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TITLE:

Assessment of Global DNA Double-strand End Resection Using BrdU-DNA Labeling Coupled With Cell Cycle Discrimination Imaging

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KEYWORDS:

BrdU foci, Immunofluorescence, DNA end resection, homologous recombination, PARP-1, PARP inhibitors, PARP inhibitor resistance, BMN673

SUMMARY:

In the present protocol, we demonstrate how to visualize DNA double-strand end resection during S/G2 phase of the cell cycle using an immunofluorescence-based method.

ABSTRACT:

The study of the DNA damage response (DDR) is a complex and essential field, which has only become more important due to the use of DDR-targeting drugs for cancer treatment. These targets are poly(ADP-ribose) polymerases (PARPs), which initiate various forms of DNA repair. Inhibiting these enzymes using PARP inhibitors (PARPi) achieves synthetic lethality by conferring a therapeutic vulnerability in homologous recombination (HR)-deficient cells due to mutations in breast cancer type 1 (BRCA1), BRCA2, or partner and localizer of BRCA2 (PALB2).

Cells treated with PARPi accumulate DNA double-strand breaks (DSBs). These breaks are processed by the DNA end resection machinery, leading to the formation of single-stranded (ss) DNA and subsequent DNA repair. In a BRCA1-deficient context, reinvigorating DNA resection through mutations in DNA resection inhibitors, such as 53BP1 and DYNLL1, causes PARPi

resistance. Therefore, being able to monitor DNA resection *in cellulo* is critical for a clearer understanding of the DNA repair pathways and the development of new strategies to overcome PARPi resistance. Immunofluorescence (IF)-based techniques allow for monitoring of global DNA resection after DNA damage. This strategy requires long-pulse genomic DNA labeling with 5-bromo-2'-deoxyuridine (BrdU). Following DNA damage and DNA end resection, the resulting single-stranded DNA is specifically detected by an anti-BrdU antibody under native conditions. Moreover, DNA resection can also be studied using cell cycle markers to differentiate between various phases of the cell cycle. Cells in the S/G2 phase allow the study of end resection within HR, whereas G1 cells can be used to study non-homologous end joining (NHEJ). A detailed protocol for this IF method coupled to cell cycle discrimination is described in this paper.

INTRODUCTION:

Modulation of DNA repair factors is an ever-evolving method for cancer therapy, particularly in DNA DSB repair-deficient tumor environments. The inhibition of specific repair factors is one of the ingenious strategies used to sensitize cancer cells to DNA-damaging agents. Decades of research led to the identification of various mutations of DNA repair genes as biomarkers for therapeutic strategy choices[1]. Consequently, the DNA repair field has become a hub for drug development to ensure a wide range of treatments, empowering the personalized medicine concept.

DSBs are repaired by two main pathways: NHEJ and HR[2]. The NHEJ pathway is error-prone, rapidly ligating the two DNA ends with little to no DNA end-processing and involving the protein kinase (DNA-PKcs), the Ku70/80 complex, 53BP1, and RIF1 proteins[3]. In contrast, HR is a faithful mechanism initiated by BRCA1[4]. An essential step in HR repair is the DNA-end resection process, which is the degradation of the broken ends leading to single-stranded (ss) DNA with 3'-OH ends. BRCA1 facilitates the recruitment of the downstream proteins that form the resectosome MRN/RPA/BLM/DNA2/EXO1, which are involved in the 5' to 3' DNA resection[5].

The initial end-resection is accomplished through the endonuclease activity of MRE11, allowing for further processing by the DNA2 and EXO1 nucleases. The generated ssDNA overhangs are quickly coated by Replication Protein A (RPA) to protect them from further processing. Subsequently, BRCA2, PALB2, and BRCA1 engage to mediate the displacement of RPA and the assembly of the RAD51 nucleofilament required for homology-directed repair mechanism. A fine balance between the usage of NHEJ and HR is necessary for the optimal maintenance of genomic integrity. The pathway choice depends on the cell cycle phase. HR is preferentially used during the S to G2 phases wherein DNA resection is at the highest level, and the sister chromatids are available to ensure proper repair.

Poly (ADP-ribose) polymerase 1 (PARP-1) is one of the earliest proteins recruited to the DSB. It regulates both resection activity and the assembly of downstream effectors involved in the NHEJ[5,6]. PARP-1 is also required for DNA single-stranded break repair during replication[7,8]. Due to its important role in DNA repair, PARP inhibitors (PARPi) are used as cancer therapies. In several HR-deficient cancers, PARPi treatment leads to a synthetic lethal response due to the incapacity of HR-deficient cells to repair the accumulated damage via an alternative pathway[9,10].

There are currently four FDA approved PARPi: Olaparib, Rucaparib, Niriparib, and Talazoparib (also called BMN 673), which are used for various breast and ovarian cancer treatments[11]. However, PARPi resistance is common, and one potential cause arises through the reacquisition of HR proficiency[12]. Loss or inhibition of PARP-1 in the presence of irradiation dysregulates the resectosome machinery, leading to the accumulation of longer ssDNA tracts[13]. Therefore, an in-depth study of DNA resection *in vivo* is critical for a clearer understanding of the DNA repair pathways and the subsequent development of new strategies to treat cancer and to overcome PARPi resistance.

There have been several methods employed to detect DNA resection events[5]. One such method is the classical IF-based technique allowing for indirect staining and visualization of the resected DNA after stress-induced DSB by using an anti-RPA antibody. Labelling genomic DNA with 5-bromo-2'-deoxyuridine (BrdU) and detecting only ssDNA is a direct measurement of DNA resection events. It circumvents the monitoring of RPA, which is involved in multiple cellular processes such as DNA replication. In the method described here, cells incubated with BrdU for a single cell cycle allow BrdU to be incorporated into one strand of the replicating cellular DNA. Following resection, IF staining is performed under conditions allowing wherein BrdU detection only in the ssDNA form, with the use of an anti-BrdU antibody. This antibody can only access exposed BrdU nucleotides and will not detect those integrated into double-stranded DNA. Using fluorescence microscopy, the resected DNA can be visualized in the form of punctate BrdU/ssDNA foci. The nuclear intensity of these foci can be used as a readout to quantify resection following DNA damage. This paper describes step-by-step the processes of this method, which can be applied to most mammalian cell lines. This method should be of broad utility as a simple way of monitoring DNA end resection *in cellulo*, as a proof of concept.

PROTOCOL:

1. Cell culture, treatments, and coverslip preparation

NOTE: All cell plating, transfections, and treatments, aside from irradiation, should take place under a sterile cell-culture hood.

1.1. Day 1

1.1.1. In a 6-well plate, place a single coverslip in each well for as many conditions as needed. Plate ~150,000 Hela cells for transfection or drug treatment, as desired.

NOTE: If transfecting, it is recommended to do a reverse transfection at the time of plating, or it is possible to do a forward transfection several hours after plating to allow for adherence. A reverse transfection is accomplished by adding the transfection mix to the coverslip before the cells are added; thus, transfection begins before the cells start adhering. A forward transfection, by contrast, is the addition of the transfection mix post-adherence to the surface usually on the following day. However, it is possible to do this on the same day, provided enough time is given for the cells to adhere.

1.1.2. For this method, use 4 wells: Well 1: siRNA control (termed siCTRL); Well 2: siRNA against PARP-1 (siPARP-1); Well 3: untreated; Well 4: Cells to be treated with 5 µM BMN673 1 h prior to irradiation.

NOTE: In this protocol, all the tests were conducted under irradiated conditions (see section 1.3).

1.1.3. Incubate the cells at 37 °C in a 5% CO_2 humidified incubator overnight, although the transfection protocol allows for up to 3 days of incubation. The incubation time prior to BrdU treatment will depend on the siRNA transfection efficiency. If there is no transfection, incubation for 16 h is sufficient to allow for adherence to the coverslip and some cell growth.

NOTE: Incubator conditions can be changed according to the cell line used.

1.2. Day 2

1.2.1. Add BrdU at a final concentration of 10 µM in the appropriate culturing media, and incubate for 16 h (one cell cycle).

NOTE: The BrdU solution is prepared in dimethylsulfoxide; the stock solution used is 10 mM and is stored in aliquots at -20 °C.

1.3. Day 3

1.3.1. Irradiate the plates with a total dose of 5 Gy of X-ray irradiation (vary the dose of irradiation depending on the irradiator type, for example, small animal irradiators vs. benchtop irradiators). See the **Table of Materials** for the brand and model of irradiator used.

1.3.2. Return the plates to the incubator, and release the cells for 3 h.

1.3.3. During the 3 h incubation period, prepare the two buffers, A and B, and 4% paraformaldehyde (PFA) in 1x phosphate-buffered saline (PBS).

NOTE: Buffers A and B and the sucrose should be prepared fresh the day of fixation, using fresh sucrose solution to prevent contamination.

1.3.2.1. Prepare buffer A (Pre-Extraction Buffer), according to the order (30 mL) described in **Table 1**.

NOTE: The 1M sucrose should be prepared the day of use to prevent contamination.

1.3.2.2. Prepare buffer B (Cytoskeleton Stripping Buffer) according to the order described in **Table 1** (30 mL).

NOTE: Buffer B must be prepared in the cited order to prevent precipitation of Tween-20 and sodium deoxycholate solution.

1.3.2.3. Prepare 4% PFA, 2 mL per condition (10 mL), under a chemical hood (**Table 1**).

2. Pre-extraction and fixation

NOTE: All pre-extraction, fixation, and staining steps are performed with the coverslips remaining in the tissue culture plate on ice; the coverslip is only lifted in the last step of mounting (see discussion).

2.1. Pre-extraction

2.1.1. Aspirate the medium, carefully wash the cells twice with 1x PBS, and remove the PBS. Add 2 mL of Pre-extraction Buffer A and immediately incubate at 4 °C for 10 min.

NOTE: It is important that incubation time in buffer A is not extended. Extended incubation in the pre-extraction buffers will result in an increased number of detached cells from the coverslip. Alternatively, instead of incubation at 4°C, incubation can be done on ice.

2.1.2. Remove Buffer A through aspiration.

NOTE: Do not wash the coverslips after removing buffer A. Proceed to the next step.

2.1.3. Add 2 mL of Cytoskeleton Stripping Buffer B and immediately incubate at 4 °C for 10 min. Carefully aspirate Buffer B, and carefully wash the cells once with 1x PBS. Carefully aspirate the 1x PBS.

2.2. Fixation

2.2.1. Fix the cells by adding 2 mL of 4% PFA under the chemical hood.

NOTE: PFA is toxic and must be manipulated under a chemical hood.

2.2.2. Incubate the cells at room temperature for 20 min. Wash the coverslips twice with 1x PBS, and aspirate the excess.

2.2.3. Cover the coverslips with 100% cold methanol, and incubate the coverslips at -20 °C for 5 min. Wash twice with 1x PBS.

NOTE: The protocol can be paused here. The coverslips can be stored at 4 °C in 1x PBS and the plate wrapped in aluminum foil if necessary. The cells should not be kept more than 5 days before continuing the IF protocol.

3. Permeabilization

3.1. Incubate the cells with 2 mL of 1x PBS containing 0.5% Triton X-100 at room temperature for 15 min. Wash the coverslips three times with 2 mL of 1x PBS.

4. Immunostaining

4.1. Blocking step

4.1.1. Prepare enough fresh blocking buffer (3% BSA in 1x PBS) to use 2 mL per coverslip.

NOTE: Prepare an extra 5mL of blocking buffer to make the antibody solutions.

4.1.2. Add 2 mL of blocking buffer to each well and incubate at room temperature for 1 h.

4.2. Primary antibody incubation

4.2.1. Prepare the primary antibody solution in fresh blocking buffer: BrdU RPN202 1:1000 and proliferating cell nuclear antigen (PCNA) 1:500, 100 µL per coverslip.

NOTE: To preserve the antibody, a smaller volume can be used, 75–100 µL for a 22 x 22 mm coverslip, 50–75 µL for an 18 x 18 mm coverslip.

4.2.2. On each coverslip, add 100 µL of primary antibody solution. Cover the coverslips with a square of parafilm using tweezers, and carefully position the parafilm to not create bubbles.

4.2.3. Cover the plate in aluminum foil, and incubate the primary antibody overnight at 4 °C in a humidified chamber.

4.2.4. Remove the parafilm squares, and wash the coverslips three times with 2 mL of sterile 1x PBS.

4.3. Secondary antibody incubation

4.3.1. Prepare enough secondary antibody solution in fresh blocking buffer: anti-mouse 488 fluorescent secondary A11011 (for BrdU) dilution 1:800 and anti-rabbit 568 fluorescent secondary A11011 (for PCNA) dilution 1:800.

NOTE: The colors used can be changed to suit the experimental needs. The specific brand used can be found in the **Table of Materials**.

4.3.2. Add on each coverslip 100 µL of secondary antibody solution, cover the coverslips with a square of parafilm using tweezers, and carefully position the parafilm without bubbles.

4.3.3. Incubate the secondary antibody at room temperature for 1 h.

4.3.4. Wash the coverslips three times with 2 mL of 1x PBS.

4.4. Nuclear staining

4.4.1. Prepare a volume of 2 mL per coverslip of 1x PBS containing 4',6-diamidino-2-phenylindole (DAPI) at a final concentration of 1 µg/mL (1:1000).

4.4.2. Add on each coverslip 2 mL of the DAPI solution. Incubate the coverslips at room temperature for 10 min. Wash the coverslips twice with 1x PBS.

4.4.3. Cover the coverslips with 1x PBS, and use either a needle or fine tweezers to lift the coverslip from the bottom of the well. Carefully blot the off the excess liquid by tapping one edge gently on a paper towel.

NOTE: Be careful not to drop the slide or allow the flat surface with the adherent cells to touch the paper.

4.4.4. Mount the coverslips on slides using 10–20 µL of IF-specific mounting media.

5. Image acquisition and analysis

NOTE: Image acquisition can be done on several types of fluorescent microscopes. An epifluorescence microscope with a 63x oil objective was used; see the **Table of Materials** for the brand and model. Z-stacks are not required, although they may be of use depending on the cell line and level of mitochondrial staining.

5.1. Image analysis

5.1.1. For each image, create multiple tiff files; merge all planes, but keep the color channels separate. Import these files into Cell Profiler (The Broad Institute https://cellprofiler.org/home) and analyze using the Speckle Counting pipeline (**Supplemental Video 1**).

5.1.2. Upload the images (**Supplemental Figure S1A**).

5.1.2.1. To begin creating the project, load the speckle counting pipeline into the program and the images to be analyzed in the provided area.

5.1.2.2. Use the **NamesAndTypes** module to assign a meaningful name to each image by which other modules will refer to it—C01: Representing BrdU Channel; C02: Representing PCNA Channel; C03: Representing DAPI Channel.

NOTE: These identifiers will depend on the microscope; there should be an identifier in the file

name to distinguish between the channels.

5.1.3. Identify which file will be used to identify the nuclei and name it (change this name as required) (see **Supplemental Figure S1B** and **Supplemental Figure S1C**).

5.1.3.1. Select the diameter of the object between 50 and 300 pixel units, which is the acceptable size range for nuclei, but change the value to fit the nuclei in the image.

NOTE: It may be that 50 is too large a value and prevents smaller nuclei from being identified; likewise, 300 may allow for large groupings of nuclei to be counted as 1.

5.1.3.2. Discard objects outside of the diameter range to ensure only those that match the criteria will be counted.

5.1.3.3. Discard objects touching borders to remove cells that may only be partially in the field.

5.1.3.4. Apply the threshold to fit the specific images. Note the following settings as an example. Change the thresholding correction factor based on how stringent the thresholding strategy will be. Other settings include threshold strategy: Global; thresholding method: Otsu; two class or three class thresholding: Two class; threshold smoothing scale: 1; threshold smoothing factor: 1; lower and upper bounds on threshold: 0.0 and 1.0; method to distinguish clumped objects: Shape; and method to draw dividing lines between clumped objects: Propagate.

NOTE: For each parameter, cell profiler provides complementary definitions and available possibility for the variable setting.

5.1.4. Identify the primary object to identify the foci (**Supplemental Figure S2A**).

5.1.4.1. Use the following settings: select the input image: Maskedgreen; named the primary object to be identified: BrdUFoci; typical diameter of the object: 1-15; discard the object outside the diameter range: Yes; discard the object touching the border of the image : No; threshold strategy: Adaptative; thresholding method: Otsu; two class or three class thresholding: Three class; threshold smoothing scale: 1; threshold smoothing factor: 3; and size of smoothing filter: 4.

5.1.5. Measure intensity (**Supplemental Figure S2B**).

5.1.5.1. Select the image to be measured: OrigGreen.

5.1.5.2. Click on **Add another image**. Select the image to be measured: PCNA. Select objects to measure: Nuclei.

NOTE: This will be used to measure the BrdU and PCNA intensity—the final data that will be graphed.

REPRESENTATIVE RESULTS:

In this protocol, the bromodeoxyuridine (BrdU)-based assay was used to quantitatively measure the resection response of HeLa cells to irradiation-induced damage. The generated ssDNA tracks are visualized as distinct foci after immunofluorescence staining (**Figure 1A**). The identified foci were then quantified and expressed as the total integrated intensity of the BrdU staining in the nuclei (**Figure 1B**, **Supplemental Figure S1**, **Supplemental Figure S2**, and **Supplemental Figure S3**). It is possible to measure the foci number or mean foci intensity, although this can be less reliable than the total nuclear intensity, largely in part to the variable size of the BrdU foci.

To differentiate between the short-range resection as a result of NHEJ and the long-range resection of HR, co-staining was performed using an anti-PCNA antibody to identify cells going through S-phase (**Figure 1** and **Supplemental Figure S4**). PCNA constitutes the DNA clamp that acts as a processivity factor for DNA polymerase and is essential for replication. PCNA is prominent in the nucleus and reaches maximal expression during the S-phase of the cell cycle. Hence, in early S-phase, the PCNA signal is low and has a granular distribution. In contrast, in late S-phase, the PCNA staining is quite strong (**Figure 1A**). When first analyzing the PCNA (S-phase) signal, the nuclei must be identified and the PCNA intensity measured.

The resulting values are then plotted as a scatter plot to best determine the cut-off intensity to discriminate between the PCNA-positive and PCNA-negative nuclei (**Supplemental Figure S4A**). The PCNA-negative results will then be removed from the data set to allow for the BrdU intensity-based analysis because of the low BrdU signal regardless of the condition (**Supplemental Figure S4B**). In these experimental conditions, the PCNA-negative nuclei harbor a basal integrated intensity of BrdU foci of 900 arbitrary units (A.U.). However, this value reaches 1800 A.U. in PCNA-positive nuclei (**Figure 1B**). This represents a 100% increase in the amount of the integrated signal of the BrdU foci. Increase in BrdU intensity can be then correlated to an increase in DNA resection, which is more pronounced and efficient when cells go through S and G2 phases and are irradiated.

In this protocol, to demonstrate an increase in DNA resection after irradiation (5 Gy), PARP-1 activity was modulated either through the loss of PARP-1 using an siRNA knockdown condition or after potent inhibition of PARP-1 using Talazoparib (BMN673) (**Figure 2** and **Supplemental Figure S5**). The amounts of resected DNA in each condition were then quantified after IF (**Figure 2B**). In unperturbed condition (siCTRL), the integrated intensity of BrdU foci per nucleus is approximately 1500 A.U. in the PCNA-positive nuclei (**Figure 2A,B**). After an efficient knockdown of PARP-1 using an siRNA (**Figure 2C**), a significant increase in the intensity of the BrdU foci to approximately 1900 A.U. was observed, which corresponds to 26% increase in intensity ($p < 0.0001$). Similarly, after inhibition of PARP-1 using BMN673 (5 μM final concentration), the intensity of foci increased from approximately 1750 to 2500 A.U. corresponding to 43% increase of the foci intensity ($p < 0.0001$). Thus, loss of PARP-1 dysregulates DNA resection as reported previously[13].

It is important to remember that BMN-673 both inhibits the activity of PARP-1 and traps it in DNA, and the effect of this treatment is much more detrimental to the cell, resulting in the greater increase of BrdU intensity than in siRNA-treated cells. The slight difference in untreated and siCTRL intensities demonstrates how any treatment, such as siRNA transfection, may affect the response and output of an assay. It can further demonstrate the importance of using the proper controls for each condition.

FIGURE AND TABLE LEGENDS:

Figure 1: BrdU foci formation is more prone to occur in PCNA-positive cells than in replicating cells. (**A**) Representative images of BrdU foci formation and PCNA staining following 5 Gy irradiation and 3 h release. A zoomed-in square showing marked BrdU foci is present in the corner of each BrdU image. Scale bars = 5 µm. (**B**) Quantification of the BrdU nuclear intensity in untreated (without BMN-673) HeLa cells. The data show the mean ± s.e.m (Mann–Whitney *U*-test). Abbreviations: BrdU = 5-bromo-2′-deoxyuridine; PCNA = proliferating cell nuclear antigen; IR = irradiation; DAPI = 4',6-diamidino-2-phenylindole; A.U. = arbitrary units; s.e.m. = standard error of the mean.

Figure 2: Poly(ADP-ribose) polymerase-1 knockdown or inhibition results in increased BrdU foci formation in replicating cells. (**A**) Representative images of BrdU foci formation and PCNA staining in untreated HeLa cells, treated with 5 µM BMN-673, siCTRL, and siPARP-1, followed by 5 Gy irradiation and 3 h release. A zoomed-in square showing marked BrdU foci is present in the corner of each BrdU image. Scale bars = 5 µm. (**B**) Quantification of the BrdU nuclear intensity in untreated HeLa cells, treated with 5 µM BMN-673, siCTRL, and siPARP-1 followed by 5 Gy irradiation, the data show the mean ± s.e.m (Mann–Whitney *U*-test). (**C**) Western blot to validate the siRNA knockdown of *PARP-1*. The F1-23 PARP-1 antibody recognizes automodified PARP-1 resulting in the smeared appearance of the siCTRL band and the solid appearance of the catalytically inhibited PARP-1 in BMN-673-treated samples. Abbreviations: PARP-1 = poly(ADP-ribose) polymerase 1; BrdU = 5-bromo-2′-deoxyuridine; PCNA = proliferating cell nuclear antigen; IR = irradiation; DAPI = 4',6-diamidino-2-phenylindole; A.U. = arbitrary units; siCTRL = siRNA control; siPARP-1 = siRNA to knock down *PARP-1*; s.e.m. = standard error of the mean.

Supplemental Figure S1: Visual representation of the Cell Profiler Software and Speckle counting pipeline part 1. (**A**) Screenshot shows the window used to identify the different channel files of the images to be analyzed. These shots show the cell profiler interface. (**B**) Screenshot of the Identify Primary Objects menu to identify the Nuclei. The highlighted values are the commonly altered values to best identify nuclei. Abbreviations: BrdU = 5-bromo-2′-deoxyuridine; DAPI = 4',6-diamidino-2-phenylindole.

Supplemental Figure S2: Visual representation of the Cell Profiler Software and Speckle counting pipeline part 2. (**A**) Screenshot of the Identify Primary Objects menu to identify the BrdU foci within the nuclei. (**B**) Screenshot of Measure Object Intensity menu to measure the BrdU and PCNA nuclear intensity. Abbreviations: BrdU = 5-bromo-2′-deoxyuridine; PCNA =

proliferating cell nuclear antigen.

Supplemental Figure S3: Visual representation of the Cell Profiler Software and Speckle counting pipeline part 3. (**A**) Representation of the nuclei identification by cell profiler following optimization. (**B**) Representation of the BrdU foci identification. (**C**) Screenshot is a zoom-in on one cell within the initial window.

Supplemental Figure S4: Scatter plot of PCNA-positive and PCNA-negative cells. (**A**) An example of a PCNA scatter plot used to distinguish PCNA-positive from PCNA-negative cells. The PCNA intensity from a single condition was plotted and the distinction between the two populations identified. The green highlights the PCNA-positive cells, the red represents the PCNA-negative cells. This is done for each individual experiment and condition. (**B**) Quantification of the BrdU nuclear intensity in PCNA-negative untreated HeLa cells, treated with 5 µM BMN-673, siCTRL, and siPARP-1; the data show the mean ± s.e.m (Mann–Whitney *U*-test). Abbreviations: PARP-1 = poly(ADP-ribose) polymerase 1; BrdU = 5-bromo-2'-deoxyuridine; IR = irradiation; PCNA = proliferating cell nuclear antigen; A.U. = arbitrary units; siCTRL = siRNA control; siPARP-1 = siRNA to knock down *PARP-1*; s.e.m. = standard error of the mean.

Supplemental Figure S5: BrdU signal without irradiation. (**A**) Representative images of BrdU foci formation and PCNA staining without irradiation. Scale bars = 5 µm. A zoomed-in square showing marked BrdU foci is present in the corner of each BrdU image. (**B**) Quantification of the BrdU nuclear intensity in PCNA-positive untreated Hela cells, treated with 5 µM BMN-673, siCTRL, and siPARP-1; the data show the mean ± s.e.m (Mann–Whitney *U*-test). (**C**) Quantification of the BrdU nuclear intensity in PCNA-negative untreated HeLa cells, treated with 5 µM BMN-673, siCTRL, and siPARP-1; the data show the mean ± s.e.m (Mann–Whitney *U*-test). Abbreviations: PARP-1 = poly(ADP-ribose) polymerase 1; BrdU = 5-bromo-2'-deoxyuridine; PCNA = proliferating cell nuclear antigen; IR = irradiation; DAPI = 4',6-diamidino-2-phenylindole; A.U. = arbitrary units; siCTRL = siRNA control; siPARP-1 = siRNA to knock down *PARP-1*; s.e.m. = standard error of the mean.

Supplemental Video 1: Use of Cell Profiler and the Speckle Counting pipeline to analyze BrdU staining for the measurement of DNA resection. This video demonstrates the use of Cell Profiler and the Speckle counting pipeline. It shows how to import and analyze images with this tool specifically for this protocol. Abbreviation: BrdU = 5-bromo-2'-deoxyuridine.

DISCUSSION:

This paper describes a method that makes use of IF staining to measure variations in DNA resection *in cellulo*. The current standard for observing an effect on DNA resection is through RPA staining; however, this is an indirect method that may be influenced by DNA replication. Previously, another BrdU incorporation-based DNA resection IF technique has been described for classifying the resulting intensities in BrdU-positive and BrdU-negative cells. This method allowed for cells that are not undergoing HR to be counted as positive due to background or mitochondrial staining resulting in a high BrdU intensity[14-16]. The primary novelty of the method described in this paper is the addition of PCNA staining, which allows for selectivity for S and G2 phases of the cell cycle, ensuring that the resulting BrdU signal is due to resection and thus, to homology-

directed repair.

The critical step in this protocol is the pre-extraction; without this, the BrdU foci will not be visible, and if done incorrectly, the cells will detach completely. Incorrectly prepared buffers will result in incomplete pre-extraction, such as partial cytoskeletal removal or increased background signal. It is very important to respect the duration of incubation with the pre-extraction buffers, increased duration in the buffers can result in the cells detaching from the coverslip. Importantly, if this protocol is performed with poorly adherent cells, pre-treat the coverslips with polylysine to reduce cell detachment during pre-extraction. Subsequently, without methanol fixation, the PCNA signal will not be visible.

Another important variable in this protocol is the blocking buffer: incompatible blocking buffers will result in loss of BrdU signal. For example, 10% fetal bovine serum in 1x PBS blocking buffer will function when not combined with methanol fixation; however, when combined with methanol fixation as required for the PCNA staining, the BrdU signal is considerably reduced. It is possible other blocking agents and methods will work with this protocol; 3% BSA in 1x PBS provided the best results. A final essential component to this technique is the use of a DNA damage source; without this, there will be little to no nuclear BrdU signal (**Supplemental Figure S5**). Experience has shown that irradiation is an excellent source of damage for this method. However, if there is no access to an irradiator, radiomimetic drugs such as neocarzinostatin can be used instead.

Cell profiler is a useful and versatile tool that allows easy and consistent analysis of the images produced in this technique. The settings can be customized for both the size of the objects identified and the threshold at which it does so (**Supplemental Video 1**). As the nuclear intensity of the BrdU staining is the desired readout, the key customization is in the identification of the nuclei from the DAPI files. The analysis of the images from each condition must be done separately as the results are obtained in the form of a single csv file containing cell and image numbers, but not condition names. To determine if the analysis conditions are appropriate, use the **Start test mode** feature, and make sure the eye icon is open in the desired steps. Close this icon when running the actual analysis as it will result in pop-up windows and slow down the program. In the **Start test mode**, users can go back and forth, altering the settings as needed to properly identify first the nuclei and then the BrdU foci.

Once satisfied with the resulting identification, use the same settings for each condition within the same experiment; this may require the acquisition of a test image for each condition prior to analyzing the whole batch to ensure that the settings comply with each condition. It is important to note that, depending on image acquisition, there may be variability in the Cell profiler settings between users and between software versions. Hence, it is essential to go through the testing stage to determine the ideal parameters for specific experiments. When preparing to graph the results, the PCNA (S phase)-positive cells must be identified. To do this, the PCNA intensity is measured, in the same fashion as the BrdU intensity, and the resulting values plotted as a scatter plot to best visualize the cut-off intensity value for PCNA-positive cells. The PCNA-negative results will then be removed from the data set to allow for the BrdU intensity graphing. The provided

supplementary figures show screenshots from the program highlighting the most optimized settings (**Supplemental Figure S1**, **Supplemental Figure S2**, and **Supplemental Figure S3**).

A possible modification to the protocol is the PCNA antibody used; two different PCNA antibodies were successfully tested, the one listed here and another which is no longer in production—a rat monoclonal antibody (Bulldog Bio PCA16D10). Finally, users should cover the coverslips with parafilm during antibody incubation. It is not strictly necessary, although a significantly larger volume of antibody solution would be required to cover the coverslip entirely without this. Another method is to place a sheet of parafilm surrounding a glass plate, place drops of the antibody solution onto the parafilm, and carefully place the coverslip cell-side down onto the drop of antibody solution. This glass plate can then be placed in a humid chamber at 4 °C overnight for primary incubation and in the dark at room temperature for secondary incubation. This method is completely functional although it does significantly increase the time of handling the coverslips and as a result, increases the probability of dropping or breaking the coverslip.

An alternative method to measure resection is through the use of the DNA-combing SMART method; while this technique provides very clear results, it is much more complicated, requires more time, and is more expensive[17]. The SMART method requires the extraction of the DNA without damaging it, followed by stretching this DNA onto coverslips to be followed by an IF. The BrdU IF method, by comparison, is both simple and cost-effective, not requiring a greater investment than the cost of the antibody. This method provides an initial way to measure resection that is more accurate than simply measuring RPA foci formation, which can be related to its effects on many cellular functions. However, RPA is phosphorylated on specific residues during resection as part of the DNA damage response.

Single-molecule imaging recently revealed phosphorylated RPA (pRPA) as a negative resection regulator[18]. Hence, in the long term, this method could be made even more precise by coupling PCNA/phosphorylated RPA staining with BrdU imaging with the use of appropriate antibodies and optimized staining conditions. However, the method presented provides a representation of resection that is observed independently from the proteins involved in the resection process, thus negating bias that can occur from changes in their signal in response to the treatment. This technique provides not only a method to determine if a protein is involved in resection, but also to determine if cell death resulting from drug treatment is related to hypo/hyper-resection. This information can be beneficial in understanding not only the mechanistic aspects of DNA repair, but also the biological mechanism underlying the drug-induced cell death.

ACKNOWLEDGMENTS:

We thank Marie-Christine Caron for outstanding technical advice. This work is supported by funding from Canadian Institutes of Health Research J.Y.M (CIHR FDN-388879). J.-Y.M. holds a Tier 1 Canada Research Chair in DNA Repair and Cancer Therapeutics. J.O'S is an FRQS PhD student fellow, and S.Y.M is a FRQS postdoctoral fellow.

DISCLOSURES:

The authors have nothing to disclose.

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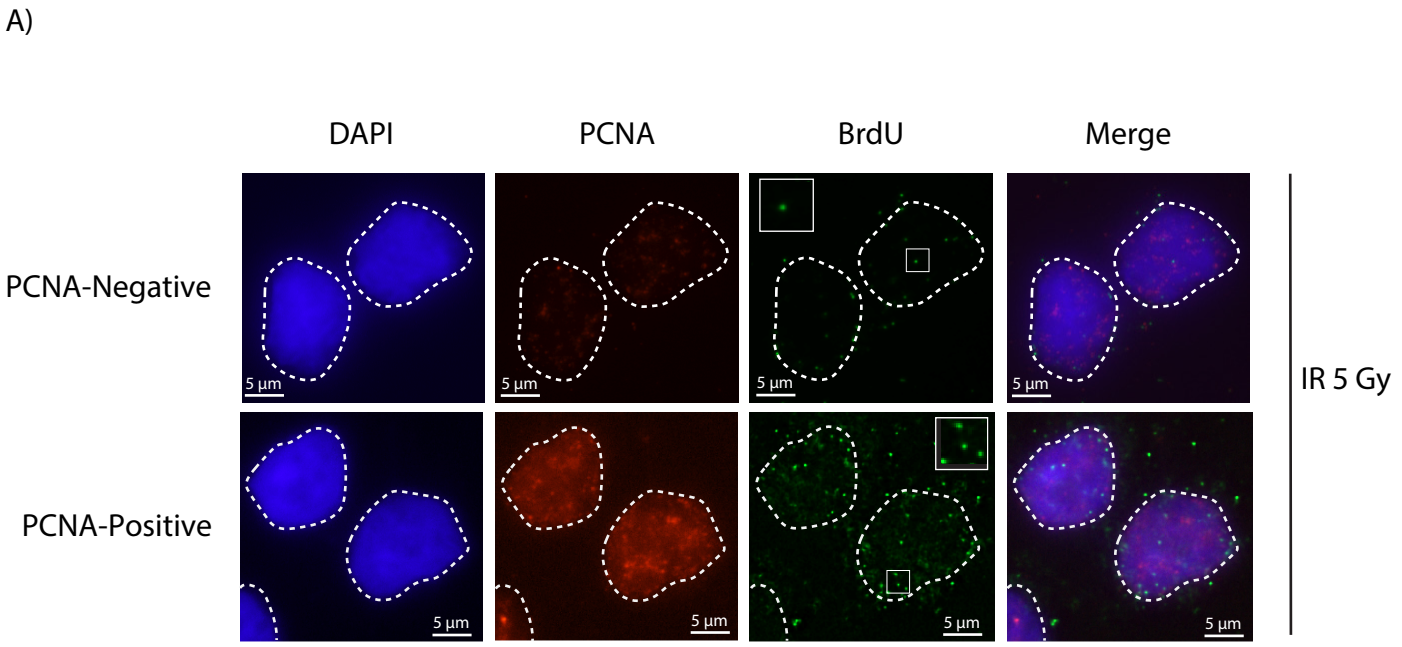
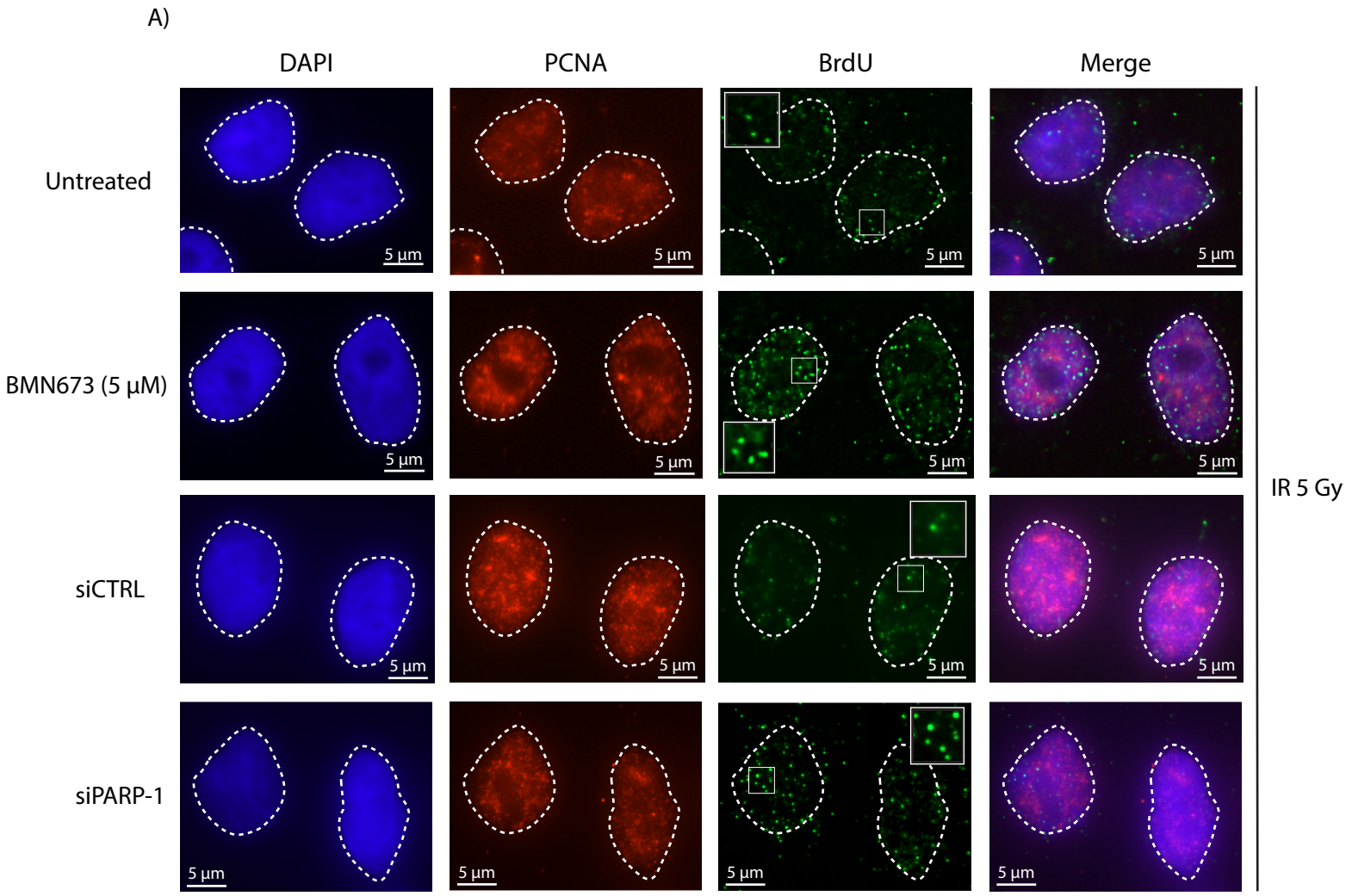
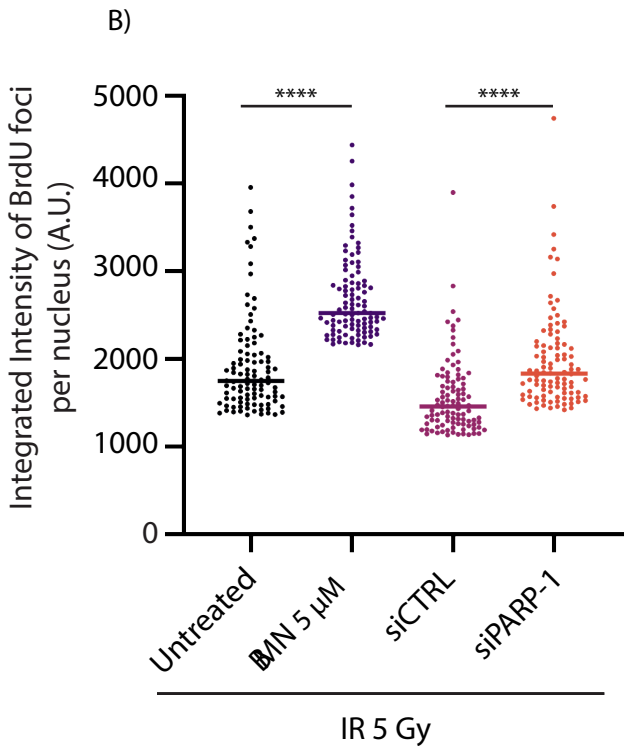


O'Sullivan, Mersaoui et al. Fig 2





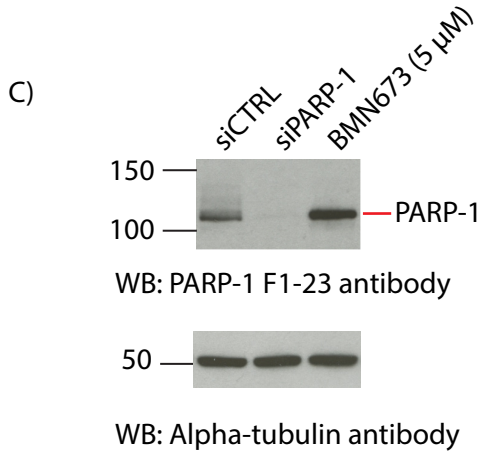


Table 1

Buffer A (Pre-Extraction Buffer) 30 mL		
Reagent	**Volume**	**Final Concentration**
H_2O	18 mL	
PIPES (pH 7, 500 mM)	600 µL	10 mM
NaCl (4 M)	750 µL	100 mM
$MgCl_2$ (1 M)	90 µL	3 mM
EGTA (500 mM)	60 µL	1 mM
Trition X-100 (10%)	1.5 mL	0.50%
Sucrose (1 M)	9 mL	300 mM

Buffer B (Cytosk
Reagent
H_2O
Tris pH 7.5 (1 M)
NaCl (4 M)
$MgCl_2$ (1 M)
Tween20 (10%)
10% Sodium Deoxycholcate

Reagent	**Volume**
16% PFA	2.5 mL
PBS 1x	7.5 mL

eleton Stripping Buffer) 30 mL	
Volume	**Final Concentration**
25.035 mL	
300 µL	10 mM
75 µL	10 mM
90 µL	3 mM
3 mL	1%
1.5 mL	0.50%

Table of Materials

Click here to access/download;Table of Materials;JoVe Source data.xlsx

Name of Material/ Equipment	Company	Catalog Number
Alexa 568 goat anti-rabbit	Molecular probes	A11011
Alexa Fluor 488 goat anti-mouse	Molecular probes	A11001
Anti PARP1 (F1-23)	Homemade	
Anti PCNA (SY12-07)	Novus	NBP2-67390
Anti-Alpha tubulin (DM1A)	Abcam	Ab7291
anti-BrdU	GE Healthcare	RPN202
Benchtop X-ray Irradiator	Cell Rad	
BMN673	MedChem Express	HY-16106
Bromodeoxyuridine (BrdU)	Sigma	B5002
BSA	Sigma	A7906
Cell profiler	Broad Institute	V 3.19
Curwood Parafilm M Laboratory Wrapping Film 4in / 250 ft	Fisher scientific	13-374-12
DAPI (4',6-Diamidino-2-Phenylindole, Dihydrochloride)	Invitrogen Life Technology	D1306
DMEM high glucose	Fisher scientific	10063542
EGTA	Sigma-Aldrich	E3889
Fetal Bovine serum	Gibco	12483-020
Fisherbrand Cover Glasses: Squares 22 x 22	Fisher scientific	12 541B
Fluorescent microscope	Leica	DMI6000B
HeLa	ATCC	CCL-2
HERACELL 160I CO_2 INCUBATOR CU 1-21 TC 120V	VWR	51030408
$MgCl_2$	BioShop Canada	MAG520.500
NaCl	BioShop Canada	SOD002.10
Needle		
PBS 1x	Wisent Bio Products	311-010-CS
PFA 16%	Cedarlane Labs	15710-S(EM)
PIPES	Sigma-Aldrich	P6757-100G
ProLong Gold Antifade Mountant	Invitrogen Life Technology	P-36930
RNAiMAX	Invitrogen	13778-075
siPARPi	Dharmacon	
siRNA control	Dharmacon	
Sodium Deoxycholcate	Sigma-Aldrich	D6750-100G
Sucrose	BioShop Canada	SUC507.5
Tris-base	BioShop Canada	TRS001.5
Trition X-100	Millipore Sigma	T8787-250ML
Tween20	Fisher scientific	BP337500
Tweezers		

Comments/Description
1:800
1:800
1:2500
1:500
1:100000
1:1000

https://cellprofiler.org/

63x immersion objective

37% CO_2

AAG AUA GAG CGU GAA GGC GAA dTdT
UUCGAACGUGUCACGUCAA

Dear Editor,

We would like to thank all the three reviewers for their comments and time. We believe that these comments will significantly improve the quality of our manuscript. Therefore, we join in this rebuttal a point-by-point answer for each reviewer.

Reviewer #1:

Manuscript Summary:

O'Sullivan et al. presented a technique suitable to monitor DNA end resection by immunofluorescence assay coupled to cell cycle discrimination. The method described is of great interest to the scientific community, for those studying DNA damage repair in particular. While utilizing BrdU foci as a tool to monitor DNA end resection is not new, the cell cycle discrimination makes the work presented unique. I recommend the manuscript for publication with minor revision to address the following concerns.

Reply: We would like to thank the reviewer for her/his comments. This will improve the quality of our manuscript.

Major Concerns:

1. For Figure 1, since all cells are irradiated, and within the figure, no other conditions are compared, "untreated" is not needed. PCNA positive and PCNA negative is enough.

Reply: The word ‘’Untreated’’ has been removed from the figure for more clarity.

However, since all cells in Figures 1 and 2 are irradiated, non-irradiated control cells have to be presented.

Reply: We have added another supplemental figure with representative images for the untreated condition without irradiation, in both PCNA positive and negative cells (figure S5-A). We have also added graphs depicting the analysis results of all the conditions in PCNA positive and negative cells without irradiation (figure S5- B-C). As you can see there is very little signal without damage induction. It reads as follows in the text : “A final essential component to this technique is the use of a DNA damage source, without this, there will be little to no nuclear BrdU signal (Figure S5).”

2. In Figure 2A, despite low PCNA expression level, cells in siCTRL treated with 5Gy show similar BrdU foci as PCNA positive cells in the untreated group, which are technically identical, unless siCTRL induces BrdU foci in cells with low PCNA expression. This contradicts the result presented in Figure 1, which shows cells with low PCNA exhibiting significantly reduced BrdU foci and raises a concern about the assay's specificity.

Reply: We agree that the selected image for the final figure should be representative of the overall quantification signal. Therefore, we have made the requested modifications with new images that represent the final results. We present 2 PCNA positive cells for each condition (please see updated figure 2A).

Furthermore, PCNA negative or positive cells should be selected and compared under different conditions. As currently presented, it is unclear whether the comparison is between PCNA negative and positive or other conditions such as 5Gy IR treatment. Thus, the authors may present separate figures for PCNA positive and PCNA negative cells to compare untreated and 5Gy IR with (+BMN673, +siCTL, and +siPARP-1).

Reply: Thank you for these comments.

In all the represented data only the PCNA positive cells were we analysed and graphed, we have added a graph for the PCNA negative results of these conditions in the supplemental figure 4.b. but as can be seen in there is little difference amongst the conditions (untreated versus BMN-treated and siCTRL versus siPARP-1) in the PCNA negative cells.

As mentioned previously we do not observe a significant signal for nuclear BrdU staining without irradiation in comparison with the condition with irradiation where the staining is significantly increased.

3. Previously, it has been shown that BrdU can induce DNA damage and prolong the persistence of double-strand break (Nusser et al. Strahlenther Onkol 2002, and Masterson and O'Dea, Anti-Cancer Drugs 2007). What is the impact of BrdU induced DNA damage and delayed repair on the experiments that involve monitoring repair efficiency?

Reply: Incorporation of BrdU in the genomic DNA can make the DNA more fragile and induce spontaneous DNA damage. However, in this context the effect of the incorporation of the BrdU is consider similar and equal in all the conditions. Furthermore, the non-irradiated condition the background level of damage induced through BrdU incorporation is relatively similar in all conditions and results in very low levels of intensity.

Could a PCNA expression coupled with RPA foci, particularly DNA damage specific phosphorylated-RPA32, more appropriate for such experiments?

Reply: This is a possible alternative as the induction of phosphorylated-RPA32 has been linked to DNA resection. We added this suggestion in the conclusion and it reads as follows : “RPA is phosphorylated on specific residues during resection as part of the DNA damage response. Using single-molecule imaging it was recently shown that phosphorylated RPA (pRPA) as a negative resection regulator18. Hence, in long term, this method could be even more precise by coupling PCNA/ phosphorylated RPA staining with BrdU imaging with the use of appropriate antibodies and optimized staining conditions. However, the method presented provides a representation of resection that is observed independently from the proteins involved in the resection process, thus negating bias which can occur from changes in their signal in response to the treatment.”

4. During replication or DNA end resection, the role of RPA is to protect the exposed single-stranded DNA. Similarly, following BrdU incorporation, the single-stranded DNA resulted from replication or DNA end resection can be detected by anti-BrdU under native conditions. The authors claim that RPA foci are less suitable because of their replication and DNA end resection role. However, for the reason provided above, it is not clear how BrdU-based assay could overcome the overlap.

It is correct that BrdU incorporated single-stranded DNA would also be exposed during replication, however as the BrdU is only incorporated for a single cell cycle only half the DNA should have BrdU and as demonstrated in our non-irradiated (undamaged) graphs the level of BrdU signal is incredibly low, as such the probability of the results being skewed by replication is low. This is compared to RPA which can bind all readily available ssDNA thus there is a higher likelihood for an increased signal in undamaged conditions which may have a greater effect on the results. Furthermore, BrdU-incorporation removes any possible bias due to RPA modulation which results as a secondary effect from the treatment being tested.

Minor Concerns:
1. The method described does not represent in vivo monitoring. Thus, it is more appropriate if the word "in vivo" (line 52) replaced by "in vitro" or "in cellulo" as used in line 508.

Reply: Changed, thank you.

2. In figure 1A, to demonstrate the consistent correlation between PCNA expression and BrdU foci, it could be helpful if more representative nuclei are presented for -/+PCNA groups.

Reply: We have changed the images used for the PCNA negative condition showing a clearer difference in the PCNA signal between the two conditions.

3. Consistently incorporating figure information for the data being described could simplify following the results. For example, line 437-38, "In our experimental condition, the PCNA negative nuclei harbour a basal integrated intensity of BrdU foci of 900…" Figure 1B?

Reply: Thank you, for this comment we have modified the text accordingly to better incorporate the figures.

4. While the foci size variability is a problem, the integrated intensity could also vary depending on resection size and the repair's extent. Thus, both could have similar limitations. To this end, it is helpful if a time-dependent BrdU foci resolving is presented.

Reply: It is true that the intensity could also be affected by the resection size, however in using the software to analyse the images we introduce less bias in choosing to measure intensity over foci number in this case. A time-course could be useful though it would be more in determining if the resection is resolved in the same time-frame rather than determining if there is more or less resection occurring.

Reviewer #2:
Manuscript Summary:
In this manuscript, the authors carefully describe a method to study DNA end resection by combining the detection of ssDNA by IF with an anti-BrdU staining after a long pulse with BrdU and cell cycle profiling using an antibody against PCNA. Both IFs methodologies have been extensively used in the past, either for DNA end resection (BrdU) or cell cycle analysis, mainly S phase detection (PCNA). The added value of this protocol is, indeed, the combination of both that provides a reliable way to study resection, as eliminates G1 cells that will show some BrdU background. Additionally, the authors describe in detail an automatic method to analyze both

stainings using a software, what also increase the potential of the protocol. All combined, the authors present in a very clear way a reliable method to study DNA end resection that is easy to implement but, at the same time, will have some added value over a simply IF of BrdU or RPA. The method is well presented, easy to follow, and the amin caveats and pitfalls are clearly described. Overall, I consider that this is an interesting publication and have no real concerns, thus I gladly support its publication in the actual format.

Major Concerns:
None

Minor Concerns:
None

Reply: Thank you.

Reviewer #3:
Manuscript Summary:
Use of non-denaturing BrdU staining to measure a step of DNA break end resection is a valuable tool to study the DNA damage response. Yet, a detailed protocol has not been published in the literature, and it is a somewhat complicated protocol. Thus, this protocol/method paper will be of significant interest to the field. The techniques are described with sufficient detail, and I also really liked that described use of Cell Profiler to examine the cell staining intensity - this will be exceptionally useful for trainees. The video was also really clear. The Discussion describes some of the critical steps of the protocol, which will also be helpful for trainees. Finally, the importance of this assay to the field of the DNA damage response was clearly/accurately described.

Major Concerns:
None

Minor Concerns:
Three things 1) For Step 2 (Pre-extractions and Fixations) I recommend an addition to the text, to clarify the following: "All pre-extraction, fixation, and staining steps are performed with the coverslips within the tissue culture plate; the coverslip is only lifted at the last step of mounting (see Discussion)." or an equivalent statement; 2) It is unclear why the authors didn't include all the details of the secondary antibodies in the protocol - adding this would be useful; 3) for "paraffin" do you mean "parafilm" they are probably interchangeable, but maybe do "(e.g. parafilm)."

Reply: Thank you for your feedback, these issues have been fixed in the text. We are very glad you enjoyed the accompanying cell profiler video, as we have experienced the frustration of using it without clear instructions.

Editorial comments:
Changes to be made by the Author(s):

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues. Please define all abbreviations at first use. **Done**

2. Please revise the following lines to avoid overlap with previously published work: 107-109; 111-112; 113-116; 117-118 **Done**

3. JoVE cannot publish manuscripts containing commercial language. This includes trademark symbols (™), registered symbols (®), and company names before an instrument or reagent. Please remove all commercial language from your manuscript (abstract, main text, figure legends; the only place brand names and sources can appear is in the Table of Materials; use generic terms instead in the manuscript. Enter the generic description used in the text in the comments column of the Table of Materials.
For example: RNAiMAX transfection; Bulldog Bio PCA16D10; CellRad X-ray irradiator (Precision X-Ray); Triton X-100; Tween-20; Alexa; Leica DMI6000B etc **Done**

4. Please revise the text, especially in the protocol, to avoid the use of any personal pronouns (e.g., "we", "you", "our" etc.). **Done**

5. Please ensure that all text in the protocol section is written in the imperative tense as if telling someone how to do the technique (e.g., “Do this,” “Ensure that,” etc.). The actions should be described in the imperative tense in complete sentences wherever possible. Avoid usage of phrases such as “could be,” “should be,” and “would be” throughout the Protocol. Any text that cannot be written in the imperative tense may be added as a “Note.” However, notes should be concise and used sparingly. Please include all safety procedures and use of hoods, etc. **Done**

6. The Protocol should contain only action items that direct the reader to do something. Please move the discussion about the protocol to the Discussion. **Done**

7. Please note that your protocol will be used to generate the script for the video and must contain everything that you would like shown in the video. Please add more details to your protocol steps. Please ensure you answer the “how” question, i.e., how is the step performed? Alternatively, add references to published material specifying how to perform the protocol action. Please add more specific details (e.g., button clicks for software actions, numerical values for settings, etc) to your protocol steps. There should be enough detail in each step to supplement the actions seen in the video so that viewers can easily replicate the protocol. **Done**

8. The Protocol should be made up almost entirely of discrete steps without large paragraphs of text between sections. Please simplify the Protocol so that individual steps contain only 2-3 actions per step and a maximum of 4 sentences per step. **Done**

9. After including a one-line space between each protocol step, highlight up to 3 pages of protocol text for inclusion in the protocol section of the video. This will clarify what needs to be filmed. **Done**

10. Please consider providing solution composition as Tables in separate .xls or .xlsx files uploaded to your Editorial Manager account. These tables can then be referenced in the protocol text. **Done**

11. Please include a scale bar for all images taken with a microscope to provide context to the

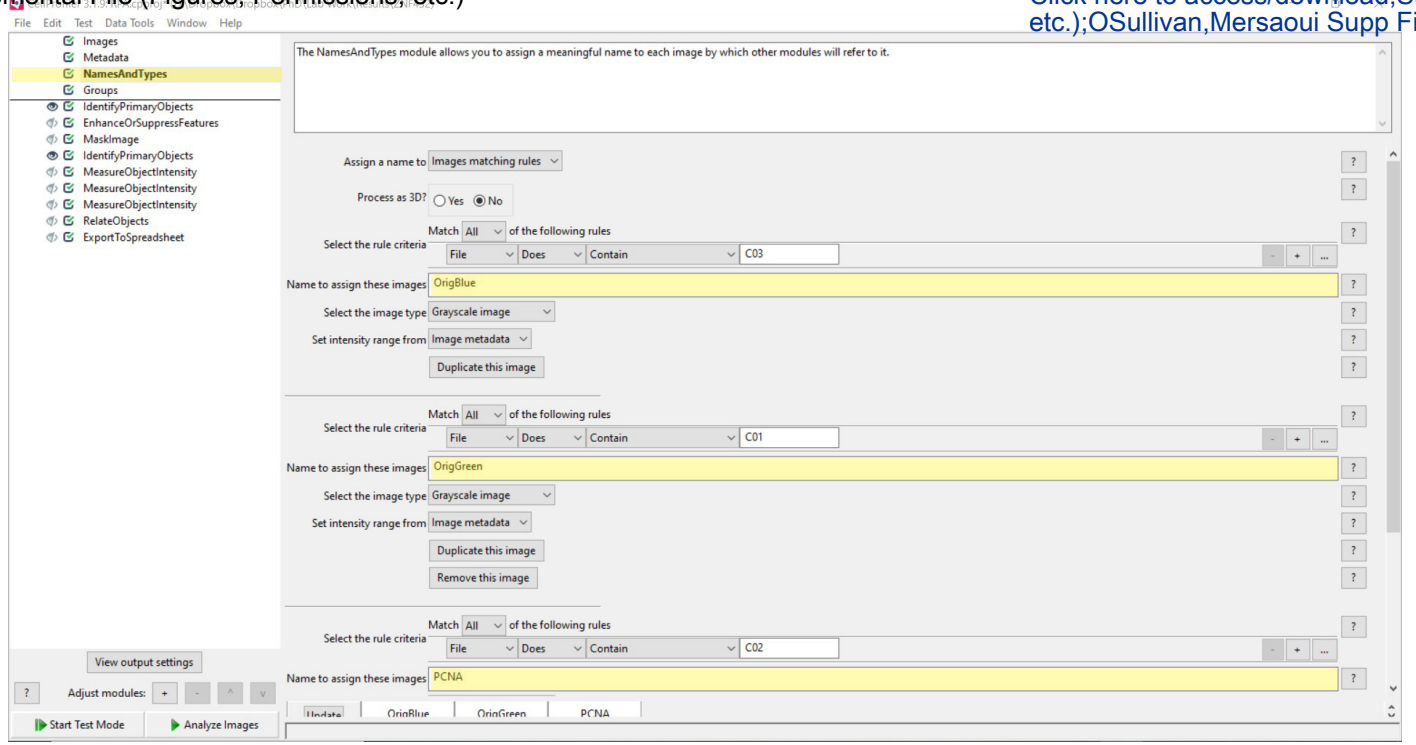
magnification used. Define the scale in the appropriate Figure Legend. Please add a space between numbers and units in all images. **Done**

12. Please sort the Materials Table alphabetically by the name of the material. **Done**

13. Please ensure that the references appear as the following: [Lastname, F.I., LastName, F.I., LastName, F.I. Article Title. Source (italics). Volume (bold) (Issue), FirstPage–LastPage (YEAR).] For more than 6 authors, list only the first author then et al. Please include volume and issue numbers for all references. Please do not abbreviate journal names, and use title case for journal names. **Done**

14. Please let us know how you would want to use the video file uploaded to the dropbox folder. We will be making the video with the highlighted section of the protocol. If you would like to show some of the analysis steps, we would prefer that you please highlight these in the text so we can incorporate them into the video to make a cohesive story instead of uploading a separate video. **We highlighted the analysis steps so we can do this as part of the video when JOVE films the method.**

A)

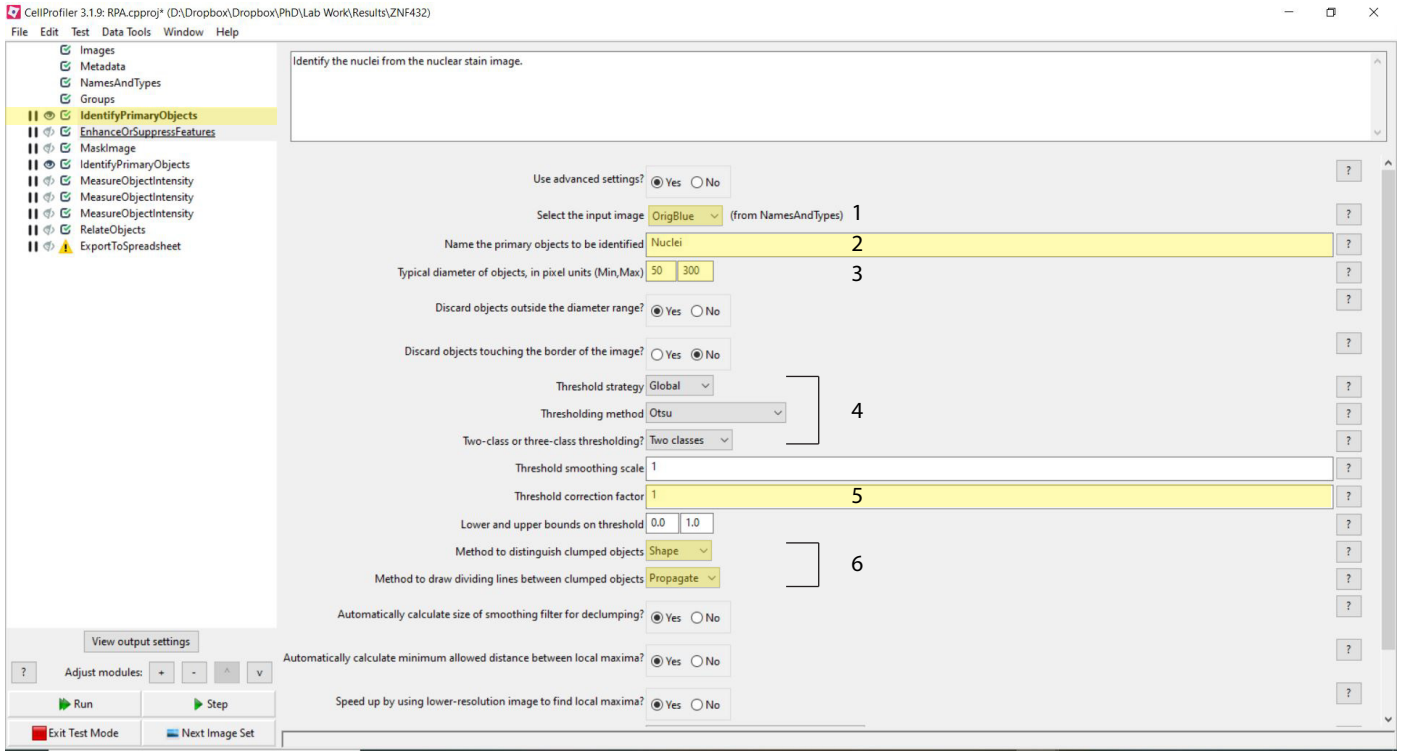


Representing the DAPI channel

Representing the BrdU channel

Representing the PCNA channel

B)



1) The channel used to identify the nucleus in this case is the DAPI

2) The label assigned the objects identified

3) The acceptable size range for the nuclei, which should be changed to best fit the cells in your images.

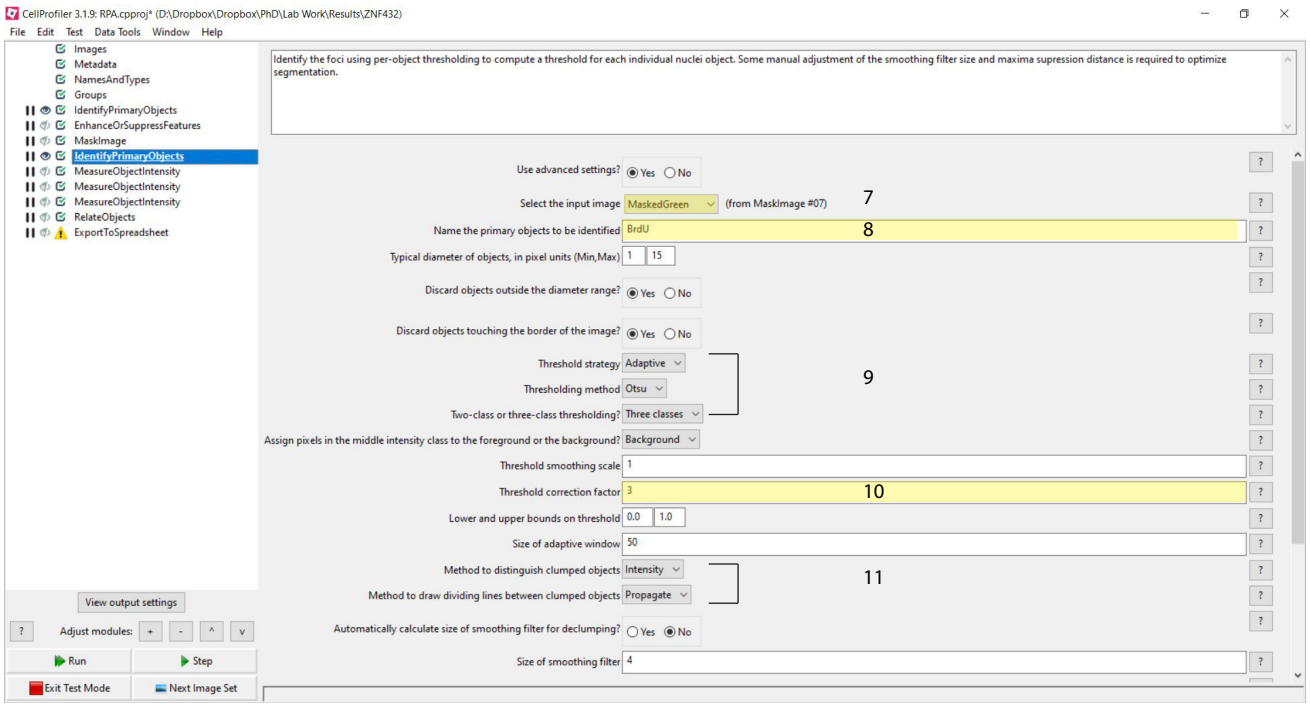
4) The thresholding strategy

5) The thresholding correction factor, i.e. how stringent the the thresholding strategy will be. This is the setting that will be most altered to fit cell shape and distribution.

6) How the cells are differentiated from each other by the program

O'Sullivan, Mersaoui et al. Supplemental Fig 2

A)



7) The channel used to identify the foci is the processed BrdU channel.

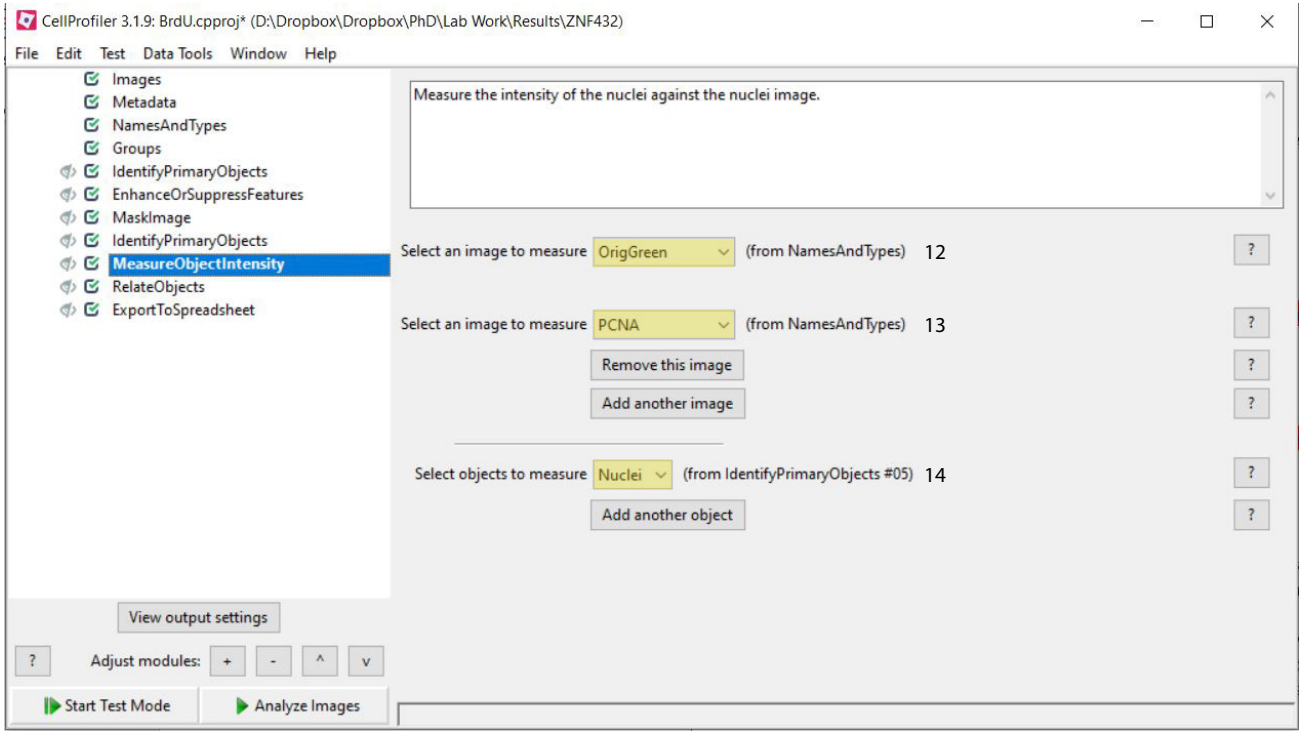
8) The name that will be given to data resulting from this section of analysis, which can be changed without issue.

9)The threshold strategy to identify and differentiate individual foci.

10) The threshold level, this will be changed to increase or decrease the number of objects identified as foci.

11) How the foci are differentiated from each other by the program

B)



12) The unprocessed channel whose intensity will be measured, in this instance the green BrdU channel.

13) The unprocessed channel whose intensity will be measured, in this instance the red PCNA channel.

14) The area that is to be measured, either nuclei or foci.

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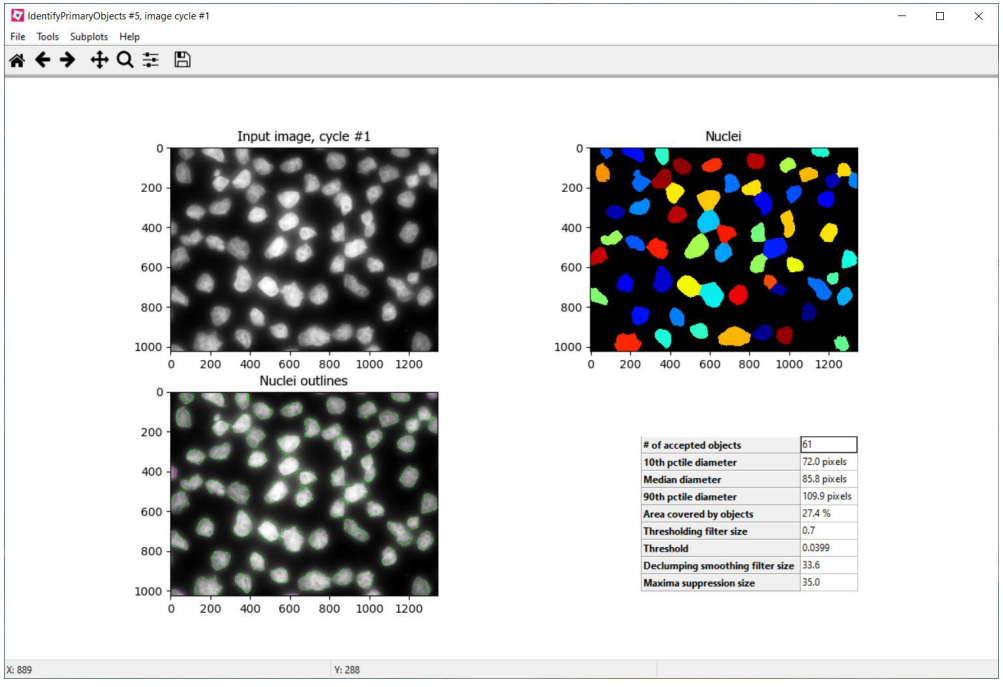
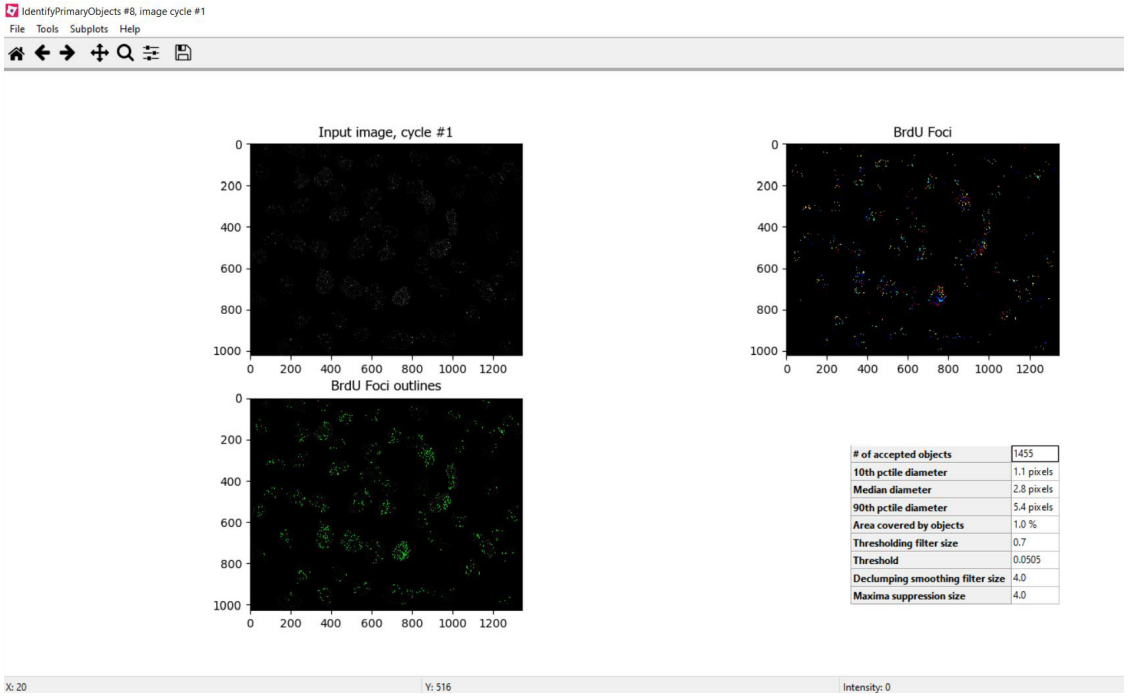
A)

Example of nuclei identification

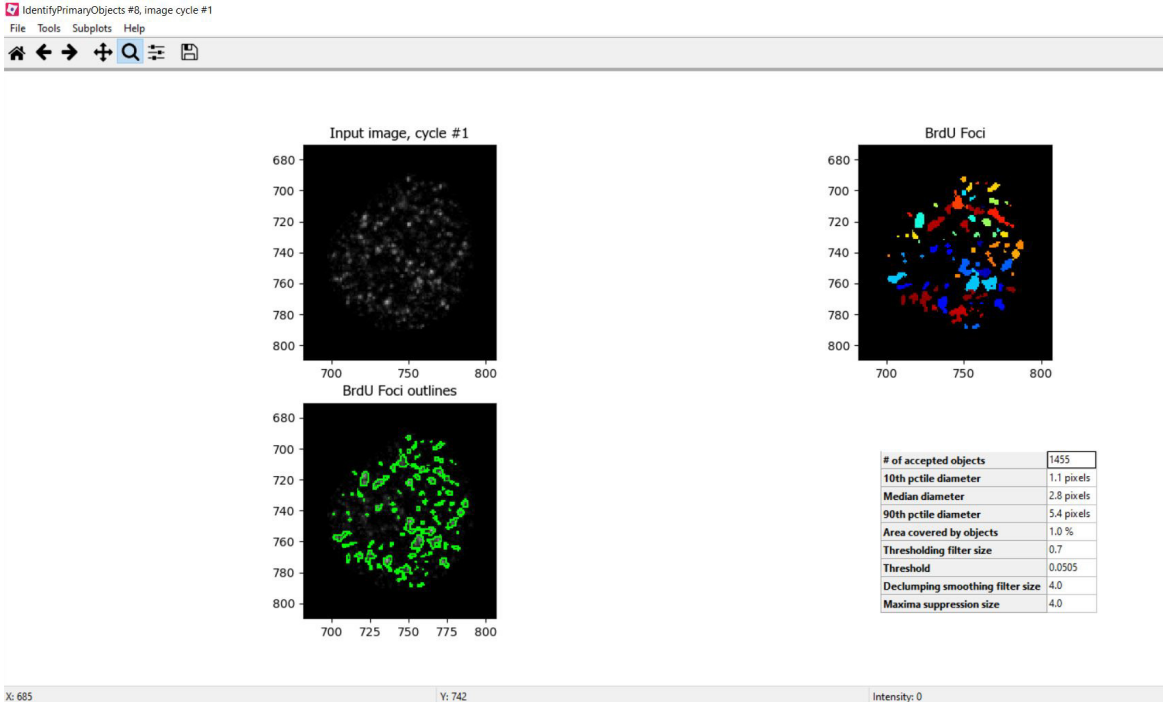


B)

Example of foci identification



C)



Zoom-in of foci identification

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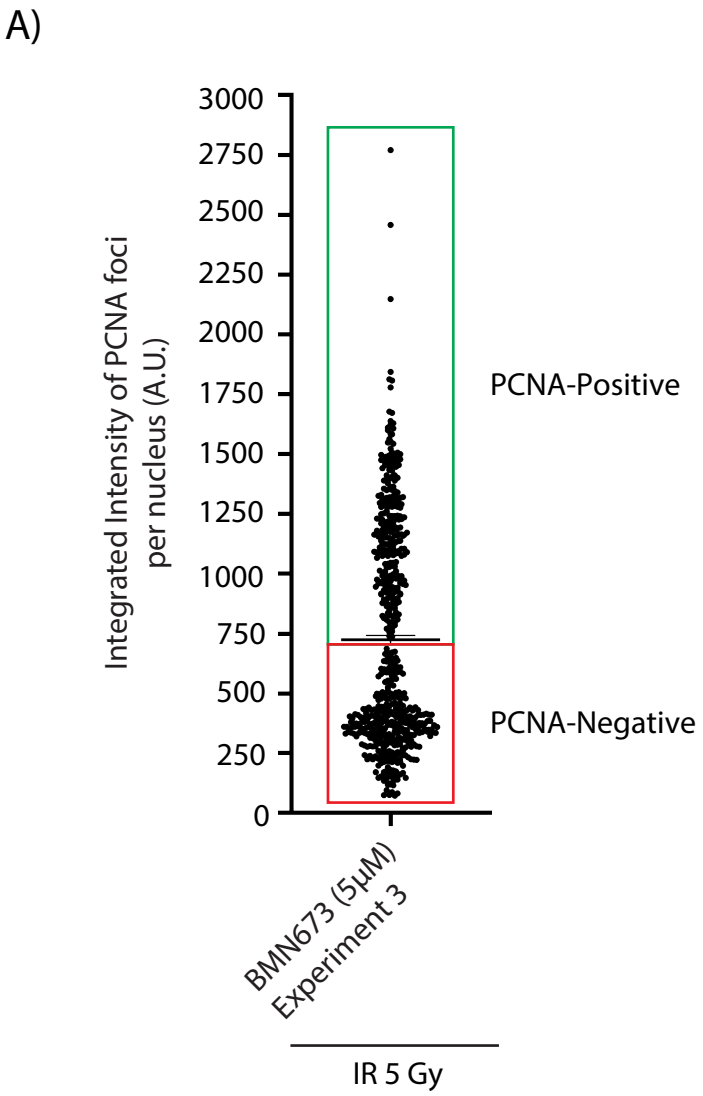

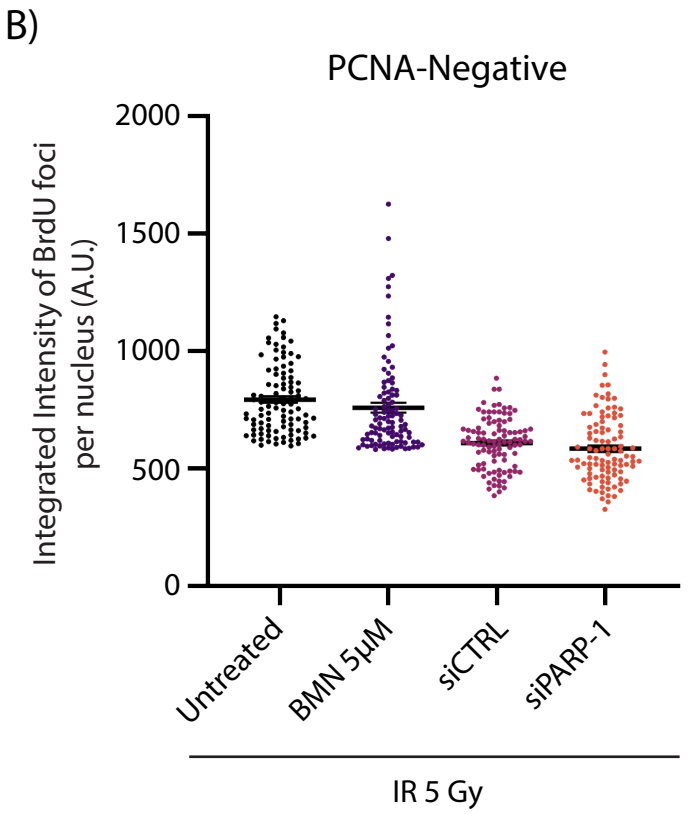

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