Video Article

Detection and Visualization of DNA Damage-induced Protein Complexes in Suspension Cell Cultures Using the Proximity Ligation Assay

Mahnoush Bahjat[1], Timon A. Bloedjes[1], Amélie van der Veen[1], Guus de Wilde[1], Chiel Maas[1], Jeroen E. J. Guikema[1]

[1]Department of Pathology, Academic Medical Center, University of Amsterdam, Lymphoma and Myeloma Center Amsterdam (LYMMCARE)

Correspondence to: Jeroen E. J. Guikema at j.e.guikema@amc.uva.nl

URL: https://www.jove.com/video/55703
DOI: doi:10.3791/55703

Keywords: Biochemistry, Issue 124, DNA damage, DNA repair, ATM, p53, suspension cell culture, BCR-ABL+ B-cell acute lymphoblastic leukemia, protein-protein interaction, *in situ* proximity ligation assay

Date Published: 6/9/2017

Citation: Bahjat, M., Bloedjes, T.A., van der Veen, A., de Wilde, G., Maas, C., Guikema, J.E. Detection and Visualization of DNA Damage-induced Protein Complexes in Suspension Cell Cultures Using the Proximity Ligation Assay. *J. Vis. Exp.* (124), e55703, doi:10.3791/55703 (2017).

Abstract

The DNA damage response orchestrates the repair of DNA lesions that occur spontaneously, are caused by genotoxic stress, or appear in the context of programmed DNA breaks in lymphocytes. The Ataxia-Telangiectasia Mutated kinase (ATM), ATM- and Rad3-Related kinase (ATR) and the catalytic subunit of DNA-dependent Protein Kinase (DNA-PKcs) are among the first that are activated upon induction of DNA damage, and are central regulators of a network that controls DNA repair, apoptosis and cell survival. As part of a tumor-suppressive pathway, ATM and ATR activate p53 through phosphorylation, thereby regulating the transcriptional activity of p53. DNA damage also results in the formation of so-called ionizing radiation-induced foci (IRIF) that represent complexes of DNA damage sensor and repair proteins that accumulate at the sites of DNA damage, which are visualized by fluorescence microscopy. Co-localization of proteins in IRIFs, however, does not necessarily imply direct protein-protein interactions, as the resolution of fluorescence microscopy is limited.

In situ Proximity Ligation Assay (PLA) is a novel technique that allows the direct visualization of protein-protein interactions in cells and tissues with unprecedented specificity and sensitivity. This technique is based on the spatial proximity of specific antibodies binding to the proteins of interest. When the interrogated proteins are within ~40 nm an amplification reaction is triggered by oligonucleotides that are conjugated to the antibodies, and the amplification product is visualized by fluorescent labeling, yielding a signal that corresponds to the subcellular location of the interacting proteins. Using the established functional interaction between ATM and p53 as an example, it is demonstrated here how PLA can be used in suspension cell cultures to study the direct interactions between proteins that are integral parts of the DNA damage response.

Video Link

The video component of this article can be found at https://www.jove.com/video/55703/

Introduction

DNA damage triggers a highly regulated series of events involving protein-protein interactions and post-translational modifications that ensures the efficient and rapid repair of DNA, thereby safeguarding genomic integrity[1]. Typically, DNA repair is studied in cells exposed to ionizing radiation by monitoring the formation of so-called ionizing radiation-induced foci (IRIF) by (confocal) fluorescence microscopy. Many DNA repair and DNA damage-sensing proteins form IRIFs, which represent protein complexes that nucleate at chromatin sites sustaining DNA damage[2,3]. The location and resolution of IRIFs over time gives important insight into spatiotemporal organization of DNA repair, and may indicate the involvement of different DNA repair pathways. The nature of the DNA damage and the cell-cycle stage in which the damage is attained determines which DNA repair pathway is activated. For instance, in cells actively engaged in DNA replication (S-phase), homologous recombination (HR) is the dominant DNA repair pathway, whereas in cells in the G1- or G2/M-phase of the cell-cycle, the non-homologous end-joining (NHEJ) repair pathway predominates. One of the earliest events following DNA damage is the activation of the DNA damage-sensing kinases Ataxia Telangiectasia-Mutated protein (ATM), which is mostly active in the G1- and G2/M-phases of the cell-cycle and regulates NHEJ, and Ataxia Telangiectasia and Rad3-related protein (ATR), which acts in S-phase by activating HR. Both ATM and ATR are very pleiotropic kinases that phosphorylate many proteins that are involved in DNA repair, cell death and survival[4]. Both kinases have been shown to phosphorylate and activate the tumor-suppressor protein p53 following the exposure to genotoxic stress, indicating that these kinases are upstream mediators of a pivotal tumor suppressive signaling axis[5,6].

The formation and composition of IRIFs is typically assessed by determining co-localization of different proteins using dual-color immunofluorescence staining and microscopy, however, not all proteins that are part of repair protein complexes form IRIFs, which limits the applicability of this approach. Moreover, (confocal) immunofluorescence microscopy is limited by the diffraction properties of light, resulting in a rather poor spatial resolution of about 200-300 nm, exceeding the size of most subcellular structures, which essentially prohibits the direct interrogation of protein-protein interactions at the molecular level. As such, the co-localization of immunofluorescence staining patterns as detected by (confocal) fluorescence microscopy is not necessarily indicative for direct protein-protein interactions. Recently, new super-resolution technologies have been developed, such as three-dimensional structured illumination microscopy (3D-SIM)[7], which was successfully used to

study 53BP1 and BRCA1 IRIF formation at nano-scale detail, revealing the spatial distribution characteristics of these proteins that could not detected by confocal laser scanning microscopy[8].

Several other methods can be used to detect protein-protein interactions *in vivo*, such as co-immunoprecipitation, pull-down methods and yeast two-hybrid screening approaches. However, these techniques are rather cumbersome, require large amounts of cells or proteins or involve overexpression of proteins, which introduces experimental artifacts. More recently, a novel technique has been developed that allows the visualization and quantification of protein-protein interactions *in situ* (*i.e.* in cells and in tissues), which is termed Proximity Ligation Assay (PLA)[9,10]. Primary antibodies that recognize two proteins of interest are detected by secondary antibodies that are conjugated to oligonucleotides (so-called PLA probes). If the two different secondary antibodies are sufficiently close due to interactions between the proteins recognized by the primary antibodies, the conjugated oligonucleotides hybridize and can be ligated to form a closed circular DNA substrate. This circular substrate is subsequently amplified by rolling circle amplification, and visualized with fluorochrome-conjugated complementary oligonucleotides. Using PLA, the subcellular localization of the protein-protein interaction is preserved as the fluorescently labeled rolling circle amplification-product remains attached to the PLA probes. The resolution of this assay is <50 nm, based on the finding that the diameter of an antibody is approximately 7-10 nm[11]. Rolling circle amplification can only take place in case two pairs of antibodies (primary + secondary) physically interact within the perimeter that is defined by their size (10 + 10 + 10 + 10 = 40 nm). The signal amplification step increases the sensitivity of the PLA assay and enables the detection of interactions of scarcely expressed proteins. PLA generates punctate, foci-like signals patterns that can be quantified on a per cell basis, by which the intra- and inter-cellular variation in protein-protein interactions can be assessed.

The formation and composition of DNA repair complexes and IRIFs is mostly studied in adherent cell lines such as the human bone osteosarcoma epithelial cell line U2OS, the human embryonic kidney cell line HEK293 and the retinal pigment epithelial cell line RPE-1, which are fast-growing and easy to transfect. Suspension cell cultures such a lymphoid and myeloid cell lines are used less frequently, as these are less amenable to transfection and generally do not adhere to coverslips, thus requiring additional/alternative steps for imaging. The resolution of DNA damage is however very relevant in the context of lymphoid and myeloid malignancies, as the DNA damage response is frequently affected by genomic (driver) aberrations in these tumors, playing a pivotal role in the malignant transformation of normal lymphoid and myeloid (progenitor) cells[12,13,14].

This protocol describes how PLA can be used to assess and quantify protein-protein interactions following the induction of DNA damage in suspension cell cultures. Here, PLA is performed to determine and visualize the interactions between ATM and p53 upon DNA damage in human B-cell leukemia cells that are induced to undergo a G1-phase cell-cycle arrest. Of note, the protocol presented here is not restricted to studying ATM and p53 interactions in G1-arrested leukemia cells, but can also be used to visualize other protein-protein interactions in various cell types and suspension cell cultures.

Protocol

1. Treatment of Cells and DNA Damage Induction

1. Culture the human BCR-ABL+ B-cell acute lymphoblastic cell lines BV173 or SUP-B15 in IMDM supplemented with 20% FCS, 50 µM β-mercaptoethanol, 2 mM L-glutamine, 100 U/mL penicillin and 100 µg/mL streptomycin at 37 °C in an atmosphere of 5% CO_2. Count cells and plate at 2 x 10^6 cell/mL in 6-wells plates, at 5 mL/well.
   NOTE: Optionally, suspension cell lines of various origin can used.
2. **To arrest the BCR-ABL+ B-ALL cells in the G1-phase of the cell-cycle, add 5 µM of imatinib methanesulfonate (STI571), and incubate overnight.**
   1. Optionally, omit this step when studying protein-protein interactions throughout the cell-cycle or in BCR-ABL-negative cell types.
3. **Induce DNA damage by adding 50 ng/mL neocarzinostatin (NCS) to the culture, and incubate for 2 h.**
   1. Alternatively, induce DNA damage by irradiating the cells using a radioactive source [^{137}Cs] at 0.5 Gy/min, at a dose of 5 Gy. After irradiation, let the cell recover for 2 h at 37 °C in an atmosphere of 5% CO_2.
4. Optionally, to assess the involvement of ATM kinase activity in response to DNA damage, add 5 µM of the ATM kinase inhibitor KU55933 prior to addition of NCS or the irradiation treatment.
5. Prepare 1x PBS + 10% BSA by layering 5 g of fraction-V BSA on top of 50 mL of 1x PBS in a beaker or Erlenmeyer flask, and let sit at RT until BSA is dissolved. Do not shake or stir as this will cause extensive foaming. Slowly filter through a 0.22 µm filter and keep on ice.
6. Harvest cells and wash twice with ice-cold 1x PBS. Count the cells and resuspend at 2 x 10^6/mL in 1x PBS + 10% BSA. Keep cells on ice.

2. Cytocentrifugation, Fixation and Permeabilization

1. Prepare the 4% paraformaldehyde solution in 1x PBS freshly. Add 2 g of PFA to 50 mL of 1x PBS and incubate in a 55 °C shaking water bath until the solution is clear. Filter the solution through a 0.22 µm filter and store at RT until use.
   NOTE: Optionally, 4% PFA fixative can be stored frozen at -20 °C. After freezing, thaw only once for use. Otherwise, the 4% PFA solution in PBS can be purchased directly.
2. Carefully polish 76 mm x 26 mm microscopy slides with lint-free tissue and degrease them by placing in 96% ethanol in a Coplin jar for 5 min. Let the microscopy slides air-dry for 10 min.
3. Assemble microscopy slides into the cytospin/cytology funnels with an area of 6 mm. Pre-coat slides by pipetting 50 µL of 1x PBS + 10% BSA into each funnel and centrifuge for 1 min at 500 rpm.
4. Add 100 µL of the cell-suspension (equaling 0.2 x 10^6 cells) to each funnel and centrifuge for 5 min at 500 rpm. For each antibody combination, at least 4 preparations are required (dual-antibody experiment; two single-antibody controls; no antibodies control).
5. **Carefully disassemble the funnels, and make sure not to touch the spots. Proceed to sample fixation immediately.**

1. Optionally, let slides air-dry for at least 30 min. After air-drying, wrap slides individually in aluminum foil and store at -20 °C until use. When using frozen preparations, make sure to thaw the slides wrapped in a paper towel to prevent overt condensation onto the slides.

6. Draw a circle (approximate diameter 15-20 mm) around the spot using a PAP pen liquid blocker (5 mm tip).
7. Add 50 µL of 4% PFA fixation solution to each spot and incubate for 30 min at RT.
8. Wash the cells 3 times for 5 min with 1x PBS in a Coplin jar at RT with gentle agitation.
9. Dry the slides around the spots with a lint-free tissue and remove as much liquid from the spot as possible using a pipet without touching the spot, or by tilting the slide. Add 50 µL of 0.25% Triton-X in 1x PBS to each spot and incubate for 10 min at RT without agitation.
10. Wash the slides 3 times for 5 min in ~50-60 mL of 0.05% Tween-20 in TBS (TBS-T) in a Coplin jar at RT with gentle agitation. 10 slides can be processed simultaneously in this fashion.

3. Blocking and Primary Antibody Incubation

1. Tap off TBS-T and dry the slides around the spots with a lint-free tissue. Remove as much liquid from the spot as possible using a pipet without touching the spot, or by tilting the slide. Shortly vortex the blocking solution and add one drop (~ 40 µL) to each spot. Incubate for 1 h at 37 °C in a pre-warmed humidified chamber.
2. Prepare the antibody cocktail by mixing the goat-anti-ATM at 1:1,000 and the rabbit-anti-phospho-Ser15-p53 at 1:100 in commercial antibody diluent, vortex antibody diluent before use. For control experiments, prepare antibody dilutions containing only the goat-anti-ATM and only the rabbit-anti-phospho-Ser15-p53 antibody (single-antibody controls).
3. Tap off the blocking solution but take care not to let the cells dry prior to adding the antibody dilutions. Add 30 µL of each antibody cocktail dilution and incubate in a humidified chamber overnight at 4 °C.
4. **Prepare *in situ* Wash Buffer A and Wash Buffer B by dissolving the content of one pouch of Buffer A and Buffer B mix in a final volume of 1,000 mL of water each.**
    1. Alternatively, prepare wash buffer A by dissolving 8.8 g of NaCl, 1.2 g of Tris-base and 0.5 mL of Tween-20 in 800 mL water. Adjust pH to 7.4 using HCl and add to a final volume of 1,000 mL.
    2. Alternatively, prepare Wash Buffer B by dissolving 5.84 g of NaCl, 4.24 of Tris-base and 26.0 g of Tris-HCl in 500 mL of water. Adjust pH to 7.5 using HCl. Add water to a final volume of 1,000 mL.
    3. Filter both solutions through a 0.22 µm filter.
5. Wash the slides 2 times for 5 min with 1x *in situ* wash buffer A in a Coplin jar at RT with gentle agitation.

4. PLA Probes, Ligation and Amplification

1. Prepare PLA probes by diluting the anti-goat PLUS and anti-rabbit MINUS both 1:5 in the commercial antibody diluent. Tap off 1x *in situ* wash buffer A from the slides and add 30 µL of the PLA probe-mixture to each spot. Incubate 1 h at 37 °C in a pre-warmed humidified chamber.
   NOTE: Any combination of secondary antibodies PLUS and MINUS PLA probes can be used (anti-mouse, anti-rabbit, anti-goat), as long as the applied primary antibodies are raised in different species, and the combination of PLA probes should always contain a PLUS probe and a MINUS probe.
2. Wash slides 2 times for 5 min with ~50-60 mL 1x *in situ* wash buffer A in a Coplin jar at RT with gentle agitation.
3. Prepare the Ligation-Ligase solution on ice by adding 1 µL of Ligase to 39 µL of Ligation solution and add 40 µL of Ligation-Ligase solution to each spot. Incubate 30 min at 37 °C in a pre-warmed humidified chamber.
4. Wash the slides 2 times for 5 min with ~50-60 mL 1x *in situ* wash buffer A in a Coplin jar at RT with gentle agitation.
5. Prepare the Amplification-Polymerase mixture on ice by diluting the Amplification stock solution 1:5 in water. Then add 0.5 µL of polymerase to 39.5 µL of 1x Amplification solution for each spot.
   NOTE: The amplification reagents are light sensitive and should be protected against direct exposure to light. The amplification reagent comes in four different colors (RED: excitation 594 nm, emission 624 nm; FarRED: excitation 644 nm, emission 669 nm; ORANGE: excitation 554 nm, emission 576 nm; GREEN: excitation 501 nm, emission 523 nm), make sure that the microscopy setup available has suitable excitation properties and filters to image these colors.
6. Tap off 1x *in situ* wash buffer A and add 40 µL of Amplification-Polymerase mixture per spot. Incubate for 100 min at 37 °C in a pre-warmed humidified chamber.

5. Mounting and Imaging

1. Prepare 0.01x *in situ* wash buffer B by adding 1 mL of 1x Wash Buffer B to 99 mL of water.
2. Tap off the Amplification-Polymerase mixture and wash slides 2 times for 10 min with 1x *in situ* wash buffer B in a Coplin jar at RT with gentle agitation.
3. Wash the slides 1 min in 0.01x wash buffer B.
4. Tap off excess 0.01x wash buffer B and dry the slides around the spots with a lint-free tissue. Remove as much liquid from the spot as possible using a pipet without touching the spot, or by tilting the slide.
5. Dilute DAPI-containing mounting medium 1:5 in mounting medium without DAPI to avoid overstaining of the nuclei.
6. Add 25-30 µL of 1:5 DAPI-containing mounting medium to a 24 mm x 24 mm coverslip and pick up the cover slip with the slide, apply slight pressure to ensure that there are no trapped air bubbles. Seal the coverslip with nail polish and wait at least 15 min before imaging.
7. **Analyze the slides using a (confocal) fluorescence microscope.**
    1. Count the number of punctate PLA signals per cell in at least 4 acquired image fields (20x objective, counting at least 20 cells per condition or antibody combination, based on a power calculation using an anticipated mean of 7 ± 5 signals in the treated cells, and 2 ± 2 signals in the untreated cells, with a probability of a type-I error of 0.05, and a power of 80%, as reported previously[15]).
       NOTE: The best resolution is achieved by acquisition of a Z-stack and deconvolution using software packages (*e.g.*, ImageJ, the Leica Acquisition Suite (LAS)). The signals should be readily discernible within the nuclear perimeter, as defined by the DAPI staining.

2. Do not count signals that clearly appear outside the nuclei. Typically, NCS treatment or γ-irradiation results in approximately 5-10 phosho-p53/ATM PLA signals per cell. The 'single-antibody' controls may give some background signal, but this should not exceed 1-2 PLA signals per 1 out of every 20 cells.
3. For publication and presentation purposes, capture images using a 40X or 63X immersion oil objective. Slides can be stored at 4 °C and should be protected from exposure to light.

Representative Results

Phosphorylation of p53 at residue Ser15 was shown to be dependent on ATM kinase activity[16]. To demonstrate and confirm the specificity of the PLA technique on cytospin preparations of suspension cell cultures, it is shown that induction of DNA damage by 2 h NCS treatment of BCR-ABL+ B-ALL cells arrested in the G1-phase of the cell-cycle resulted in the specific interaction between ATM and phospho-Ser15-p53, as expected. Punctate PLA signals were observed in the nucleus of the majority of cells treated with NCS (**Figure 1B**), with an average of 7 PLA signals per cell[15], whereas these signals were not detected in cells that were not treated with NCS (**Figure 1A**). Pre-treatment of the cells with the specific ATM kinase inhibitor KU55933 prior to induction of DNA damage clearly diminished the number of PLA signals to an average of 2 PLA signals/cell (**Figure 1C**). The ATM-phospho-Ser15-p53 PLA signals were exclusively detected in the nucleus, as expected. PLA experiments in which either the goat-anti-ATM or the rabbit-anti-phospho-Ser15-p53 antibody was omitted ('single-antibody' control) did not yield any specific PLA signals (**Figures 1D** and **1E**). Quantification and statistical analyses of these results are presented in **Figure 1F** (adapted from Ochodnicka *et al.* J.Immunol. 2016)[15].

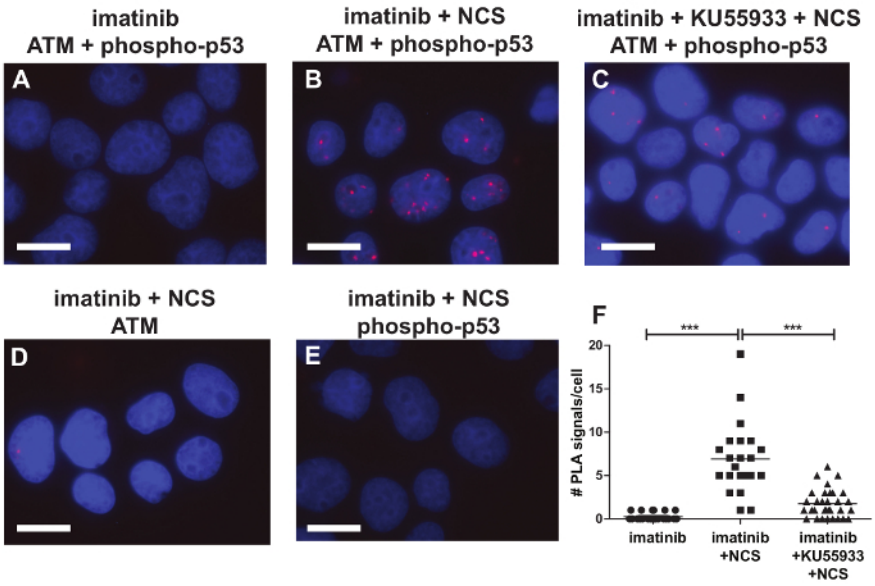

Figure 1: *In situ* PLA for ATM/phospho-Ser15-p53 Interactions in BV173 Human BCR-ABL+ Cells. (**A**) Cells were treated overnight with 5 μM imatinib (specific Abl kinase inhibitor that provokes a G1-phase cell-cycle arrest in BCR-ABL+ cells), with imatinib and 50 ng/mL NCS, which induced DNA damage (**B**), or with imatinib and pre-treated with 5 μM of the specific ATM kinase inhibitor KU55933 prior to NCS treatment (**C**). Single-antibody control PLA experiments for the goat-anti-ATM (D) and rabbit-anti-phospho-Ser15-p53 antibodies (**E**) are shown. Red fluorescence punctate signals represent *in situ* protein proximity (<50 nm) of ATM and phospho-Ser15-p53 proteins. Scale bars = 5 μm (white lines). (**F**) Quantification of the number of PLA signals in at least 20 cells treated under different experimental conditions are shown. Horizontal lines represent means; statistical significances were determined by one-way analysis of variance (ANOVA; *** p <0.001). Please click here to view a larger version of this figure.

Discussion

In this report, it is demonstrated that PLA can be used to determine and visualize the specific interaction between proteins in suspension cell cultures. Of note, the protocol described here is not restricted to the study of DNA repair complexes but also applies to visualize and quantify other protein-protein interactions in suspension cell cultures. It is shown that the ATM kinase interacts with phosphorylated p53 in G1-arrested BCR-ABL+ B-ALL cells when exposed to a DNA damage-inducing agent. Previously, the PLA technique was used by us to show that ATM and the FOXO1 transcription factor interacted in BCR-ABL+ B-ALL cells, however, this interaction most likely did not result in the ATM-dependent phosphorylation of FOXO1. In support of the PLA data, it was confirmed by immunoprecipitation of endogenous ATM/ATR-phosphorylated proteins that, in contrast to the established ATM substrates p53 and NBS1, FOXO1 is *not* phosphorylated by ATM upon induction of DNA damage[15]. In addition, the PLA approach was successfully applied to study the formation of mismatch repair complexes (*e.g.* the interaction between MSH2 and MSH6) interactions in various human B-cell lymphoma cell lines (data not shown). In these particular contexts, the PLA technique offered an alternative approach to support our findings. It is demonstrated that PLA allows the semi-quantitative assessment of protein-protein interactions between experimental conditions and gives insight into the cell-to-cell variation in these interactions.

The analysis of specific protein-protein interactions in cells (exposed to different experimental conditions) has typically been challenging and is subject to experimental artifacts. Generally, protein co-immunoprecipitation has been the method of choice but requires large numbers of cells and/or proteins, essentially prohibiting the analysis of protein-protein interactions in rare cell types or between proteins that are expressed at low levels. Moreover, overexpression of proteins in (irrelevant) cell line models often leads to overexpression artifacts, and results can be very difficult to confirm/validate *in vivo*, or in the relevant cell types. Also, co-immunoprecipitation requires lysis of cells and solubilization of proteins, which can result in the loss of signal when protein-protein interactions are weak. Importantly, protein co-immunoprecipitation and yeast-two-hybrid approaches are unable to detect intra- and inter-cellular variation in protein-protein interactions. The PLA technique offers an attractive and easy alternative for these techniques, and provides important additional insight. PLA can be used in a standard (diagnostic) laboratory setting as it does not require highly specialized skills beyond the capability to perform immunohistochemistry.

The development of super-resolution microscopy has allowed the in-depth analysis of the molecular architecture of protein complexes. This technique, however, is not readily available and involves a dedicated research infrastructure. PLA offers an accessible, simple and cheap alternative approach for the study of protein complex formation, with a comparable resolution. An obvious limitation of the PLA technique is the availability of specific primary antibodies raised in different species. However, several companies now offer dedicated (and validated) antibody combinations that streamline the use of the PLA technique.

Studies on the involvement of proteins in DNA damage repair and the DNA damage response rely heavily on the analysis of IRIF formation and composition. However, it must be noted that co-localization of proteins in IRIFs does not necessarily imply direct protein-protein interactions. The PLA technique offers a unique opportunity to study DNA repair proteins and the formation of repair complexes in more detail. The spatiotemporal analysis of the formation of such complexes can be mapped in greater detail using PLA. Moreover, it will allow researchers to assess the formation of repair complexes in tissues and clinical specimens of patients that have been treated with DNA damaging agents or therapies, which may provide important insight into the efficacy of these treatment modalities and may even aid in the selection of patients that will benefit most from these treatments.

Disclosures

The authors have nothing to disclose.

Acknowledgements

Research in the Guikema laboratory is funded by the Innovational Research Incentives Scheme from The Netherlands Organization for Scientific Research (VIDI grant 016126355) and the 'Stichting Kinderen Kankervrij' KiKA (project 252).

References

1. Maréchal, A., & Zou, L. DNA Damage Sensing by the ATM and ATR Kinases. *Cold Spring Harb Perspect Biol* . **5** (9), a012716 (2013).
2. Huen, M. S. Y., & Chen, J. Assembly of checkpoint and repair machineries at DNA damage sites. *Trends Biochem Sci.* **35** (2), 101-108 (2010).
3. Bekker-Jensen, S., & Mailand, N. Assembly and function of DNA double-strand break repair foci in mammalian cells. *DNA Repair.* **9** (12), 1219-1228 (2010).
4. Matsuoka, S., *et al.* ATM and ATR substrate analysis reveals extensive protein networks responsive to DNA damage. *Science.* **316** (5828), 1160-1166 (2007).
5. Barlow, C., Brown, K. D., Deng, C. X., Tagle, D. A., & Wynshaw-Boris, A. Atm selectively regulates distinct p53-dependent cell-cycle checkpoint and apoptotic pathways. *Nature Genet* . **17** (4), 453-6 (1997).
6. Tibbetts, R. S., *et al.* A role for ATR in the DNA damage-induced phosphorylation of p53. *Genes Dev.* **13** (2), 152-7 (1999).
7. Schermelleh, L., *et al.* Subdiffraction Multicolor Imaging of the Nuclear Periphery with 3D Structured Illumination Microscopy. *Science.* **320** (5881), 1332-1336 (2008).
8. Chapman, J. R., Sossick, A. J., Boulton, S. J., & Jackson, S. P. BRCA1-associated exclusion of 53BP1 from DNA damage sites underlies temporal control of DNA repair. *J Cell Sci.* **125** (Pt 15), 3529-3534 (2012).
9. Gullberg, M., *et al.* A sense of closeness: protein detection by proximity ligation. *Curr Opin Biotechnol.* **14** (1), 82-6 (2003).
10. Söderberg, O., *et al.* Characterizing proteins and their interactions in cells and tissues using the in situ proximity ligation assay. *Methods* . **45** (3), 227-32 (2008).
11. Dong, Y., & Shannon, C. Heterogeneous immunosensing using antigen and antibody monolayers on gold surfaces with electrochemical and scanning probe detection. *Anal Chem.* **72** (11), 2371-6 (2000).
12. Jackson, S. P., & Bartek, J. The DNA-damage response in human biology and disease. *Nature.* **461** (7267), 1071-1078 (2009).
13. Economopoulou, P., Pappa, V., Papageorgiou, S., Dervenoulas, J., & Economopoulos, T. Abnormalities of DNA repair mechanisms in common hematological malignancies. *Leuk Lymphoma.* **52** (4), 567-582 (2011).
14. Rendleman, J., *et al.* Genetic variation in DNA repair pathways and risk of non-Hodgkin's lymphoma. *PloS One.* **9** (7), e101685 (2014).
15. Ochodnicka-Mackovicova, K., *et al.* The DNA Damage Response Regulates RAG1/2 Expression in Pre-B Cells through ATM-FOXO1 Signaling. *J Immunol.* (2016).
16. Banin, S., *et al.* Enhanced phosphorylation of p53 by ATM in response to DNA damage. *Science.* **281** (5383), 1674-1677 (1998).

