To: Dr Alisha DSouza,
Senior Review Editor

Re: Response to reviewer comments **JoVE61447**

Dear Editor,

Thank you for giving us the opportunity to submit a revised draft of our manuscript titled "Visualization of DNA repair proteins interaction by immunofluorescence". We appreciate the time and effort that you and the reviewers have dedicated to provide valuable feedback on our manuscript and we are providing here the modified version, which answers all points.

We have significantly modified wording as per reviewer 2 request, and are hopeful it will make it an easier, and more informative, read. Technical details and background information have been added to make the manuscript more suitable for its format: easier to reproduce for the audience, and more manageable for the filming crew.
We believe that we have addressed all comments raised by the three reviewers, and hope you will find the manuscript ready in its present state.
Due to the extensive editing, we have chosen to indicate changes by a vertical line in the margin rather than track changes.
The text to be used for filming is also highlighted. While we are happy to provide a picture of the irradiator if needed, its access is restricted and we will not be able to allow visitors into the room. As readers will have to familiarize themselves with their local irradiator, limited amount of information has been included in this regard.

Comments from Reviewer #1:
Major Concerns:
Both short abstract and long abstract have not represented the background and purpose of the study
Answer: We thank this reviewer for this comment. We are very limited in number of words allowed in the summary. However, we have included the following statement at the end of summary, to state more clearly the purpose of the method presented "Here, we describe the method of indirect immunofluorescence as a mean to detect DNA repair proteins, analyze their spatial and temporal recruitment, and help interrogate protein-protein interaction at the sites of DNA damage." (lines 15-17).
We have significantly expanded the abstract (lines 24-28) as well as the introduction (lines 46 to 107 encompass all edited text and adds on), to better introduce the background (DNA repair) and the purpose of conducting indirect immunofluorescence studies (DNA damage signaling; DNA repair efficiency, etc.).

Please write in sentence what is the purpose of this study.
Answer: The last paragraph of the introduction now reads: "The purpose of indirect immunolocalization is to assess the efficiency of DNA damage repair in cell lines, following IR like

in this study, or after exposure to various stresses in cell, from DNA crosslinking to blockage of the replication fork (a list of DNA damaging agents is provided in Table 1). [insert Figure 1, Table 1]" (lines 126-129).

Please add more references to the discussion.

Answer: We would like to thank you for raising this important point. As we expanded and detailed the introduction, the representative results, and the discussion, we have added a significant amount of references in the introduction and results. Unfortunately, most caveats of the experiments and possible pitfalls we mention in the discussion are in the category of "negative results: known fact but poorly documented". We have added available references whenever available.

Minor Concerns:

Consistent with the writing such writing Gamma H2Ax and Gamma H2AX with symbol Gamma

Answer: Thank you for pointing this out. We have looked for and modified all non-standard nomenclature, and replaced it by "γH2AX" all throughout the manuscript.

Comments from Reviewer #2:

Major Concerns:

This reviewer expressed concerns about the overall quality of the manuscript, with the results and discussion lacking precision and the presence of typos or convoluted sentences throughout.

Answer: We have addressed these concerns by carefully proofreading the manuscript, rewriting the introduction, simplifying sentences, and adding statement and references where the writing did not appear sufficiently precise. See below from the minor comment section for details.

The manuscript does not effectively convey the rationale for performing the representative experiments

Answer: Please see new version of the manuscript attached: the introduction and results sections have been heavily edited, to clearly highlight and describe the aims of these experiments. Changes addressing this comment are found on lines 120-129.

Minor Concerns:

This reviewer made a very comprehensive list of suggested edits.

Answer: We would like to thank the reviewer for their time spent commenting on the document and suggesting specific edits. While the word count precluded the expansion of the summary, we have incorporated comments in the short abstract/summary (page 1, lines 14-17) included all comments and rephrasing suggestions in the long abstract (sentences 4-6, page 1, lines 24-28), introduction (page 2, lines 46-72) as we feel these really help with clarity.

In addition, this reviewer suggested expanding the introduction of DDR proteins, their role in damage sensing and resolution and methods description.

Answer: Abstract and introduction have been expanded (see introduction page 2, line 74 to page 3, line 107) to incorporate a more comprehensive presentation of DNA damage actors and their

mechanism of action. In addition, literature cited has been updated to include more recent references, and additional citations where appropriate.
Major changes are indicated in the text by vertical lines in the margin and blue font.

Figure 1 should be referred to at an appropriate point during the introduction (i.e. earlier). The legend for figure 1 includes rather vague wording and does not mention the specific factors illustrated in the figure. Is this figure taken from a specific journal? If so it needs to be referenced.
Answer: Figure 1 is now referred on page 2 (paragraph 3, line 75) in the introduction. Figure legend (page 9, line 357) has been modified to include key DDR factors. As we assembled Figure 1, no citation is required.

Representative results section: 'western blotting' should be 'Western blotting'
Answer: This has been changed accordingly (line 276).

Representative results section paragraph 2: 'O2' should be written as 'oxygen'
Answer: This was changed (line 282).

Representative results section para 3 sentence 1 rephrasing suggestion: 'breaks and insults that occur due to endogenous stresses…'
Answer: Thank you for this suggestion. We have modified this sentence (lines 283-285).

In figure 3C the authors describe pre-apoptotic cells exhibiting "solid" gH2A.X staining throughout the nucleus. Whilst some of these cells may be pre-apoptotic, it is important to note that S-phase cells also exhibit this pan-nuclear staining pattern.
Answer: This is a very valid and important comment. We have clarified our statement to reflect pan-nuclear staining of cells in S-phase. See new phrasing on lines 286-291.

Representative results section para3 sentence 2 typo: '…foci formation increase as a…' should be 'increases'
Answer: Thank you for the thorough review. We have corrected the spelling error (line 297).

Representative results section para3 sentence 5: the sentence 'In cells depleted for a DNA repair gene…' is too general - a persistence of DSBs at late time points is a characteristic of cells with a defect in 'slow kinetics' HR. Cells with a defect in 'fast kinetics' NHEJ typically show elevated foci only at early time points. This important difference should be explained with appropriate references.
Answer: This reviewer raised an interesting point. In the first submitted version, we tried to avoid diving too deep into the details of the DNA damage repair, which is a complicated topic and could only be covered by a long review. While we wanted this article to be a beginner-friendly guide for scientists who have not done foci before, and thus might be novice in DNA repair, we fully agree with this reviewer that providing specific details on the relationship between foci and cell cycle will benefit the audience. We have accordingly extended the content (lines 295-317).

Representative results section para3, last sentence needs re-phrasing. Co-localization indicates that 2 proteins spatially and temporally associate within a discrete area. The authors note that 'It can be quantified as a percentage of the protein population co-localizing, as well as the area of the foci co-localizing.' The actual co-localization data derived from the experiment shown in Figure 5 should be presented in this figure.

Answer: The data included in Figure 5 are now presented in this section. The additional text has been added to the representative results, paragraph 5, on lines 319-346.

Discussion section: the authors mention that 30 to 300 nuclei represents 'good sampling'. Much better sampling would involve the automated imaging of a coverslip, which can be set up with many microscopy packages and allows many thousands of cells to be imaged automatically. This should perhaps be mentioned as a more advanced method for foci quantification, especially if using a DNA damaging agent that results in a much more heterogeneous range of foci numbers between cells in the same treatment.

Answer: Thank you for this suggestion. It has been included (lines 431-432).

Discussion section: referring to figure 6 - the phrase 'it is important to present the data in a light that readers can understand…' is too vague. Also, is this data a quantification of the images in figure 4?

Answer: Sentence has been changed to "For this reason, it is important that raw data is summarized, processed, analyzed and presented in an effective format in a light that readers can understand; facilitating comparison and revealing trends and relationships within the data." in paragraph 6 (lines 438-441). Figure 6 (now B in Figure 4) is in fact the quantification of γH2AX foci formation shown in Figure 4 and the figure legend (page 10, lines 376-382) has been modified to clarify this.

Table 1 legend title is written as 'Genotoxics' - this should be 'Genotoxins'. 'Example of…' should be 'Examples of…'

Answer: Thank you for the thorough review. We have corrected this (line 350).

Figure 1 legend title is better as 'DNA repair pathways' rather than 'DNA repair'

Answer: We agree. Figure legend title was changed (line 357).

Figure 3 legend rephrasing suggestion: '…breaks occurring due to endogenous sources are not repaired'

Answer: Figure legend modified accordingly (line 370).

Figure 6 legend phrasing 'convey the clearest message to the reader' is too vague and should be reconsidered

Sentenced has been changed to "Depending on the biological question raised and the type of data acquired, different options are available to present the raw data, in order to facilitate comparison and critical understanding." Now part of Figure 4 legend (lines 378-380).

Figure 3 panel B and C, as well as figure 4 T=0 DAPI appear to be overexposed.
Answer: The panels have been modified to include lower exposure.

Comments from Reviewer #3:

Major Concerns:
My only concern of medium not major importance is that the authors do not really engage into the methodologies used so far for colocalization of DNA repair proteins especially. See for example the Pclc method which is free to use and has been applied in a variety of radiations Free Radic Res. 2016 Nov;50(sup1):S64-S78. Epub 2016 https://www.ncbi.nlm.nih.gov/pubmed/27593437. Also here for a recent review https://www.ncbi.nlm.nih.gov/pubmed/31739493 The authors need to explain better the overall advances in the field without major verbalism and the need for quantification of the level of complexity via localization of DNA Damage response proteins. For example gammaH2AX and 53 BP1 should be very high. Do they verify it?
Answer: We would like to thank the reviewer for this comment. In our first version, the co-localization was merely mentioned, and it indeed involves complex analysis. We have modified the text to better introduce the concept of colocalization, and to propose two pipelines for analysis (Cell Profiler, which will be used in the video, and ImageJ with plugin). Since the Pclc method is extremely well explained in the references provided and allows detection of clustered DNA lesions, we decided to include these two references that will be of great interest to any readers interested in immunolocalization at DNA damage.
We have expanded the colocalization description in the representative results (lines 319-346), and are now presenting quantifications of Figure 5, with indeed a high level of co-localization, as expected in irradiated control cells.

Minor Concerns:
1.Why the authors have used such a high dose of 4 Gy? Details about the radiation and irradiation should be given.
Answer: 4 Gy is a high dose of irradiation, but we routinely subject the cells to 1, 2, or 4 Gy in order to follow DNA damage repair. While this might seem an excessive dose for monitoring γH2AX, it provides robust stimulation of foci formation, including by proteins difficult to detect. While we feel that it is important to provide conditions with which the "first time" user is more likely to succeed in observing foci, we have now modified the text to state that 4 Gy is indeed a high dose, and that other methods of damage can be used to damage DNA in a more controlled fashion (introduction: lines 98-103).

2. In the Table with different types of stress, the authors need to include various radiations of different LET. It cannot be conceived that a method of colocalization is only in the 2020 only for X rays or gamma rays.
Answer: This is again a very valid point. In this method, we aim at providing a method for staining and imaging protein foci, and are focusing more on the protein recruitment to damages than the quality and number of damages induced.

However, the table provides an opportunity to include more stresses as an option for studying DNA damages. The authors do not feel competent in using radiations of different LET. For this reason, this is now mentioned in the table but not detailed in the text, and citations have been included for reference.

3. In the repair process foci do not drop even after several hrs. Why? This is an artifact. It relates to the high dose and excess number of foci. The dose should d be lower 2 Gy. What about lower dose?

Answer: We agree that in our example (former Figure 6), the repair at 4h did not appear evident. This is an artifact indeed, that we cannot explain: the cell lines we use in the laboratory show efficient repair at 4h post irradiation, even after 4 Gy.

While 2 Gy, and 1 Gy would induce less damage and faster repair, we choose to show massive irradiation for the reasons mentioned earlier (foci formation by low-abundance proteins or low-abundance PTM events). Updated quantification of γH2AX foci resolution is provided in the new Figure 4.

We would like to thank again the reviewers for raising important points, as the manuscript has been made stronger by their suggestions.

In addition to the above modifications, the text has been proofread and spelling and grammatical errors have been corrected.

We look forward to hearing from you in due time regarding out submission and to respond to any further questions and comments you may have.

Sincerely,

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