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# Immunofluorescence Imaging of DNA Damage and Repair Foci in Human Colon Cancer Cells

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Cover Letter

February, 28th, 2020

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Dr. Ronald Myers
Science Editor
*JOVE*

Dear Dr. Myers,

I am pleased to submit a protocol for the Tumor Microenvironment Methods Collection entitled 'Analysis of DNA damage and repair mechanisms by immunofluorescence technique after neutron mixed-beam irradiation used in boron neutron capture therapy in colon cancer cells' for consideration for publication in JOVE.

In this manuscript, we show that radiation-induced foci of repair proteins are useful markers for immunofluorescent detection of DNA repair and damage activated by boron neutron capture therapy (BNCT). Mechanisms of DNA repair activated by BNCT have not been fully determined. We present immunofluorescence assay in colon cancer cells after irradiation by the neutron-mixed beam.

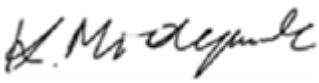
We believe that this manuscript is appropriate for publication by JOVE because it demonstrates immunofluorescence procedure which will allow biological effects at the cellular level in protein levels at DNA-double strand breaks sites not only in BNCT therapy but also could be useful in the evaluation of biological effects in other anti-cancer therapies. Moreover, the immunofluorescence method of detection of DNA damage response and DNA repair could be a general potential method for assessing tumor detection.

This manuscript has not been published and is not under consideration for publication elsewhere. We have no conflicts of interest to disclose.

Thank you for your consideration!

Sincerely,

Kamila Maliszewska-Olejniczak



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**TITLE:**

# Immunofluorescence Imaging of DNA Damage and Repair Foci in Human Colon Cancer Cells

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**KEYWORDS:**

BNCT, DNA-DSBs, radiation-induced foci, DNA damage, repair pathways, mixed-beam

**SUMMARY:**

Radiation-induced DNA damage response activated by neutron mixed-beam used in boron neutron capture therapy (BNCT) has not been fully established. This protocol provides a step-by-step procedure to detect radiation-induced foci (RIF) of repair proteins by immunofluorescence staining in human colon cancer cell lines after irradiation with the neutron-mixed beam.

**LONG ABSTRACT:**

The purpose of the manuscript is to provide a step-by-step protocol for performing immunofluorescence microscopy to study the radiation-induced DNA damage response induced by neutron-gamma mixed-beam used in boron neutron capture therapy (BNCT). Specifically, the proposed methodology is applied for the detection of repair proteins activation which can be visualized as foci using antibodies specific to DNA double-strand breaks (DNA-DSBs). DNA repair foci were assessed by immunofluorescence in colon cancer cells (HCT-116) after irradiation with the neutron-mixed beam. DNA-DSBs are the most genotoxic lesions and are repaired in mammalian cells by two major pathways: non-homologous end-joining pathway (NHEJ) and homologous recombination repair (HRR). The frequencies of foci, immunochemically stained, for commonly used markers in radiobiology like γ-H2AX, 53BP1 are associated with DNA-DSB number and are considered as efficient and sensitive markers for monitoring the induction and repair of DNA-DSBs. It was established that γ-H2AX foci attract repair proteins, leading to a higher concentration of repair factors near a DSB. To monitor DNA damage at the cellular level, immunofluorescence analysis for the presence of DNA-PKcs representative repair protein foci from the NHEJ pathway and Rad 52 from the HRR pathway was planned. We have developed and

introduced a reliable immunofluorescence staining protocol for the detection of radiation-induced DNA damage response with antibodies specific for repair factors from NHEJ and HRR pathways and observed radiation-induced foci (RIF). The proposed methodology can be used for investigating repair protein that is highly activated in the case of neutron-mixed beam radiation, thereby indicating the dominance of the repair pathway.

## INTRODUCTION:

Radiation-induced DNA damage response activated by neutron mixed-beam used in boron neutron capture therapy (BNCT) has not been fully determined. This protocol provides a step-by-step procedure to perform the detection of radiation-induced foci (RIF) of repair proteins by immunofluorescence staining e.g., in human colon cancer cell line after irradiation with the neutron-mixed beam.

Ionizing radiation (IR) induces a multitude of different types of DNA damage (DNA-DSBs, DNA-SSBs, DNA base damage) out of which DNA double-strand breaks are the most genotoxic DNA lesions[1]. Unrepaired breaks can cause cell death, while misrepaired breaks raises the probability of chromosome rearrangement, mutagenesis, and loss of decisive genetic information. The damage response pathways responding to DSBs include pathways of DNA repair like non-homologous end joining (NHEJ), a mechanism that requires Ku 70/80, DNA–PKcs, Xrcc4, and DNA ligase IV as key factors[2–6]. In mammals, the second main DNA repair pathway is homologous recombination repair (HRR) pathway which requires key components - the Rad52 epistasis gene family– Rad51, Rad52, Rad 54, Rad55, Rad57 and Rad 58[7]. Rad51 and Rad54 are key human recombination factors involved in repair mechanisms related to the replication stress and DNA breaks in eukaryotes. Interestingly, it was observed that the downregulation of the HRR pathway enhances the NHEJ pathway which is error-prone pointing at HR pathway relevance for the genome stability[8].

The first step of DSBs formation is the phosphorylation of the γ-H2AX histone at Ser-139 by Ataxia telangiectasia mutated kinase (ATM) from the PI3 kinase family[7,8]. Interestingly, H2AX phosphorylation can be visualized easily by immunofluorescence technique as foci (γ-H2AX foci) using antibodies specific for phosphorylated H2AX[6]. There is a 1:1 relationship between the number of DSBs and γ-H2AX foci, therefore, DSB marker, γ-H2AX, is studied extensively through the characterization of foci formation, size, and quantity[6-11]. Formation of γ-H2AX foci leads to the recruitment and accumulation of DNA damage response (DDR) proteins and chromatin-modifying factors, such as 53BP1 (p53 binding protein 1), MDC1 (mediator of DNA damage checkpoint), BRCA1, Mre11/Rad50/Nbs1, PARP-1, and many others repair factors thus forming radiation-induced foci (RIF). All these proteins co-localize with γ-H2AX through direct or indirect binding[1,11,12].

It is important to detect DNA damage and repair with a proper sensitive test, therefore, more attention is paid to the development of highly precise techniques[13]. In the context of DNA damage and repair studies, the methodology is crucial for sensitive detection of genome DNA damage, description of damage category, and quantification of DNA damage and repair mechanisms[13]. To detect single cells with damaged DNA, comet assay is used commonly in

radiobiological studies[14]. Other available cytogenetic methods recognize chromosomal aberrations, including dicentrics, translocations, acentric fragments, rings, and chromatid type aberrations, and micronucleus chromosomal damage (Mn). The most frequently used method in radiobiology, especially in biological dosimetry, is the dicentric chromosome assay due to its high specificity for radiation[15]. For example, PCR, a classic molecular method, cannot recognize the type of detected DNA damage. In this case, the immunometric methods pass the sensitivity level because reactions are specific between the antigen and the antibody. Immunofluorescence imaging provides visual evidence for the appearance of different proteins in distinct foci in response to DNA damaging agents like ionizing radiation[16]. However, the activation levels of damage and repair proteins' mRNA levels are easily detectable by real-time PCR which is a proper quantitative method for further molecular studies in the context of DNA damage response[17].

Taking into account that γ-H2AX foci attract repair factors[18], to monitor DNA damage and repair at the cellular level, we have developed a reliable immunofluorescence staining procedure, based on the analysis of the representative repair protein foci from the NHEJ pathway (DNA-PKcs) and Rad52 from the HRR pathway.

Here, we propose the use of immunodetection for these proteins as the efficient and sensitive procedure for monitoring DNA-DSB induction and repair. Up till now, there have been no available data on DNA-DSBs based on foci of repair proteins at the cellular level after the neutron mixed-beam irradiation for BNCT, except γ-H2AX and 53BP1 markers[19]. We suggest the adaptation of the HCT-116 colon cancer cell line, as it is rich itself in DSBs foci, as a standard cell line for DNA damage analysis, because RIFs are easily detectable. This adherent cell line is easy to maintain and proper for irradiation procedures. The proposed procedure is based on a huge amount of previous studies related to the general immunofluorescence procedure of γ-H2AX staining. However, it includes all details regarding the selection of appropriate antibodies with tested dilutions for each representative protein belonging to each repair pathway. Moreover, it describes the utilization of a unique neutron-mixed beam used in BNCT therapy. However, we recommend to extend the studies with both methods, immunofluorescence staining firstly and then, with high-cost molecular analysis as performed previously[4,17].

**PROTOCOL:**

## 1. Preparation of cell culture and experimental set-up

1.1. Maintain human colon cancer cells, HCT-116 as recommended by the supplier, in monolayers in 75 $cm^2$ culture flasks containing 10 mL of formulated McCoy's 5a Medium Modified, supplemented with 10% fetal bovine serum and 1% antibiotic antimycotic solution.

1.2. Grow cells in a humidified 5% $CO_2$ environment at 37 °C until 70% of confluency with medium renewal every 2 to 3 days.

1.3. To gain BNCT beam (e.g., neutron beam plus $^{10}$BPA) and BNCT effect of killing cancer cells as heavy particles and to deposit most of their energy within the boron-containing cancer cells,

the day before irradiation, add boron delivery agent to the cell culture medium – e.g., $^{10}$BPA, 4-Borono-L-phenylalanine (10 µg $^{10}$B/mL, 0.925 mM final) 12–16 h (maximum uptake of boron) before the irradiation, as previously studied for colon cancer cells and thyroid cancer cells[20,21].

NOTE: If using another cell line, for which no data exists in the case of BNCT study, test the boron uptake for each cell line to obtain optimal boron concentration. Measure the $^{10}$BPA cellular uptake by inductively coupled plasma optical emission spectroscopy (ICP-OES) as performed by Dagrosa group[21].

1.4. After 12-16 h of boron delivery, aspirate the medium and wash the cells with 10 mL of 1x PBS. Then trypsinize the cells by adding 2 mL of Trypsin-EDTA solution to the cells and incubate for 5 min at 37 °C to enhance the cell detachment. When cells start to detach, stop the trypsin action by adding 10 mL of complete medium.

1.5. Aspirate the cell suspension in the 15 mL tube and count the cells using an automated cell counter by adding 10 µL of 0.4% trypan blue to 10 µL of cells, mix, and aliquoting 10 µL on a counting slide. Dilute the cell suspension to a concentration of 1 x $10^6$ cells/mL of the medium. Aliquot 1 mL of the cell suspension per cryotube.

1.6. Prepare thermoluminescent dosimeters (TLDs) for the measurement of gamma-ray doses and gold foils for neutron fluencies and attach TLDs to the cryotubes or cell culture flask before the irradiation.

1.7. Irradiate the 1 x $10^6$ cells/mL with the neutron-mixed beam at recommended minimal thermal neutron flux of 5.9 x $10^{11}$ (n/cm$^{-2}$ min$^{-1}$)[4] in culture flasks or cryotubes depending on the facility capabilities.

NOTE: Set up the time of irradiation before to obtain recommended neutron flux.

1.8. After irradiation, seed 1 mL of irradiated cells (1 x $10^6$ cells/mL) on 22 x 22 mm coverslips in 35 mm Petri dishes or 6 well plates, supplement with 2 mL of required medium. Incubate cells for a maximum of 3 h at 37 °C in a humidified 5% $CO_2$ environment to let them attach to the coverslips. Observe the attachment of cells from time to time under an inverted microscope with 20x objective.

NOTE: For HCT-116 cells minimum density is 600,000 cells to seed. For quick further staining procedure use chamber slides, reduce cell density accordingly.

## 2. Fixation of cells

2.1. After irradiation and incubation of cells, remove the medium from the attached cells and wash cells once with 2.5 mL of PBS.

2.2. Fix cells with 1 mL of 70% ethanol for 10 min at RT.

NOTE: Alternatively use 1%-3.7% paraformaldehyde (PFA) for fixation as recommended previously[19]. The protocol is paused here. Store fixed cells in ethanol in a freezer at -20 °C for not more than a few weeks for further analysis and immunofluorescence staining.

## 3. Permeabilization of cells

3.1. After fixation remove ethanol from the Petri dish and wash with 2.5 mL of 1x PBS.

NOTE: Do not let the cells dry between the rinsing steps.

3.2. Gently add 1 mL of 0.2% of Triton X-100/PBS to cover the coverslips with cells in Petri dishes.

3.3. Incubate for 5 min at RT.

3.4. Wash the cells 3x with 2.5 mL of 1x PBS.

NOTE: Increase the percentage of Triton X-100 in PBS up to 0.5% if needed (more foci detectable, dependent on the tested cell line). Perform additional washes with PBST (PBS with 0.5% Tween) to avoid unspecific binding.

3.5. Block permeabilization step with 1 mL of 2% BSA (Bovine Serum Fraction V albumin) diluted in PBS and incubate for a minimum of 30 min or 1 h with 1% BSA.

NOTE: This step can be omitted and is dependent on the type of antibody used. Alternatively, use 5% FBS as recommended in ref[4].

## 4. Immunofluorescence staining

4.1. Add the proper amount of primary antibody (Anti-γ-H2AX, Anti-DNA-PKcs, and Anti-Rad52) diluted in PBS with BSA (2% Bovine Serum Fraction V albumin) as recommended (see **Table 1** with recommended dilutions) (100 µL is needed per sample).

[Place **Table 1** here]

4.2. Cover the Petri dish to maintain the humidity in a plastic box with moisturized lignin using distilled water and incubate for 30 min at 37 °C in the incubator.

NOTE: The protocol can be paused here. Incubation can be performed at 4 °C for overnight.

4.3. After incubation perform 3 washes with 2.5 mL of PBS.

NOTE: Do not allow the slide to dry during rinsing steps.

4.4. Add the proper amount of secondary antibody (anti-mouse IgG FITC, Goat Anti-Rabbit IgG (Alexa Fluor 488) diluted in PBS with BSA (see **Table 1**) (100 µL is needed per sample). For this and the following steps work in the dark.

4.5. Cover the Petri dish again to maintain the humidity in a plastic box with moisturized lignin and incubate for 30 min minimum at 37 °C in the incubator.

4.6. After incubation perform 3 washes with 2.5 mL of PBS.

4.7. Add 100 µL of DAPI diluted in PBS to a final concentration of 1 µg/mL to counterstain the nuclei.

4.8. Incubate shortly, for a maximum of 2 min at RT.

4.9. After incubation perform 3 washes with 2.5 mL of PBS.

4.10. Remove the PBS and gently put a coverslip on the top of the mounting medium, avoiding the formation of air bubbles and seal edges of the coverslip with nail polish. Wait until the varnish will dry and paint the coverslip around.

4.11. Wait for the hardening of the mounting medium (up to 3 h) before image analysis under fluorescence microscopy.

NOTE: The protocol can be paused here. Store glass slides in a plastic box at 4 °C in the dark.

## 5. Image acquisition and analysis

5.1. Acquire images with a fluorescence microscope under 100x objective with immersion oil.

5.1.1. Analyze foci in the nuclei with a fluorescence microscope equipped with the following filters for: Alexa Fluor 488: excitation⁄emission (nm): 496/519, emission color green; DAPI: excitation⁄emission (nm): 358⁄461, emission color blue.

NOTE: Analysis of DSBs along single-particle tracks may require advanced microscopy like confocal laser scanning microscopy with higher resolution.

5.2. Save acquired images and process with appropriate imaging software e.g., ImageJ or Image Pro[3,20].

NOTE: Use of individual macros and plugins developed for automatic analysis or development/purchase of individual bioinformatic software is recommended.

5.3. If using Image-Pro software for foci analysis, process images in TIFF files: select

**Count/Size** Button, then click **Types** [choose the type of measurement (e.g., diameter or area)], click **Ranges** [set range of the measurement], click **Split objects if necessary**. To adjust brightness, click **Bright** [adjust treshold]. Then click **Count** [red button].

5.4. To export data, click **Data Table | Statistics | Export to Excel.** In the spreadsheet software, draw graphs with the required statistical analysis.

**REPRESENTATIVE RESULTS:**

Firstly, we performed an analysis of a standard marker of detection of DNA-DSBs, γH2AX foci in colon cancer cells, non-radiated, and irradiated with the neutron-mixed beam. γ-H2AX foci appear as distinct fluorescent dots and show the formation of DNA-DSBs (as each γ-H2AX fluorescent dot represents a single DSB) (see **Figure 1**).

[Place **Figure 1** here].

Interestingly, in irradiated cells with the neutron-mixed beam (N+BPA) at a dose of approximately 2.6 Gy (1,33 Gy (γ) + 1,26 Gy (N)), we observed higher diameter levels of γ-H2AX foci (**Figure 1B**). Moreover, a single track of high LET α-particle was detected (because of BNCT nuclear reaction) crossing the nuclei of the cell (yellow arrow) (**Figure 1A**). Different types of radiation could cause different effects: the higher the LET radiation, the more complex DNA-DSBs, the greater the γ-H2AX foci and the higher area levels of repair proteins visible as radiation-induced foci[18].

Therefore, we tested levels of representative repair proteins of DNA-PKcs (from NHEJ repair pathway) and Rad52 (from HR pathway) by immunofluorescence microscopy in the same conditions, which was not performed before in the case of BNCT beam. We were able to detect the high mean value of the foci diameter of DNA-PKcs at DNA breaks after a neutron-mixed in comparison with control cells (no radiation) (see **Figure 2**).

[Place **Figure 2** here].

In this case, we have observed clustered DNA-DSBs in irradiated HCT-116 cells by the neutron-mixed beam as DNA-PKcs is specific for more complex foci. In irradiated cells, by neutron-mixed beam, these foci were observed only within the cell nucleus as more complex, larger, and clustered with higher intensity (**Figure 2A,B**). In the case of Rad52, the effect was not as strong as for DNA-PKcs which indicates the dominance of the NHEJ pathway in this type of radiation. Moreover, based on the literature data, complex DSBs are slowly repaired and DNA-PKcs is only recruited to longer-lived complex DSBs which indicates that the neutron-mixed beam leads to the formation of complex DSBs and repair through DNA-PKcs, however, more research is needed at a molecular level to confirm these previously obtained results[22].

**FIGURE AND TABLE LEGENDS:**

**Table 1: Recommended dilutions and notes for the usage of antibodies used in section 4.**

**Figure 1: Representative images of patterns of γ-H2AX foci in colon cancer cells of the cell line HCT-116 (without radiation) and after neutron-mixed beam irradiation.** Neutron-mixed beam irradiation was performed at a dose of 2.6 Gy in cells treated the day before with BPA (N+BPA). (**A**) The left panel represents the DAPI-staining of nuclei. The right panel, green foci (Alexa 488), corresponds to the immunodetection of γ-H2AX. The yellow arrow indicates track of α-particle crossing the nucleus (scale bar = 10 µm). (**B**) Representative diagrams illustrating the observed increase in the size of radio-induced foci. The mean of γ-H2AX foci diameter was performed automatically by Image-Pro software.

**Figure 2: Representative images of patterns of DNA-PKcs and Rad52 repair foci in colon cancer cells of the cell line HCT-116 (without radiation) and after neutron-mixed beam irradiation.** Neutron-mixed beam irradiation was performed at a dose of 2.6 Gy in cells treated the day before with BPA (N+BPA). (**A**) The left panel represents the DAPI-staining of nuclei. The middle panel represents the detection of radiation-induced foci. The right panel is the merged image. (**B**) Representative diagrams illustrating the observed increase in the size of radio-induced foci. The mean of Rad52 and DNA-PKcs foci diameter was performed automatically using the image analysis software.

**DISCUSSION:**

The frequencies of foci, immunochemically stained for γ-H2AX and 53BP1 are commonly used in radiobiology and are associated with DNA-DSB number and are considered as efficient and sensitive markers for monitoring the induction and repair of DNA-DSBs[19]. The co-staining procedure of γ-H2AX and 53BP1 is a standard procedure for the detection of DNA-DSBs. Formation of γ-H2AX foci is associated with the recruitment of 53BP1, a regulator of the DNA damage response[23]. However, different cell lines may vary in the background levels of γ-H2AX /53BP1 foci [11]. It has been shown that γ-H2AX foci attract repair factors[18], accumulating a higher concentration of repair proteins close to a DSB site. The more complex DNA-DSBs, the greater the γ-H2AX foci and the higher the levels of repair proteins. It activates a cascade of repair pathways, if repair factors are accumulating in a higher concentration in a DSB site, they are easily detectable using specific antibodies and the proposed protocol allows them to be visualized easily. Here we demonstrate a methodology to study the biological effects at the cellular level for BNCT therapy the impact of the neutron-mixed beam on DNA repair using immunofluorescence technique in human colon cancer cells. The authors developed and introduced step-by-step reliable protocol with specific antibodies for detecting DNA repair pathways based on immunofluorescent staining with an antibody specific for repair factors from NHEJ and HRR pathway and observed radiation-induced foci (RIF). Moreover, the authors propose the use of HCT-116 colon cancer cell line as a standard cell line for DNA damage analysis and as a control cell line for the test of DNA repair antibodies because this cell line is itself rich in DSBs foci. DNA-DSBs are easily detectable, and different cell lines, especially cancer cells like the cervical cell line represent different background levels of H2AX foci, and intensity[24].

There are two major critical steps in the protocol, fixation of cells, and immunostaining procedure. The authors recommend the fixation of cells in 70% ethanol as it preserves cells for a

long time, and it is incubated in the freezer not more than a few weeks. Also, this step is critical because this can be pause point after irradiation procedure is performed e.g., in another institution/building/another day. Another critical step is the proper concentration of the antibody. The authors have attached in the table the tested concentrations of primary and secondary antibodies.

The presented procedure provides a step-by-step protocol to obtain reliable results, however, some parameters could change the results of the experiments, like the proper choice of antibodies used, different reagents used for the fixation and permeabilization steps, time of incubations, critical percentage of reagents, washing steps, work in the dark, and the proper fluorescent microscope. The authors explain how to obtain reliable and repeatable results in the notes.

A minor limitation of this technique is to have access to the source of radiation like neutron source, however, the protocol can be used as a general protocol for the detection of DNA repair pathways obtained after different types of radiation and can be treated as universal protocol for various types of radiation e.g., comparing low-LET and high-LET radiation repair pathways activation.

We provide a universal, ready-to-use methodology that can be used at the cellular level to analyze biological effects in the context of BNCT therapy. In BNCT, non-radioactive boron-10 (e.g., after treatment with 4-Borono-L-phenylalanine, BPA – boron delivery agent) is irradiated with low‑energy thermal neutrons and as a result of the nuclear reaction alpha particles and lithium-7 nuclei with high LET are produced[25,26]. Therefore, our protocol can be useful for the analysis of biological effects at a cellular level also for the radiation represented by other high LET beams like protons used in proton beam therapy[27], and carbon-ions used in hadron therapy[28]. We have observed that more detailed research is needed in the field of high-LET radiation and mixed beams, and knowledge of the DNA repair process is required to develop effective anti-cancer therapies, therefore, we have developed a protocol that allows researching radiation-induced DNA damage response activated by the neutron-mixed beam. Moreover, the immunofluorescence method of detection of DNA damage response and DNA repair could be a potential method for assessing and detection of tumors.

**ACKNOWLEDGMENTS:**

Neutron-mixed beam composed of neutron/gamma radiation was accessed from the Maria research reactor in the National Centre for Nuclear Research in Poland. K.M.-O. was supported by the National Science Centre, Poland (Miniatura 2) grant no. #2018/02/X/NZ5/02849.

**DISCLOSURES:**

The authors have nothing to disclose.

**REFERENCES:**

1. Carter, R. J. et al. Complex DNA Damage Induced by High Linear Energy Transfer Alpha-Particles and Protons Triggers a Specific Cellular DNA Damage Response. *International Journal of*

*Radiation Oncology, Biology, Physics.* **100** (3), 776–784 (2018).
2. Kondo, N. et al. DNA damage induced by boron neutron capture therapy is partially repaired by DNA ligase IV. *Radiation Environmental Biophysics*. **55** (1), 89–94 (2016).
3. Sakai, W., Sugasawa, K. DNA Damage Recognition and Repair in Mammalian Global Genome Nucleotide Excision Repair. *DNA Replication, Recombination, and Repair: Molecular Mechanisms and Pathology*. Springer, Tokyo. 155-174 (2016).
4. Rodriguez, C. et al. In vitro studies of DNA damage and repair mechanisms induced by BNCT in a poorly differentiated thyroid carcinoma cell line. *Radiation Environmental Biophysics*. **57** (2), 143–152 (2018).
5. Sollazzo, A. et al. Live Dynamics of 53BP1 Foci Following Simultaneous Induction of Clustered and Dispersed DNA Damage in U2OS Cells. *International Journal of Molecular Sciences.* **19** (2) (2018).
6. Jeggo, P., Löbrich, M. Radiation-induced DNA damage responses. *Radiation Protection Dosimetry*. **122** (1–4), 124–127 (2006).
7. Sigurdsson, S., Van Komen, S., Petukhova, G., Sung, P. Homologous DNA pairing by human recombination factors Rad51 and Rad54. *The Journal of Biological Chemistry.* **277** (45), 42790–42794 (2002).
8. Choi, E. H., Yoon, S., Hahn, Y., Kim, K. P. Cellular Dynamics of Rad51 and Rad54 in Response to Postreplicative Stress and DNA Damage in HeLa Cells. *Molecules and Cells*. **40** (2), 143–150 (2017).
9. Burma, S., Chen, B. P., Murphy, M., Kurimasa, A., Chen, D. J. ATM phosphorylates histone H2AX in response to DNA double-strand breaks. *The Journal of Biological Chemistry.* **276** (45), 42462–42467 (2001).
10. Nakamura, T. M., Du, L. L., Redon, C., Russell, P. Histone H2A phosphorylation controls Crb2 recruitment at DNA breaks, maintains checkpoint arrest, and influences DNA repair in fission yeast. *Molecular and Cellular Biology.* **24** (14), 6215–6230 (2004).
11. Kuo, L. J., Yang, L. X. Gamma-H2AX - a novel biomarker for DNA double-strand breaks. *In Vivo*. **22** (3), 305–309 (2008).
12. Sage, E., Shikazono, N. Radiation-induced clustered DNA lesions: Repair and mutagenesis. *Free Radical Biology and Medicine.* **107**, 125–135 (2017).
13. Boguszewska, K., Szewczuk, M., Urbaniak, S., Karwowski, B. T. Review: immunoassays in DNA damage and instability detection. *Cellular and Molecular Life Sciences.* **76** (23), 4689–4704 (2019).
14. Møller, P. et al. Potassium bromate as positive assay control for the Fpg-modified comet assay. *Mutagenesis.* geaa011 (2020).
15. Sommer, S., Buraczewska, I., Kruszewski, M. Micronucleus Assay: The State of Art, and Future Directions. *International Journal of Molecular Sciences.* **21** (4), e1534, (2020).
16. Bennett, B.T., Bewersdorf, J., Knight, K. L. Immunofluorescence imaging of DNA damage response proteins: optimizing protocols for super-resolution microscopy. *Methods*. **48** (1), 63–71 (2009).
17. Cheng, L. et al. Simultaneous induction of dispersed and clustered DNA lesions compromises DNA damage response in human peripheral blood lymphocytes. *PLoS One*. **13** (10), e0204068 (2018).
18. Paull, T. T. et al. A critical role for histone H2AX in recruitment of repair factors to nuclear foci after DNA damage. *Current Biology.* **10** (15), 886–895 (2000).

19. Okumura, K. et al. Relative biological effects of neutron mixed-beam irradiation for boron neutron capture therapy on cell survival and DNA double-strand breaks in cultured mammalian cells. *Journal of Radiation Research.* **54** (1), 70–75 (2013).
20. Dagrosa, M.A. et al. First evaluation of the biologic effectiveness factors of boron neutron capture therapy (BNCT) in a human colon carcinoma cell line. *International Journal of Radiation Oncology, Biology, Physics.***79** (1), 262–268 (2011).
21. Dagrosa, A. et al. Studies for the application of boron neutron capture therapy to the treatment of differentiated thyroid cancer. *Applied Radiation and Isotopes.* **69** (12), 1752–1755 (2011).
22. Reynolds, P. et al. The dynamics of Ku70/80 and DNA-PKcs at DSBs induced by ionizing radiation is dependent on the complexity of damage. *Nucleic Acids Research.* **40** (21), 10821–10831 (2012).
23. Rasche, L. et al. Analysis of Lymphocytic DNA Damage in Early Multiple Sclerosis by Automated Gamma-H2AX and 53BP1 Foci Detection: A Case Control Study. *PLoS One*. **11** (1), e0147968 (2016).
24. Banáth, J. P., Macphail, S. H., Olive, P.L. Radiation sensitivity, H2AX phosphorylation, and kinetics of repair of DNA strand breaks in irradiated cervical cancer cell lines. *Cancer Research.* **64** (19), 7144–714 9 (2004).
25. Barth, R. F. et al. Current status of boron neutron capture therapy of high grade gliomas and recurrent head and neck cancer. *Radiatiation Oncology*. **7**, 146 (2012).
26. Mirzaei, H. R. et al. Boron neutron capture therapy: Moving toward targeted cancer therapy. *Journal of Cancer Research and Therapeutics.* **12** (2), 520–525 (2016).
27. Vitti, E. T., Parsons, J. L. The Radiobiological Effects of Proton Beam Therapy: Impact on DNA Damage and Repair. *Cancers (Basel)*. **11** (7), E946 (2019).
28. Mohamad, O. et al. Carbon Ion Radiotherapy: A Review of Clinical Experiences and Preclinical Research, with an Emphasis on DNA Damage/Repair. *Cancers (Basel)*. **9** (6), E66 (2017).

Figure

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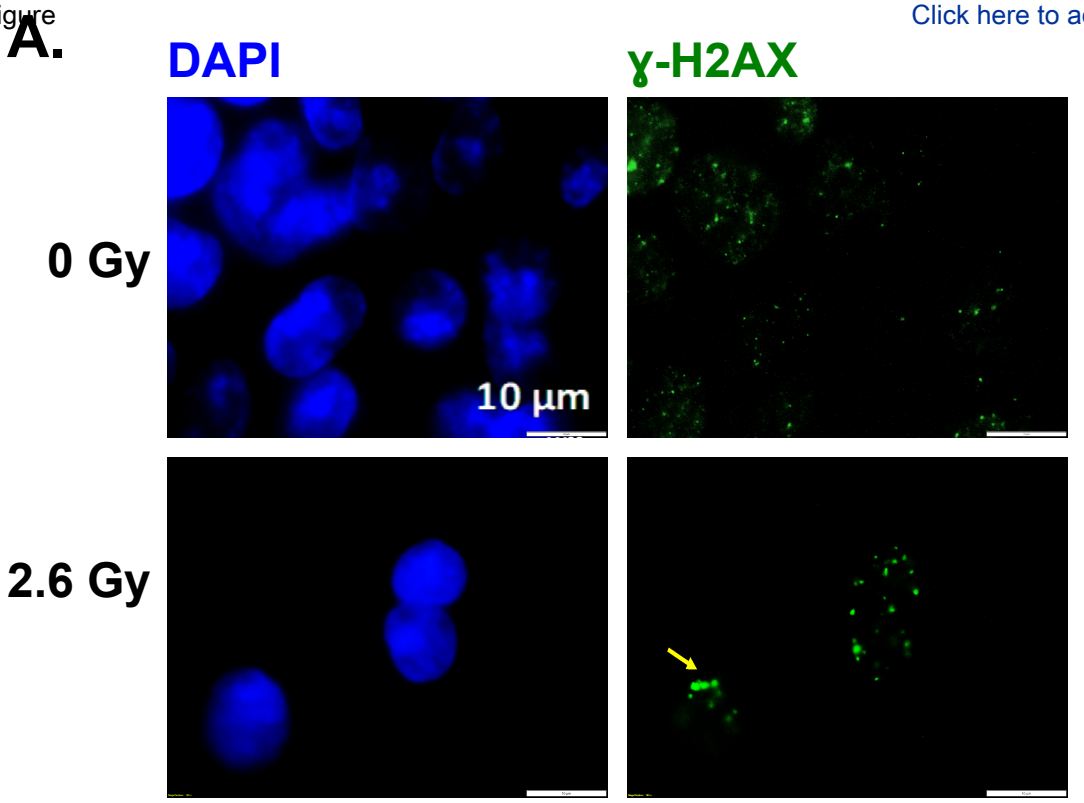
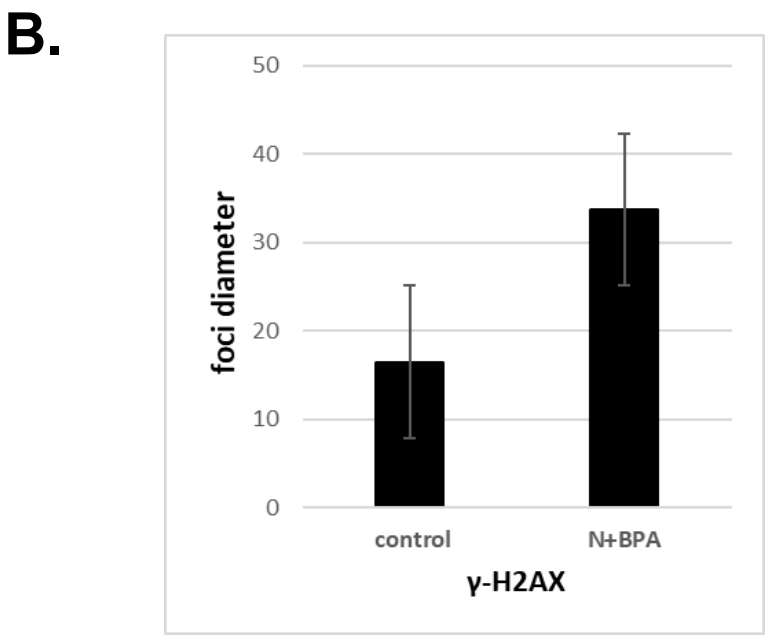
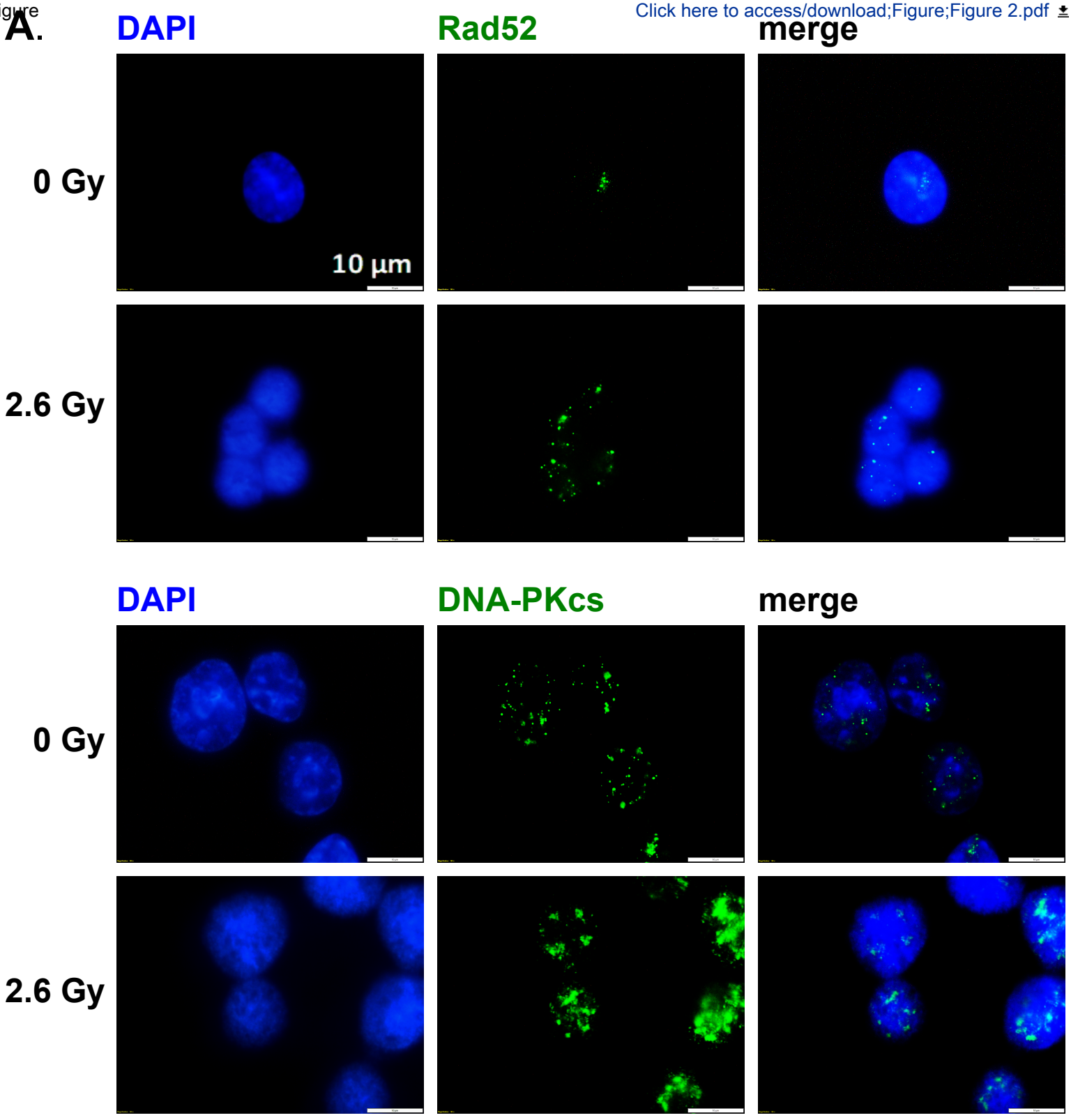


Figure

Click here to access/download;Figure;Figure 2.pdf



| **Primary antibody** |
| --- |
| Anti-ɣ-H2AX |
| Anti-DNA-PKcs |
| Anti-Rad52 |
| **Secondary antibody** |
| anti-mouse IgG FITC |
| Goat Anti-Rabbit IgG H&L (Alexa Fluor 488) |

| Function | Recommended dilution |
|---|---|
| detection of DNA-DSBs | 1:1000 (PBS-BSA) |
| detection of RIFs of DNA-PKcs protein belonging to NHEJ | 1:200 (PBS-BSA) |
| detection of RIFs of Rad52 protein belonging to HRR | 1:200 (PBS-BSA) |
| | |
| γ-H2AX foci | 1:400 (PBS-BSA) |
| for NHEJ and HRR repair proteins foci | 1:500 (PBS-BSA) |

| storage [°C] |
| --- |
| 4 |
| -20 |
| -20 |
| **in the dark** |
| 4 |
| -20 |

**Name of Material/ Equipment**

12 mm Coverslips

35 mm Petri dishes

4-Borono-L-phenylalanine

Antibiotic-Antimycotic (100X)

Anti-DNA PKcs (phospho S2056) antibody - ChIP Grad

Anti-Mouse IgG (whole molecule)–FITC antibody produced in goat

Anti-phospho-Histone H2A.X (Ser139) Antibody, clone JBW301

Anti-RAD52 antibody

Bovine Serum Albumin Fraction V (BSA)

DAPI (4',6-Diamidino-2-Phenylindole, Dihydrochloride)

Fetal Bovine Serum (Heat Inactivated)

Goat Anti-Rabbit IgG H&L (Alexa Fluor 488)

HCT-116 cell line

ImageJ

Image Pro

LUNA II Automated Cell Counter

McCoy's 5A Medium (Modified, with L-glutamine and sodium bicarbonate)

microscope slides

Phosphate Buffered Saline (PBS)

Triton X-100

Trypan Blue Stain, 0.4%

Trypsin-EDTA solution 0.25%

| Company | Catalog Number |
|---|---|
| VWR | 89015-725 |
| Sarstedt | 7.183.390.000 |
| SIGMA-ALDRICH | 17755 |
| Gibco | 15240062 |
| Abcam | AB18192 |
| SIGMA-ALDRICH | F0257 |
| MerckMillipore | 05-636 |
| Abcam | AB117097 |
| Roche | BSAV-RO |
| ThermoFisher SCIENTIFIC | D1306 |
| SIGMA-ALDRICH | F9665 |
| Abcam | AB150077 |
| ATCC | CCL-247™ |
| National Institute of Health (NIH) | https://imagej.nih.gov/ij/ |
| Media cybernetics | http://www.mediacy.com/imagepro |
| Logos Biosystems | L40002 |
| SIGMA-ALDRICH | M9309 |
| ThermoFisher SCIENTIFIC | B-1198 |
| Hirszfeld Institute of Immunology and Experimental Therapy, PAS | 20.59.52.0 |
| SIGMA-ALDRICH | X100 |
| Logos Biosystems | T13001 |

**Comments/Description**

**Response to the Editorial's revise:**

**1. The editor has formatted the manuscript to match the journal's style. Please retain and use the attached version for revision.**

At the editor's request, the author retained and used the attached version.

**2. Please address all the specific comments marked in the manuscript.**

a) To address the Editor's request, the author has checked and reworded the title. We propose the final title: '*Immunofluorescence Imaging of DNA Damage and Repair Foci Induced by Neutron Mixed-Beam in Human Colon Cancer Cells*'.
b) The author removed the information about the HCT-116 cell line about the deletion of the mismatch repair gene. It is not significant in this case.
c) We agree with the Editor's comment about the Introduction – we added significance and rationale for using the immunostaining method over other methods. The author reworded the Introduction:
   - The author added missing citations.
   - At the Editor's request, the author added to the Introduction section different detection methods presented in the literature.
   - To address the Editor's request, the author moved part of the text from the discussion to the introduction, and from the introduction to the discussion.
   - The author included the missing information in the Introduction section about DNA DSB and repair and the explanation of why the HCT-116 cell line was chosen.
d) We cannot agree that Steps 1.3 and 1.7 are important for filming purposes because we do not propose it as the main crux of the protocol in JOVE Tumor Microenvironment Methods Collection. The main crux of the protocol is the preparation of the cancer cells after irradiation – whole immunostaining procedure with the following steps: seeding after irradiation, fixation, permeabilization, staining, and visualization of radiation-induced foci. The second argument for this is that we cannot film the irradiation procedure in the reactor because, firstly, the whole irradiation procedure takes place inside the reactor hall at the core. Due to the risk of contamination, we cannot allow unauthorized persons without appropriate training including the JOVE crew. Moreover, some procedures in the nuclear reactor are secret. Finally, the procedure for performing a full experiment takes 3 days, so even if we had another source of radiation available directly in the laboratory, it could not be filmed in one business day.
e) For thermal neutron flux, we used the same units: $n/cm^2\ min^{-1}$ as in the published work of Rodriguez et al. 2018 mentioned in the protocol. We agree with the Editor that there is a mistake and we corrected as $n/cm^{-2}\ min^{-1}$.
f) To address the Editor's request, we expanded the journal-title in the Bibliography section and added volume and pages where necessary.

**3. Please ensure that there is a minimum of 1 page of highlighting. Also please ensure that the highlighted text reflects the crux of the manuscript.**

The author confirms that the highlighted text is of 1.5-page volume. The highlighted text reflects the crux of the manuscript – the procedure of immunostaining of the cells after irradiation.

**4. Please copyedit and proofread well before submission.**

At the editor's request, the author proofread the manuscript before submission.

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**Supplemental Coding Files**

Figure 1.svg