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TITLE:

Visualization of DNA Repair Proteins Interaction by Immunofluorescence

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SUMMARY:

Following DNA damage, human cells activate essential repair pathways to restore the integrity of their genome. Here, we describe the method of indirect immunofluorescence as a means to detect DNA repair proteins, analyze their spatial and temporal recruitment, and help interrogate protein-protein interaction at the sites of DNA damage.

ABSTRACT:

Mammalian cells are constantly exposed to chemicals, radiations, and naturally occurring metabolic by-products, which create specific types of DNA insults. Genotoxic agents can damage the DNA backbone, break it, or modify the chemical nature of individual bases. Following DNA insult, DNA damage response (DDR) pathways are activated and proteins involved in the repair are recruited. A plethora of factors are involved in sensing the type of damage and activating the appropriate repair response. Failure to correctly activate and recruit DDR factors can lead to genomic instability, which underlies many human pathologies including cancer. Studies of DDR proteins can provide insights into drug response and cellular mechanisms of drug resistance.

There are two major ways of detecting proteins in vivo: direct observation, by tagging the protein of interest with a fluorescent protein and following it by live imaging, or indirect immunofluorescence on fixed samples. While visualization of fluorescently tagged proteins allows precise monitoring over time, direct tagging in N- or C-terminus can interfere with the protein localization or function. Observation of proteins in their unmodified, endogenous version is preferred. When DNA repair proteins are recruited to the DNA insult, their concentration increases locally and they form groups, or “foci”, that can be visualized by indirect immunofluorescence using specific antibodies.

Although detection of protein foci does not provide a definitive proof of direct interaction, co-localization of proteins in cells indicates that they regroup to the site of damage, and can inform of the sequence of events required for complex formation. Careful analysis of foci spatial overlap in cells expressing wild type or mutant versions of a protein can provide precious clues on functional domains important for DNA repair function. Last, co-localization of proteins indicates possible direct interactions that can be verified by co-immunoprecipitation in cells, or direct pulldown using purified proteins.

INTRODUCTION:

Human cells are constantly exposed to a variety of DNA damaging agents of various origins. Exogenous sources mostly consist of exposure to radiations, chemicals (including chemotherapeutic agents and some antibiotics), and viruses, while the main endogenous sources include errors in DNA replication and oxidative stress. The direct effects of genotoxic exposure can range from a modified base to a potentially lethal DNA double-strand break (DSB), depending on the stress and the exposure dose. Ultimately, unrepaired or mis-repaired DNA damage can lead to the accumulation of mutations, genomic rearrangements, genome instability and eventually lead to carcinogenesis[1]. Mammalian cells have evolved complex pathways to recognize a specific type of DNA damage[2,3] and repair them in a timely fashion, synchronized with the cell cycle progression.

Ionizing radiation (IR) damages the DNA double helix and creates double-strand breaks (DSBs), one of the most deleterious forms of DNA damage. The MRN (MRE11, RAD50, NBS1) complex functions as a sensor of DNA ends and activates the protein kinase ataxia telangiectasia mutated (ATM)[4,5]. Following the initial activation of ATM by DNA ends, ATM triggers a cascade of DDR events at the site of the break, initiating with a key event, the phosphorylation of the histone variant H2AX[6]. H2AX phosphorylation on residue S139 activates it into γH2AX, spanning regions up to megabases around the DNA lesion[6-9]. This event increases DNA accessibility, leading to the recruitment and accumulation of other DNA repair proteins[7]. Because γH2AX is abundantly and specifically induced surrounding DSBs, it can be readily visualized using specific antibodies, and is commonly used as a surrogate marker for DSBs in the DNA repair field. Once the break is signaled, cells activate their DNA repair pathways and process the DNA damage. The protein MDC1 (mediator of DNA damage checkpoint protein 1) directly binds γH2AX[10], interacts with ATM[11] and also with NBS1[12,13]. It contributes to increasing the concentration of MRN complex at the DSB and initiating a positive ATM feedback loop. γH2AX is rapidly removed once the break is repaired, consequently, allowing the monitoring of DSB clearance. Followed by microscopy, the decrease in γH2AX over time provides an indirect measurement of residual breaks and DNA repair efficiency.

Eukaryotic cells can repair DSBs by several pathways, the two main ones being non-homologous end-joining (NHEJ) and homologous recombination (HR) (**Figure 1**). NHEJ essentially ligates DNA double-strand ends without the use of extended homology and operates throughout the cell cycle[14,15]. HR becomes predominant during S and G2 phases, and is otherwise repressed, since it requires a sister chromatid as a homologous template for repair[14,16]. Pathway choice between

NHEJ and HR not only depends on the physical proximity of the sister chromatid, but also on the extend of DNA end resection[17], which inhibits NHEJ .

Homology-dependent DSB repair initiates by nucleolytic degradation of the 5' strand from the break ends to generate 3' single-strand DNA (ssDNA) tails, a process referred to as 5'-3' resection. The MRN complex initiates DNA end resection and further resection is processed in combination with BLM/EXO1 (Bloom syndrome protein/exonuclease 1) or BLM/DNA2 (DNA replication ATP-dependent helicase/nuclease)[18-22]. DNA end resection is enhanced by CtIP (CtBP-interacting protein) through its direct interaction with MRN complex[23] and recruitment of BRCA1 (breast cancer type 1 susceptibility protein)[24,25]. Replication protein A (RPA) promptly binds to the ssDNA exposed and is then displaced by the recombinase protein RAD51 to form a nucleoprotein filament that catalyzes homologous search and strand invasion[26-28].

The initiation of resection is a critical step for repair pathway choice. Once resection has initiated, the DNA ends become poor substrates for binding by Ku70/Ku80 heterodimer (component of NHEJ pathway) and cells are committed to HR[17,29,30]. The Ku70/Ku80 heterodimer binds to DSB ends, recruiting DNA-PKcs and p53 Binding Protein 1 (53BP1)[29,30]. 53BP1 acts as a barrier to resection in G1, thus blocking HR while promoting NHEJ[31,32], but it is removed in a BRCA1-dependent manner in S phase, consequently allowing resection to occur[33,34]. Therefore, 53BP1 and BRCA1 play opposing roles in DSB repair, with 53BP1 being a NHEJ facilitator whilst BRCA1 acts enabling breaks to repair through HR.

In the laboratory, DSB formation can be induced by ionizing radiation (IR). While this example utilizes a high dose of 4 Gy, 1 Gy and 2 Gy also create a significant amount of DSBs, suitable for the analysis of foci formation by abundant proteins. It is important to note that the type and dose of radiation used can lead to different lesions in the DNA and in the cell: while IR induces DSBs, it can also cause single strand breaks or base modification (see[35,36] for a reference on irradiation linear energy transfer (LET) and type of DNA damage). To determine the kinetics of ionizing radiation-induced foci (IRIF) formation and their clearance, which indicate repair of the damage and reversal of the activated DDR[8,9,37,38], foci formation can be monitored at different time points after ionizing radiation. Timing of activation and clearance of all major DNA damage proteins is known[39], and many are investigated as surrogate markers of key events. For example, pRPA, which possesses high affinity for ssDNA is used as a surrogate of the break resection, MRN proteins (MRE11, RAD50, NBS1) and exonucleases can be used to assess resection efficiency too. While RAD51, BRCA1, BRCA2 (breast cancer type 2 susceptibility protein), and PALB2 (partner and localizer of BRCA2) can be monitored to evaluate HR efficiency, the presence of the Ku proteins or 53BP1, are used as markers of NHEJ (**Figure 1**).

As proteins of the DNA repair machinery recruit each other to the break and assemble in super-complexes, DNA-protein and protein-protein interactions can be inferred by following their individual localization over time and analyzing co-localization of proteins, as visualized by overlapping signals in cell[40-42]. In cell lines, the introduction of point mutations or deletion in specific DNA repair genes either through genome editing, or by overexpression of plasmid-based mutants, allows investigation of specific residues and their possible role in recognition of DNA

damage (e.g., co-localization with γH2AX) or complex assembly (co-localization with another, or several, proteins), as well as their impact on DNA repair. Here, we use indirect immunofluorescence as a mean to investigate the formation and resolution of DSBs by following γH2AX foci over time. We also present one example of foci formation and co-localization analysis by a major player in DSB repair: p53 Binding Protein 1 (53BP1)[32]. As mentioned earlier, 53BP1 is considered central to DNA repair pathway choice. Following 53BP1 accumulation and its co-localization with γH2AX provides precious information on cell cycle phase, DNA damage accumulation, and pathway used to repair DSBs. 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]

PROTOCOL:

1. HeLa cell culture

1.1. Pre-treat round glass coverslips with 1 M HCl at 50 °C for 16-18 hours. Rinse with ddH_2O and store in 100% EtOH.

1.2. Prepare cell culture medium: add 10% (v/v) FBS to DMEM medium.

1.3. Grow 4.0 x 10^4 cells/well in sterile 12-well plate with an 18 mm round glass coverslip at 37 °C and 5% CO_2 in a humidified incubator. Grow cells to 80% confluency (approximately 24 hours).

2. Cell treatment with radiation (IR)

2.1. To induce the formation of double-strand breaks, expose cells to 4 Gy γ-irradiation (control: No irradiation, t=0). In the Gamma Cell -40, this corresponds to 4.54 min, at 0.815 Gy per min.

2.2. Incubate cells at 37 °C and 5% CO_2 in a humidified incubator for the appropriate time-length (time points chosen here t=1, 2, 4, 16h).

3. Nuclear extraction and cell fixation

3.1. Prepare stock solutions: 0.2 M PIPES (pH 6.8), 5 M NaCl, 2 M sucrose, 1 M $MgCl_2$, 0.1 M EGTA (pH 8.0).

3.2. Prepare nuclear extraction buffer (NEB): dissolve 10 mM PIPES (pH 6.8), 100 mM NaCl, 300 mM sucrose, 3 mM $MgCl_2$, 1 mM EGTA (pH 8.0) and 0.5% (v/v) Triton X-100 in ddH_2O. Mix until dissolved completely.

3.3. Prepare 4% (v/v) paraformaldehyde (PFA): dissolve 10 mL of 16% PFA aqueous solution in 30 mL PBS. Mix until dissolved completely.

3.4. At appropriate time-point (t=0, 1, 2, 4, 16 h), wash cells twice with 1 mL of PBS. Remove PBS completely.

3.5. Add 200 µL of NEB to each well for cell nuclei extraction (cytoplasm is degraded, only nucleus remains) (**Figure 2**). Incubate for 2 minutes at room temperature and remove completely.

NOTE: Do not exceed 2 minutes.

[insert Figure 2]

3.6. Wash cells with 1 mL of PBS. Remove PBS completely. Add PBS carefully, cells are very fragile at this step.

3.7. Add 200 µL of 4% (v/v) PFA to each well for cell fixation. Incubate for 10 minutes at 4 °C. Remove PFA completely.

3.8. Add 1 mL of PBS.

NOTE: Cells can be stored in PBS at 4 °C.

4. Immunofluorescence staining

4.1. Prepare blocking solution: dissolve 5% BSA (w/v) in PBS and add 0.3% (v/v) Triton X-100. Mix until completely dissolved.

4.2. Prepare dilution buffer: dissolve 1% BSA (w/v) in PBS and add 0.3% (v/v) Triton X-100. Mix until completely dissolved.

4.3. For blocking, add 200 µL of blocking solution to each well. Incubate for 2 hours at room temperature or 16-18 hours at 4 °C.

NOTE: If goat antibody will be used, add 5% goat serum to blocking solution.

4.4. Dilute primary antibody in dilution buffer (1:500) and vortex until well mixed.

NOTE: If goat antibody is used, add 1% goat serum to dilution buffer.

4.5. In a humidity/incubation box, adhere a piece of parafilm. Add 10 μL of primary antibody (in a single drop). Align one edge of the coverslip with the drop and slowly lower onto the parafilm, for the liquid to spread throughout (avoid bubbles if possible). Incubate for 2 hours at room temperature.

4.6. Wash coverslips three times in PBS for 1 minute.

4.7. Dilute secondary antibody in dilution buffer (final concentration: 2 μg/mL) and vortex until well mixed.

4.8. Apply 10 μL of secondary antibody as described for the primary antibodies. Incubate for 2 hours at room temperature.

NOTE: Protect from light.

4.9. Wash coverslips three times in PBS for 1 minute.

4.10. Wash coverslips with H_2O for 1 minute.

4.11. Counterstain DNA with DAPI: apply 10 μL of 300 nM DAPI (as described for antibodies), incubate for 30 minutes at room temperature and then mount onto glass slide with a glycerol based mounting media. Alternatively, add one drop (10 μL) of commercial antifade mounting media containing DAPI onto a slide and apply a coverslip. Gently press the coverslip and remove excess fluid around it with a paper towel.

4.12. Seal coverslips with transparent nail polish and let them dry for 20 minutes.

4.13. Store slides at 4 °C.

5. Image acquisition

5.1. Place a drop of immersion oil onto the 60x objective lens. Use DAPI to locate the nuclei through eye piece.

5.1.1. For XYZ image acquisition, open acquisition software and select parameters: Scanner type: Galvano; Scanner mode: Roundtrip; Image size: 512×512; PMT mode: VBF; PMT average: frame (4 times); PMT sequential scan: line.

5.1.2. Select the dye and the detectors:
Channel (CH1), Dye (DAPI), Detector (SD1)
Channel (CH2), Dye (Alexa Fluor 488), Detector (HSD3)

Channel (CH3), Dye (Alexa Fluor 647), Detector (HSD4)

5.1.3. Select **ON** in "Z" and select **Start/End** (15 slices).

5.2. Adjust the live image. Press the **Live** button on the **Live** window.

5.2.1. Adjust the focus and set laser intensity (%), sensitivity (HV), gain and offset on "PMT" tool window.

5.2.1.1. Adjust the laser intensity (%): for brightness and bleaching. The higher the laser intensity, the stronger the signal, but the specimen will photobleach.

5.2.1.2. Adjust the sensitivity (HV): noise level. The higher the HV, the stronger the signal, but image will be noisy if too high.

NOTE: Always keep voltage constant.

5.2.1.3. Adjust the offset: background level.

5.3. Start the acquisition.

5.3.1. Select the folder to save images. Press the **LSM Start** button to start acquiring the image. Press the **Series Done** button to complete the image acquisition.

6. Data analysis

6.1 For image analysis, open the analysis software.

6.1.1. Press the **Batch** tool window and select the images to analyze.

6.1.2. Press the **Analysis** tool window and select Projection (will display the maximum intensity projection from 15 slices).

6.1.3. Under **Input/Output setting**, select the batch created and output folder.

6.1.4. Press **Process** for images to be processed.

6.1.5. Export images as .tiff files.

6.2 For nuclear foci quantification, open CellProfiler.

6.2.1. Open the γH2AX and 53BP1 foci quantification pipeline (see **Supplemental information**).

6.2.2. Graph data using a table software.

6.3 For co-localization analysis, open CellProfiler.

6.3.1. Open the **Colocalization** pipeline (see **Supplemental information**). A spreadsheet file will be created and saved in the preferred location. However, graphs themselves will not be automatically saved. It is suggested to take a snapshot of the windows to keep for record of the results.

6.3.2. Graph data using a table software.

REPRESENTATIVE RESULTS:

On day 2, or 24 h post seeding cells on coverslips, cells have undergone one division and are 80% confluent. Specific knock downs or mutations in DNA repair proteins can increase doubling time, or sensitize cells to genotoxic treatment, and seeding density as well as treatment doses should be adjusted accordingly. To determine best working conditions, timing of the DNA damage response can be established by Western blotting of γH2AX over time, and sensitivity to irradiation can be identified by colony forming assay.

Cells not treated with irradiation exhibit little, if any, γH2AX foci (**Figure 3A**). However, γH2AX foci formation can be triggered in cultured cells by various stresses inherent to the growing conditions: cells left confluent in acidified media, presence of genotoxic endotoxin in the FBS, oxygen concentration used for culture, to cite a few.

[insert Figure 3]

In addition, in cells depleted for key DNA damage proteins, such as *BRCA1* or *BRCA2* mutant cells, DNA breaks that occur as a result of endogenous stresses are not repaired as in wild-type cells, and accumulate. As a result, elevated γH2AX can be observed in HR deficient cells, even in normal growing conditions (**Figure 3B**). A sub-population of cells can exhibit "solid" nuclear staining with γH2AX (**Figure 3C**). Pan-nuclear stain can indicate that a cell is overwhelmed with damage beyond repair, and/or is pre-apoptotic. Such cells are typically characterized by enlarged nuclei, and the presence of cytoplasmic vacuoles. Additionally, γH2AX can be triggered by replicative stress in S-phase and stalled replication forks, or by G2/M arrest. If needed, cell cycle phases can be identified by staining the DNA content or co-staining with specific markers. When conducting DNA damage repair analysis, pan-nuclear can be quantified independently from individualized foci.

Following irradiation, nuclei exhibit a large number of double strand breaks to which γH2AX localizes extremely rapidly (**Figure 4**). In optimal conditions, few if any γH2AX foci are observed in the absence of irradiation. Following irradiation, formation of γH2AX foci increases sharply. If the breaks are processed and repaired, the foci count per nuclei is decreased, as well as the number of cells positive for foci. The intensity and number of foci varies depending on the cell line used and the dose of irradiation delivered. In low passage HeLa cells, grown in endotoxin-

free serum, we routinely observed a sharp increase of foci at 30 minutes and 1 h post irradiation, which peaks between 1 and 2h, then decreases progressively until 16 h. For this reason, representative time points at 0, 1, 2, 4 and 16 h post irradiation are presented here. Behavior of the break signaling, repair, and survival to irradiation can vary greatly between cell lines as well as upon depletion or mutation of genes. For this reason, the most appropriate time points will be experiment- and cell-dependent. In the experiment, at 16 h γH2AX residual foci have reached the same basal level than non-irradiated cells. Monitoring the residual foci to include later time points is suggested if working with slowly dividing cell lines.

[insert Figure 4]

Conversely, in cells deficient for DNA damage repair functions, γH2AX foci accumulate in the absence of irradiation (t=0 h) and the repair can be delayed, or absent even at long time points (t=16 h, t=24 h) post irradiation. Direct comparison between wild type and mutant cells can give precious information on the DNA repair function of the gene mutated, and hint at the type of repair. While a deficiency in NHEJ can force the break to be repaired by HR in S-phase, DSBs that have been resected and must be repaired by HR will not be repaired by NHEJ. For this reason, accumulation of damage post S-phase might indicate a HR deficiency, and high γH2AX levels post division hint at NHEJ defects.

When several proteins are investigated in the same cells, by multiplexing primary antibodies raised in different animal species and revealing these with secondary antibodies labeled with distinct fluorophores, co-localization can be investigated (**Figure 5**). Co-localization indicates whether proteins (i) are recruited to the break (ii) are recruited in a timely fashion, (iii) assemble in DNA repair complexes. When looking for co-localization, a commonly accepted qualitative method is to present results as a simple overlay of the different channels (i.e., green and red). Superposition of green and red will give rise to yellow hotspots, where the two proteins of interest are present in the same pixels (**Figure 5A**). However, this method has limitations, since it is dependent on the relative signal intensity collected in both channels, and the two fluorochromes may have differences in signal strength. Therefore, overlay methods help to generate a visual estimate of co-localization events, but are not appropriate for quantitative purposes. Quantitative analysis of co-localization can be achieved by an object-based approach (**Figure 5B** (i-iii) or by a statistic approach that performs an intensity correlation coefficient-based (ICCB) analyses (**Figure 5B** (iv)). Several tools for co-localization analysis are available, including JACoP (Just Another Co-localization Plugin; https://imagej.nih.gov/ij/plugins/track/jacop.html) and the "Colocalization" pipeline (CellProfiler) utilized here, that can be used as an ICCB tool, in order to assess the relationship between fluorescence intensities. This is mostly done by calculating the correlation coefficient (Pearson's coefficient) that measures the strength of the linear relationship between two variables. Fluorescence co-localization can be represented graphically in scatter plots, where the intensity of one fluorochrome (green) is plotted against the intensity of the second fluorochrome (red) for each pixel. Complete co-localization results in points clustered around a straight line, whose slope reflects the ratio of the fluorescence of the two colors. On the contrary, lack of co-localization results in the distribution of points into two separate groups (distributed along the axes), each showing varying signal levels of one color with

little or no signal from the other color. Pearson's coefficient value ranges from 1 to -1, with 1 standing for complete positive correlation, -1 for a negative correlation and zero indicating no correlation. Alternatively, the Pclc method can be used. This method has been applied in a variety of radiations (see[36] for details and freely available method).

FIGURE AND TABLE LEGENDS:

Table 1: Genotoxic agents. Examples of DNA damaging agents, their mechanism of action and the damage induced based on suggested working concentration.

Table 2: Antibodies used. List of antibodies used in this study.

Figure 1: DNA double strand breaks (DSB) repair pathways. DSB repair involves two major pathways: Homologous Recombination (HR, left) and Non-Homologous End-Joining (NHEJ, right). Following the break, proteins get activated to mark the break (γH2AX), participate in end resection (MRN, pRPA), promote recombination (BRCA1, PALB2, BRCA2, RAD51) or limit resection and promote NHEJ (53BP1). Other proteins participate in damage repair, but proteins listed are routinely followed by indirect immunofluorescence.

Figure 2: Nuclear extraction. Representative images of cells prior to (left) and post (right) nuclear extraction. Cytoplasm should be digested but the nuclear structure should remain intact post extraction (right). (A) 20x magnification; scale bar = 20 μm and (B) 40x magnification; scale bar = 10 μm.

Figure 3: Nuclear foci with no stress. In the absence of external stressor, very few if any γH2AX foci are observed (A). In the absence of essential DNA repair proteins, γH2AX accumulation can be observed as breaks occurring due to endogenous sources are not repaired (B). Accumulation of unrepaired breaks may lead cells to become pre-apoptotic, which is marked by a "solid" γH2AX nuclei staining (C). Scale bar = 5 μm.

Figure 4: Nuclear foci and quantification following stress (time-course). γH2AX foci formation at t=0 (no irradiation) and at the indicated time-points post irradiation (4 Gy, t=1, 2, 4, 16h). (A) Representative images are shown. DAPI indicates the nuclei. Scale bar = 5 μm. (B) Nuclear foci of γH2AX following exposure to γ-irradiation were scored by automated quantification (CellProfiler) in ≥ 100 nuclei for each time-point. 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. (i) Cloud plot shows mean ± SD. Symbol (*) indicates statistical significance relative to control, using Student's two tailed t-test: *** $p \leq 0.0001$, (ii) induction curve shows mean ± SEM, (iii) more than 10 foci per nucleus.

Figure 5: Co-localization analysis. By using several antibodies raised against different species (here, anti-mouse γH2AX (red) and anti-rabbit 53BP1 (green)), several proteins can be investigated. (**A**) Representative images are shown. DAPI indicates the nuclei. Co-localization is visualized by color and area overlap (here red + green = yellow). Scale bar = 5 μm. (**B**) Individual and co-localizing foci can be quantified individually using one of several softwares (here, CellProfiler, see other options in main text) and plotted. (i-iii) Object-based approached used to determine co-localization (iv) Correlation results obtained for co-localization, using a pixel-based approach (ICCB analysis).

DISCUSSION:

Analysis of the timing and efficiency of DNA damage repair by microscopy has proven essential to understand how the DNA repair machinery functions, in normal cells and in human pathologies such as cancer.

The development of specific antibodies that allow detection of activated proteins in their phosphorylated version (such as γH2AX, pRPA, pRAD50 and others[10,23,39,43]) has played a central role in gaining a better understanding of DNA repair timing of events and its synchronization with the cell cycle. This success is exemplified by the analysis of 53BP1 phosphorylated residues, by mass spectrometry and immunolocalization (see[32] for review), which has helped understanding the function of this heavily modified protein. With the ascent of high-resolution microscopic technique such as STORM and increased use of small antibodies (nanobodies) direct protein interaction in cells is becoming more accessible and accurate. However, indirect immunofluorescence as described here remains an essential experiment to perform when investigating a novel potential DNA repair protein and looking for timing of action and protein partners.

The success of indirect immunofluorescence rests entirely on two criteria: (i) the quality of reagents – especially antibodies used to detect phospho-residues on activated DNA repair proteins, and (ii) the timing of the experiment. Using published protocols and antibodies should be encouraged when available. When using a novel antibody or investigating a novel protein, the antibody should be validated, and specificity should be established by either knock down of target protein in cells (the signal should be lost) or protein depletion (use of purified protein to deplete specific antibodies prior to incubation, as performed in[44] for RAD51AP1).

Optimal blocking conditions and antibody dilution should be established to minimize artifacts and non-specific staining. Buffers will be prepared fresh and if nuclear extraction is performed, it must be timed to avoid damaging nuclei. In investigating the repair efficiency in human cells, quantification of nuclear foci should be performed, and the nuclear extraction can help with excluding cytoplasmic proteins. However, it can be beneficial to not perform the nuclear extraction step, for example to investigate whether a mutant protein suspected to not interact with its cellular partner fails to translocate to the nucleus upon DNA damage.

In addition, the quality of the imaging is of paramount importance to ensure that data can be properly acquired, analyzed, and accurate data plotted. Parameters to control for are many and include: specificity of the primary antibodies, spectrum of excitation of the fluorophores chosen (secondary antibodies), protecting samples against photobleaching, choice of support for seeding cells, good sampling (minimum of 30 to 300 nuclei, depending on the question), and in some cases post-treatment such as deconvolution or spectral un-mixing. When possible, automated imaging of a full coverslip, which allows thousands of cells to be imaged, is suggested. Involving imaging specialists such as a core facility prior to setting up the experiment should be considered as it will greatly enhance quality of the results.

Last, based on the cell lines used, the treatment performed, and the proteins investigated, basal levels of foci may vary greatly and make results difficult to interpret. 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 **Figure 4B**, the same quantification of foci is plotted in three separate versions: (i) total number of foci (cloud plot) after DSBs induced by irradiation (ii) induction of foci over time -kinetics- (iii) % of positive cells (>10 foci/nucleus).

Cloud plots are to be preferred whenever possible (**Figure 4B** (i)) as they show all data points without discrimination and thus offer the most comprehensive overview of the experiment. However, plotting more selective and relevant information, such as number of positive cells above an arbitrary background cut off (5 foci in most cell lines), the number of foci co-localizing, foci induction over time, or quality measurement of the foci, such as pixel size or intensity, can be more appropriate in some cases, and is the responsibility of the authors.

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DISCLOSURES:
The authors have nothing to disclose.

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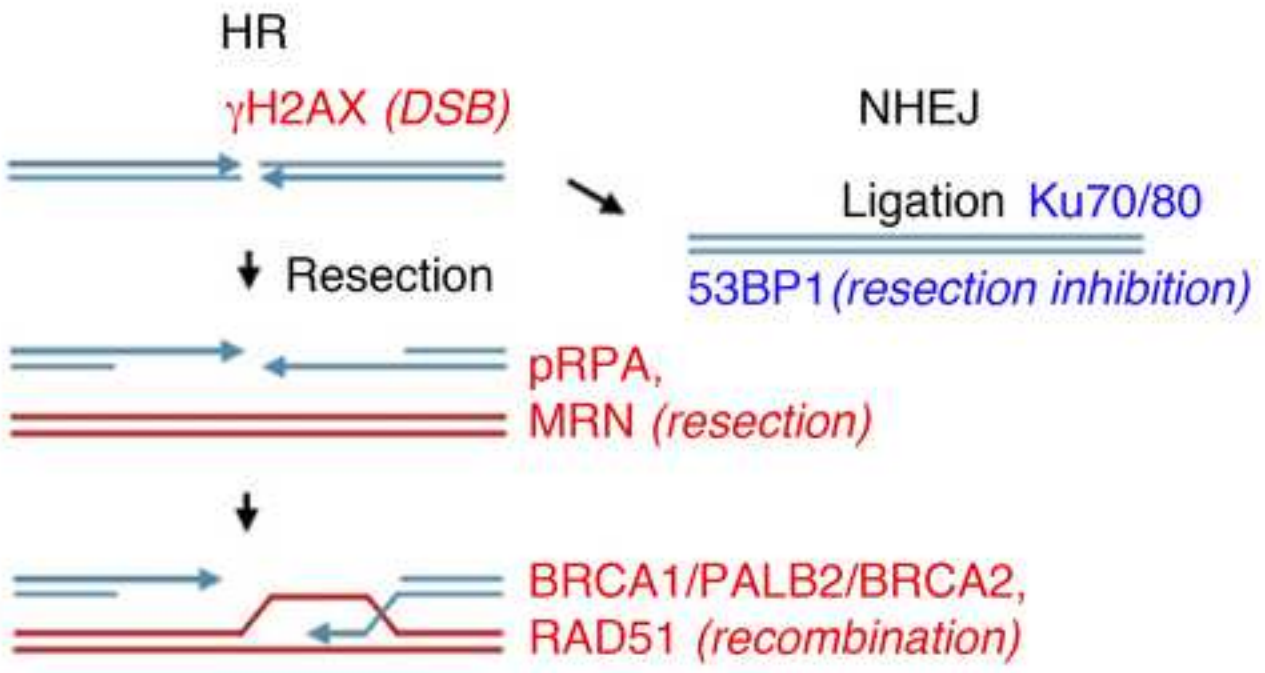
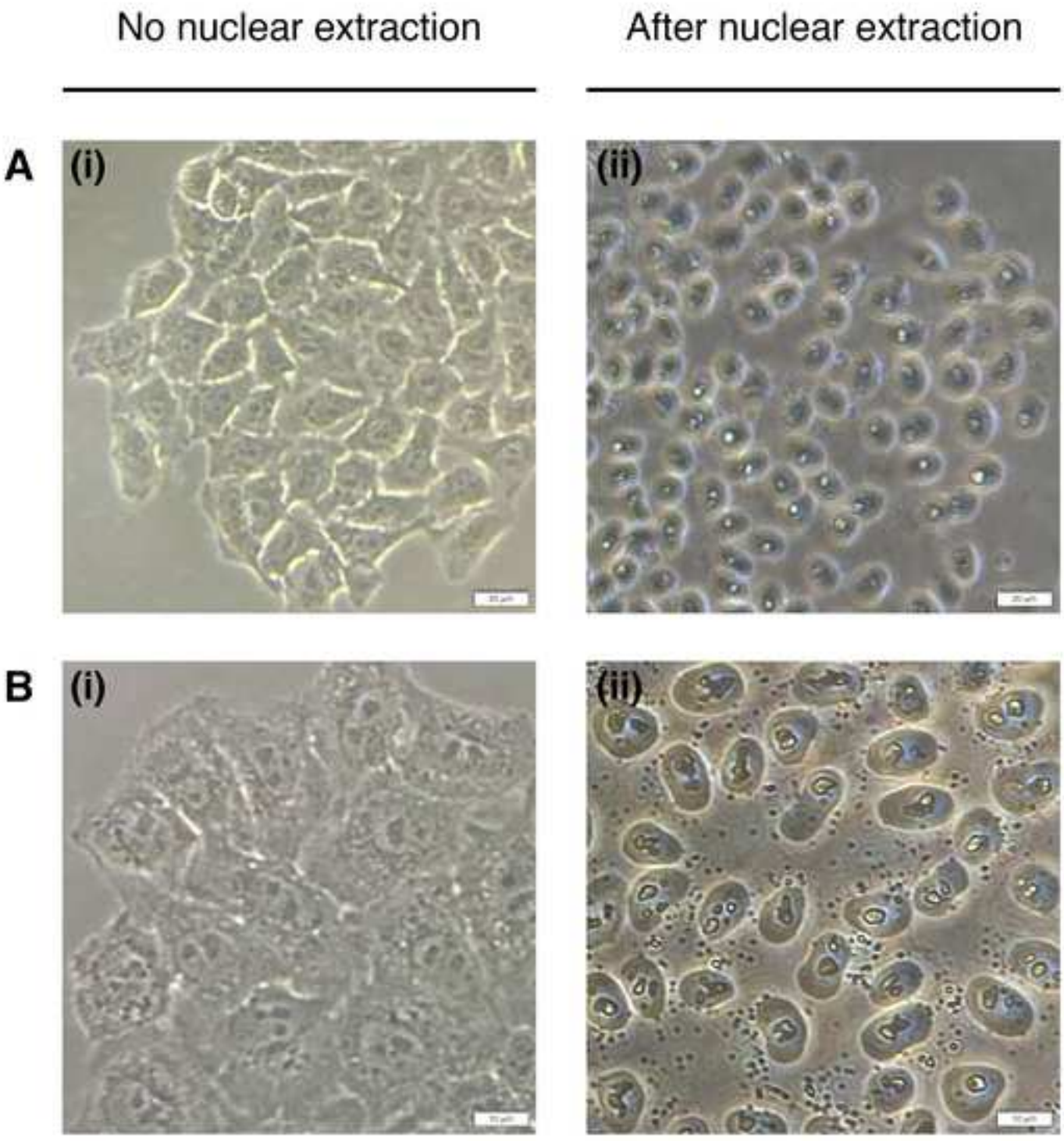
Figure 1_delaPena_2020



Figure

Click here to access/download;Figure;Figure 2 -before and after NEB- (final).psd

Figure 2_delaPena_2020



Figure

Click here to access/download;Figure;Figure 3.psd

Figure 3_delaPena_2020

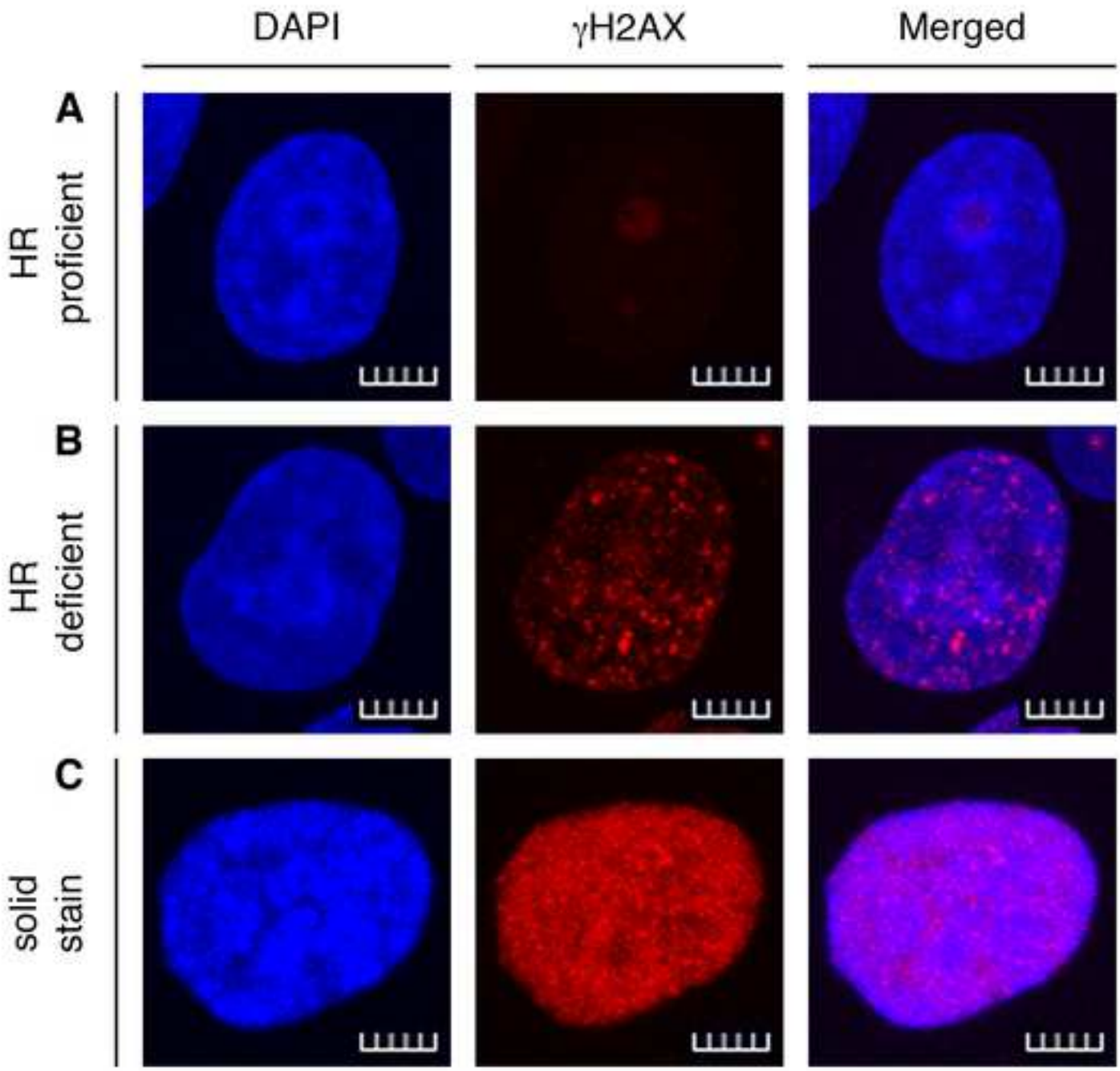


Figure 4_delaPena_2020

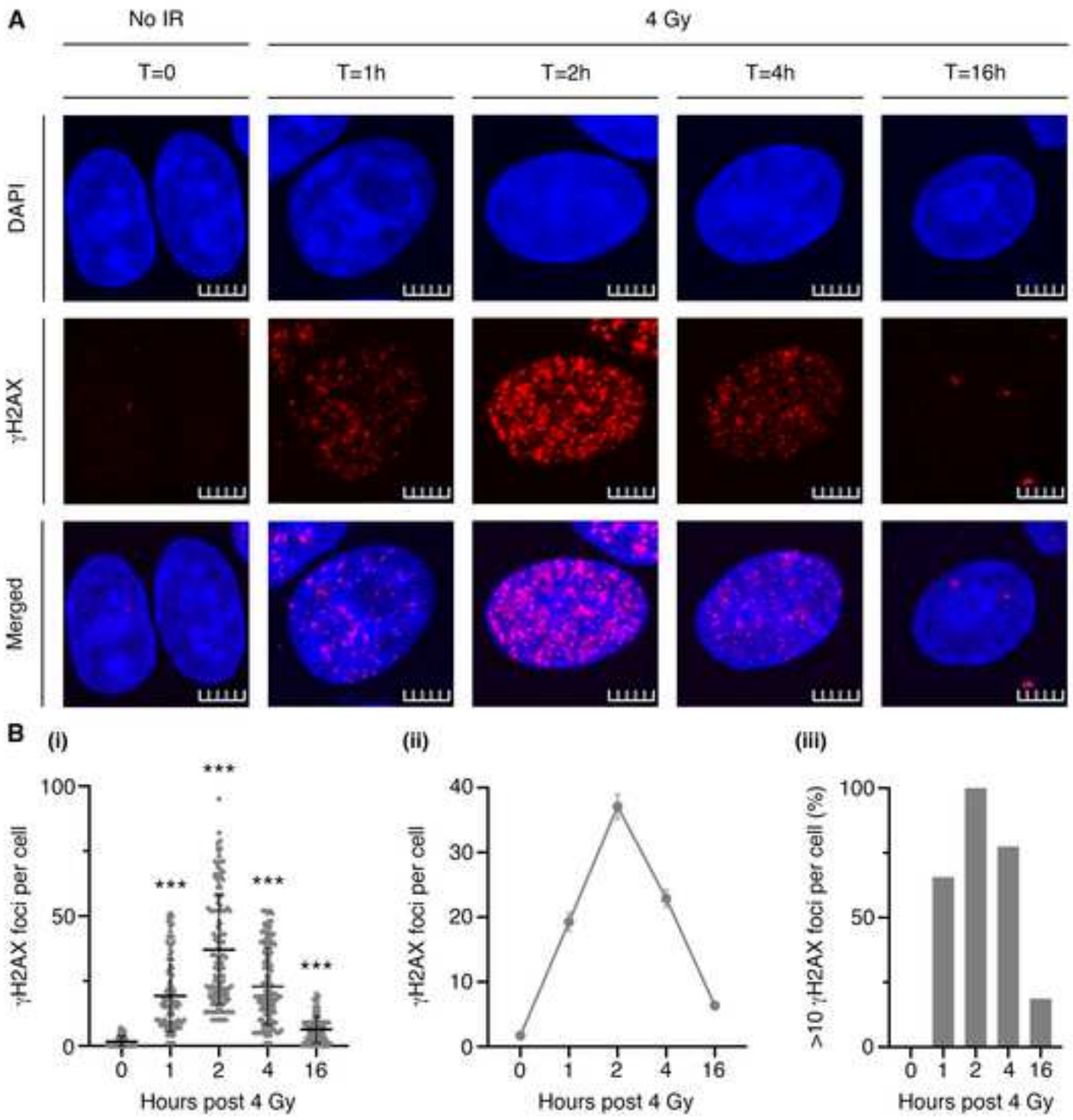
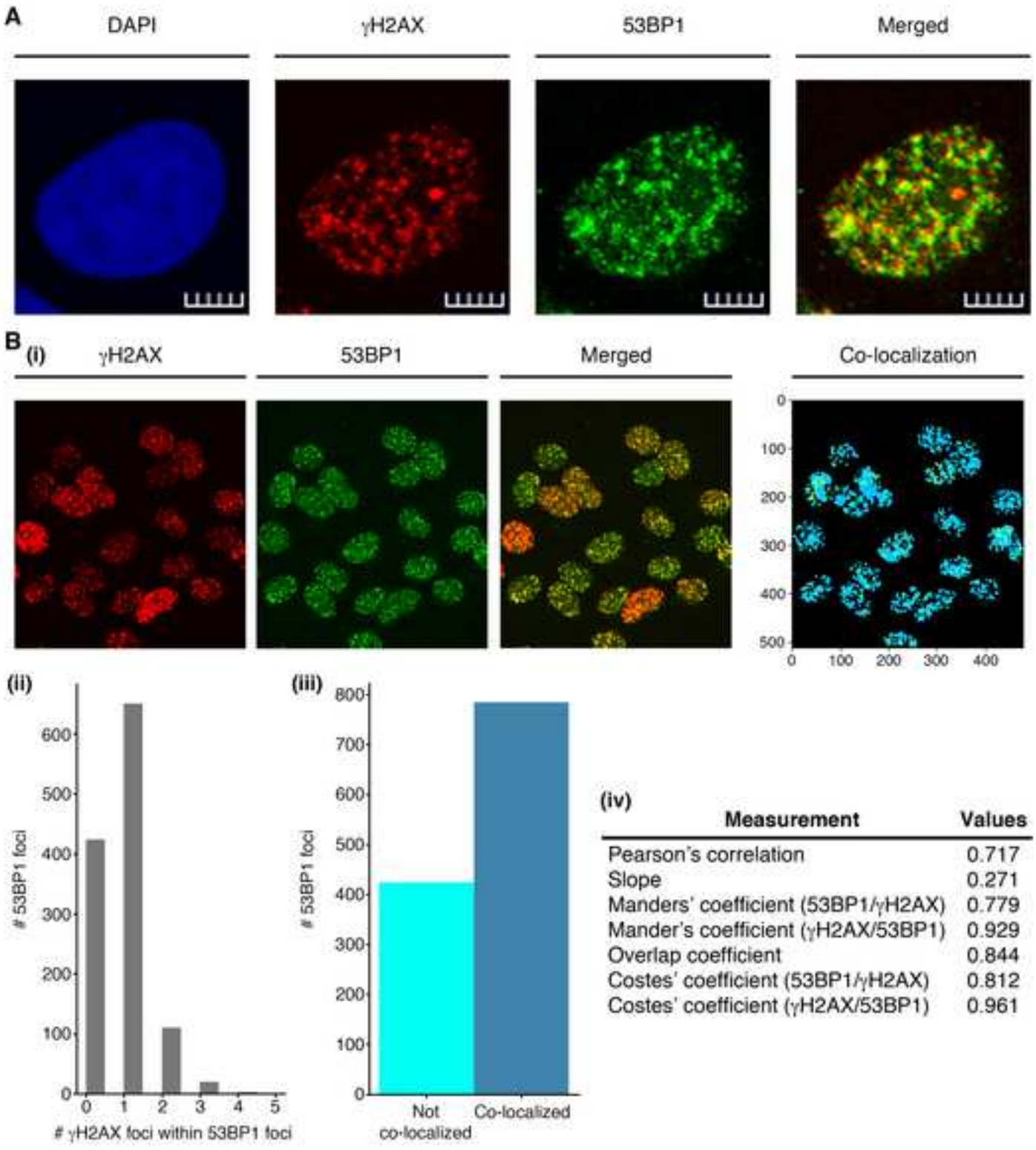


Figure 5_delaPena_2020



Measurement	Values
Pearson's correlation	0.717
Slope	0.271
Manders' coefficient (53BP1/γH2AX)	0.779
Mander's coefficient (γH2AX/53BP1)	0.929
Overlap coefficient	0.844
Costes' coefficient (53BP1/γH2AX)	0.812
Costes' coefficient (γH2AX/53BP1)	0.961

Table 1 Click here to access/download;Table;Table 1.xlsx

DNA damaging agent	Mechanism of action	Recommended dose
	Radiation	
γ-rays/X-rays	Formation of double-stranded breaks with some uncontrolled cellular effects	1-4 Gy
^{36}Ar ions	Radiation Formation of double-stranded breaks	270 keV/μm
α-particles	Radiation Formation of double-stranded breaks	116 keV/μm
Bleomycin	Inhibitor of DNA synthesis	0.4-2 μg/mL
Camptothecin	Inhibitor of topoisomerase I	10-200 nM
Cisplatin	Alkylating agent (inducing intrastrand crosslinks)	0.25-2 μM
Doxorubicin	Intercalating agent Inhibitor of topoisomerase II	10-200 nM
Etoposide	Inhibitor of topoisomerase II	10 μM
Hydroxyurea	Inhibitor of DNA synthesis (by ribonucleotide reductase)	10-200 μM
Methyl methanesulfonate	Alkylating agent	0.25-2 mM
Mitomycin C	Alkylating agent	0.25-2 μM
Ultraviolet (UV) light	Formation of thymidine dimers (generating distortion of DNA chain)	50-100 mJ/cm^2

Table 2

Antibody	Company	Reference
53BP1	Cell Signaling	4937
Anti-Mouse IgG H&L (Alexa Fluor 647)	Abcam	ab150103
Anti-phospho-Histone H2A.X (Ser139), clone JBW301	Millipore	05-636
Anti-Rabbit IgG H&L (Alexa Fluor 488)	Abcam	ab150081

Source
Rabbit
Donkey
Mouse
Goat

Name of reagent/material
16% (v/v) paraformaldehyde (PFA) aqueous solution
Bovine serum albumin (BSA)
Coverglass #1, 18 mm round (coverslips)
DMEM, High Glucose [(+) 4.5 g/L D-Glucose, (+) L-Glutamine, (-) Sodium Pyruvate]
DPBS, no calcium, no magnesium
Ethylene glycol-bis(β-aminoethyl ether)-N,N,N′,N′-tetraacetic acid (EGTA)
Fetal Bovine Serum (FBS)
Magnesium chloride ($MgCl_2$)
Piperazine-N,N′-bis(2-ethanesulfonic acid) (PIPES)
SlowFade Diamond Antifade Mountant with DAPI
Sodium chloride (NaCl)
Sucrose
Superfrost Plus Microscope Slides
TC-Treated Multiple Well Plates, size 12 wells
Triton X-100
TrypLE Express Enzyme (1X), No Phenol Red
Trypsin-EDTA (0.5%), No Phenol Red

Company	Catalog Number
Electron Microscopy Sciences	15710
Sigma-Aldrich	A3059
Neuvitro	NC0308920
Gibco	11965118
Gibco	14190144
Research Products International	E57060
Life Technologies	104370028
Research Products International	M24000
Research Products International	P40150
Invitrogen	S36973
Research Products International	S23020
Research Products International	S24060
Fisherbrand	1255015
Costar	3513
AmericanBio	AB02025
Gibco	12604021
Gibco	15400054

Comments/Description

Microscopy quality of the PFA ensures best images. If using "home-made PFA", filter prior to us
Heat-shock fraction is recommended, to avoid precipitation/background.
Coverslips need to be cleaned and sterilized prior using, with HCl or ethanol.
Adjust the growing media to the needs of cell line used.
PBS for tissue culture.
Nuclear extraction buffer.
The quality of FBS can be assessed by testing γH2AX foci formation. If traces of genotoxic endo
Nuclear extraction buffer.
Nuclear extraction buffer.
300 nM DAPI with VECTASHIELD Antifade Mounting Medium can be used instead.
Nuclear extraction buffer.
Nuclear extraction buffer.
Polysine Slides can be used instead.
Seeding on coverslips is done in 12-wells plate.
Nuclear extraction buffer.
Trypsin-EDTA can be used instead.
TrypLE can be used instead.

e.

ɔtoxin are present in the batch, γH2AX will be positive in the absence of stress.

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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