
   Note: Make sure to use proliferating cells as a positive control to ensure that the labeling and detection procedures are done appropriately.
   b. Incubate cells with BrdU for 30 min.
   c. Collect cells in FACS tubes.
      i. Abl pre-B cells:
         1) Resuspend cells briefly by pipetting and transfer cells directly to FACS tubes.
         2) Spin down cells, decant supernatant by inverting tubes quickly to pour out media and tapping the inverted tubes on a paper towel, and wash cells by resuspending them in 1mL of 1X FACS wash, followed by spinning down cells again.
         Note: Do not use vacuum to avoid loss of samples.
      ii. MCF10A cells:
         1) Remove media and wash cells with 1mL of 1× PBS.
         2) Add 300 μL of 0.25% Trypsin to a well and return cells to the incubator for 5–10 min.
         3) Inspect cells under the microscope to ensure that most cells have detached off the plate surface.
         4) Add 1 mL of MCF10A media to resuspend cells and transfer cells to FACS tubes.
         5) Spin down cells, decant supernatant, and wash cells by resuspending cells in 1 mL of 1× FACS wash, followed by spinning down cells again.
4. Fix cells in 150 μL of BD Cytofix/Cytoperm Buffer at room temperature for 20 min.
5. Wash cells with 1 mL of 1× BD Perm/Wash Buffer.
6. Permeabilize cells by one of the following methods
   a. Freeze-and-thaw method
      i. Freeze cells in 0.5 mL of 10% DMSO in FBS at -80°C.
      ii. Thaw cells at room temperature and wash freeze-thaw cells with 1 mL of 1× BD Perm/Wash Buffer.
   b. Permeabilization with detergent
      i. Resuspend cells in 150 μL of BD Permeabilization Plus Buffer and incubate on ice for 10 min.
      ii. Wash cells with 1 mL of 1× BD Perm/Wash Buffer.
7. Fix cells in 150 μL of BD Cytofix/Cytoperm Buffer at room temperature for 5 min.
8. Add 100 μL of 300 μg/mL DNase I (in 1× PBS) to each tube of cells and incubate at 37°C for 1 h.
9. Wash cells with 1 mL of 1× BD Perm/Wash Buffer.
10. Stain cells with 100 μL of 1:50 diluted FITC-anti-BrdU antibody (in 1× BD Perm/Wash Buffer) at room

temperature for 1 h.

11. Wash cells with 1 mL of 1× BD Perm/Wash Buffer.
12. Add 15 µL of 7-AAD to each tube of cells and incubate at room temperature for 3 min.
13. Add 300 µL of 1× PBS to each tube.
    Note: Do not wash off 7-AAD. Add 1× PBS directly to each tube and process flow cytometry analysis directly.
14. Record DNA content (7-AAD) and BrdU incorporation (FITC) on BD FACS Fortessa X-20 or an equivalent flow cytometer.
15. Analyze data using FlowJo (Figure 1).

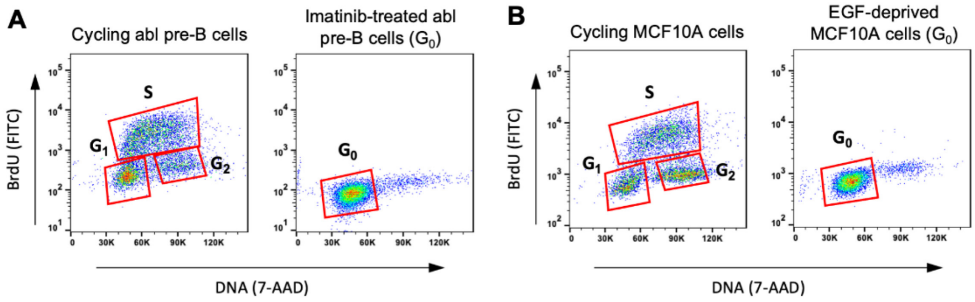

Figure 1. Flow cytometric analysis of cycling and G_0-arrested abl pre-B cells and MCF10A cells following BrdU labeling.
Dot plots depicting cell cycle profiles of cycling and cells arrested in G_0 phases by **(A)** imatinib treatment (abl pre-B cells) or **(B)** EGF withdrawal (MCF10A) cells after BrdU labeling and flow cytometry analysis for BrdU incorporation (FITC) and DNA content (7-AAD). S (BrdU-positive), G_1 (BrdU-negative, 2N DNA), and G_2 (BrdU-negative, 4N DNA) cells in cycling cultures and G_0 cells (BrdU-negative, 2N DNA) in imatinib-treated abl pre-B cell and EGF-deprived MCF10A cultures are shown in red polygon gates.

B. Monitoring DNA end resection by flow cytometry-based chromatin-bound RPA assay after irradiation in G_0-arrested and G_1 phase cells in proliferating cultures

1. Cell preparation
   a. G_0 abl pre-B cells: Treat abl pre-B cells with imatinib as indicated in Step A3. Aliquot 1 mL of imatinib-treated cells in wells of a 24-well plate for irradiation.
   b. G_0 MCF10A cells: Synchronize MCF10A in EGF-free MCF10A media in a 12-well plate as indicated in Step A3 for irradiation.
   c. Proliferating abl pre-B cells: Resuspend cells in pre-warmed DMEM media at 2×10^6 cells/mL and aliquot 1 mL of cells in each well of a 24-well plate for irradiation.
   d. Proliferating MCF10A cells: Plate 5×10^5 MCF10A cells 5 mL of MCF10A media in a well of a 6-well plate for 24 h before irradiation.
2. For analysis of G_1 phase abl pre-B or MCF10A cells from proliferating cultures, incubate cells with 10 µM EdU for 1 h before irradiation.
3. Irradiate cells in XRAD 320 irradiator.
   Note: The dosage of irradiation and the time of sample collection after IR were determined empirically, based on the distinct RPA staining intensities between 53BP1-proficient (normal DNA end protection, basal levels of resection) and 53BP1-deficient (impaired DNA end protection).
   a. Imatinib-treated (G_0) abl pre-B cells: 15Gy IR; cell collected 3–18 h after IR
   b. EGF-deprived (G_0) MCF10A cells: 30 Gy IR; cell collected 4 h after IR
   c. Proliferating abl pre-B cells: 5 Gy IR; cell collected 3 h after IR
   d. Proliferating MCF10A cells: 25 Gy IR; cell collected 6 h after IR
4. Collect cells in FACS tubes, spin down, and wash with 1 mL of FACS wash.
5. Pre-extract cells with 150 mL of cold Triton X-100 in 1× PBS on ice for 10 min

a. Imatinib-treated (G_0) abl pre-B cells: 0.05% Triton X-100
b. EGF-deprived (G_0) MCF10A cells: 0.5% Triton X-100
c. Proliferating abl pre-B cells: 0.2% Triton X-100
d. Proliferating MCF10A cells: 0.5% Triton X-100

6. Wash cells with 2 mL of FACS wash.
7. Fix cells in 150 µL of BD Cytofix/Cytoperm Buffer at room temperature for 20 min.
8. Wash cells with 1 mL of FACS wash.
9. Stain cells 100 µL of 1:500 diluted anti-RPA32 and 1:1,000 diluted anti-phospho-H2AX (S139) antibodies (in 1× BD Perm/Wash Buffer) at room temperature for 2 h.
10. Wash cells with 1 mL of 1× BD Perm/Wash Buffer.
11. Stain cells 100 µL of 1:500 diluted Alexa Fluro 488 goat anti-rat IgG and Alexa Fluro 647 goat anti-mouse IgG (in 1× BD Perm/Wash Buffer) at room temperature for 1 hour, protected from light.
12. Wash cells with 1 mL of 1× BD Perm/Wash Buffer.
13. For analysis of G_1-phase cells in EdU-pulsed proliferating cultures:
    Note: Click-iT Plus EdU imaging kit is used in place of BrdU labeling kit because DNase I treatment in the procedures of BrdU labeling kit results in poor RPA staining compared to untreated cells.
    a. Prepare Click-iT Plus reaction cocktail according to the manufacturer's instructions.
    Note: Use Click-iT Plus reaction cocktail within 15 min of preparation.
    b. Resuspend cells in each tube in 250 µL of Click-iT Plus reaction cocktail and incubate at room temperature for 30 min, protected from light.
    c. Wash cells with 2 mL of 1× BD Perm/Wash Buffer.
14. Add 15 µL of 7-AAD and incubate at room temperature for 3 min.
15. Add 300 µL of 1× PBS to each tube.
16. Record DNA content (7-AAD), RPA (Alexa Fluro 488), and phospho-H2AX (S139) or EdU (Alexa Fluro 647) on BD FACS Fortessa X-20 or equivalent flow cytometer.
    Note: The choice of fluorophores should be based on the availability of reagents and the flow cytometers. As we use Alexa Fluro 647 for visualizing phospho-H2AX (S139) and EdU in our RPA assay, we typically do not perform phospho-H2AX (S139) staining in the analysis of proliferating cells.
17. Analyze data using FlowJo (Figure 2).

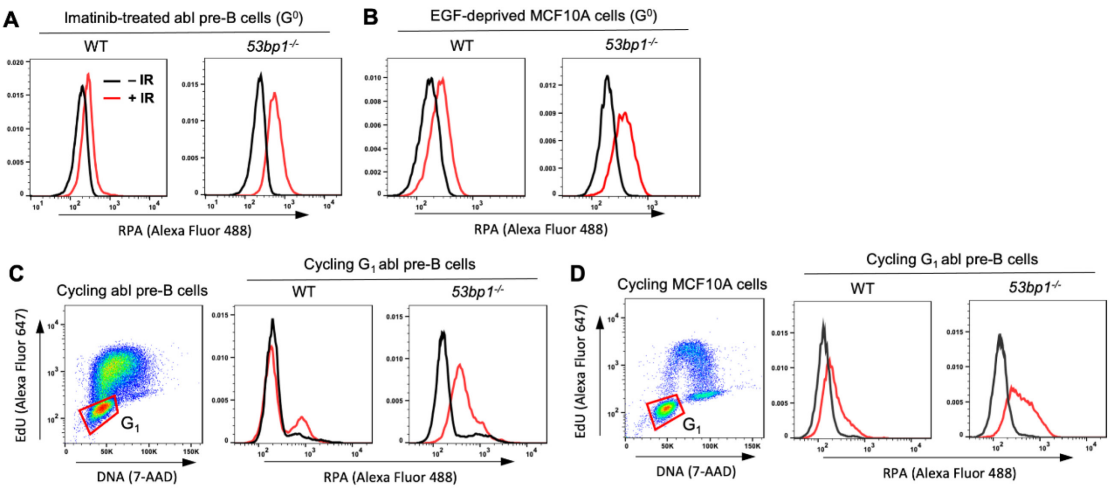

Figure 2. Flow cytometric analysis of chromatin-bound RPA levels after irradiation.
(A, B) Histograms showing chromatin-bound RPA levels (Alexa Fluor 488) in G_0-arrested WT or 53BP1-deficient abl pre-B **(A)** or MCF10A **(B)** cells after IR. **(C, D)** The dot plots on the left show EdU incorporation (Alexa Fluor 647) and DNA content (7-AAD) in cycling abl pre-B **(C)** and MCF10A **(D)** cells, with G_1 cells shown in the red polygon gates. The histograms on the right show chromatin-bound RPA levels (Alexa Fluor 488) in G_1-phase WT or 53BP1-deficient abl pre-B **(C)** or MCF10A **(D)** cells, with red polygon gated cells in the dot plots, after IR.

Data analysis

1. When working with imatinib-treated abl pre-B cells and EGF-deprived MCF10A cells, generate a histogram with 7-AAD as the X-axis to identify cells with 2N DNA contents (using cycling cells as the control to determine cells with 2N and 4N DNA content) to specifically analyze levels of chromatin-bound RPA in G_0 cells (with 2N DNA).
2. To identify G_1-phase cells in a proliferating population for analysis of chromatin-bound RPA, generate a dot plot with Alexa Fluro 647 (EdU) as the Y-axis and 7-AAD as the X-axis to identify EdU-negative cells with 2N DNA content.

Recipes

1. DMEM media (for abl pre-B cells)

Reagent	Final concentration	Amount
DMEM, high glucose, no glutamine	-	860 mL
Heat-inactivated FBS	10%	100 mL
L-glutamine (200 mM)	2 mM	10 mL
Sodium pyruvate (100 mM)	1 mM	10 mL
MEM non-essential amino acids (100×)	1×	10 mL
Penicillin/streptomycin (10,000 U/mL)	100 U/mL	10 mL
2-mercaptoethanol (14.3 M)	57 mM	4 mL
Total	n/a	1,000 mL

2. MCF10A media

Reagent	Final concentration	Amount
DMEM/F-12	-	470 mL
Horse serum	5%	25 mL
EGF (100 µg/mL)	20 ng/mL	100 µL
Hydrocortisone (1 mg/mL)	0.5 µg/mL	250 µL
Cholera toxin (1 mg/mL)	100 ng/mL	50 µL
Insulin (10 mg/mL)	10 µg/mL	500 µL
Penicillin/streptomycin (10,000 U/mL)	100 U/mL	5 mL
Total	-	500 mL

3. FACS Wash

Reagent	Final concentration	Amount
Heat-inactivated FBS	2%	10 mL
10× PBS	1×	50 mL
MilliQ H_2O	-	440 mL
Total	-	500 mL

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Competing interests

The authors declare no competing interests.

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