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.**