Chapter 19

Proximity Ligation Assay for Detection of R-Loop Complexes upon DNA Damage

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Abstract

In situ Proximity Ligation Assay (PLA) can be used to detect the close proximity (less than ~40 nm) of two biological molecules of interest in cells. Here we report the application of this method for the specific detection of R-loop interacting proteins and RNA modifications in close proximity to R-loops in non-damage and ionizing radiation (IR) induced DNA damage conditions.

Key words Proximity Ligation Assay, R-loops, RNA modification, DNA Damage, RNA:DNA hybrids, Double Strand breaks

1 Introduction

Understanding molecular interactions is vital to elucidate how biological pathways occur. Methods to detect protein–RNA interactions have become increasingly important as our understanding of RNA functionality has expanded. R-loops are RNA structures with an RNA:DNA hybrid and a single strand DNA, which can have opposing roles in the cell, as both a genomic threat and as important functional components in processes such as transcription [1] and in the regulation of gene expression [1, 2]. It is also becoming apparent that R-loops are important in the repair of the lethal DNA lesions double strand breaks (DSB) [3]. R-loops can form at DSBs and act as functional platforms in repair through the recruitment of repair proteins, to regulate both homologous recombination (HR) [4] and non-homologous end joining (NHEJ) [5]. R-loops are also now known to be modified, with the RNA strand carrying N6-methyladenosine (m^6A) [6] and 5-methylcytosine (m^5C) [7]. Although the presence of R-loops at DSBs has been shown by many, their exact role in DNA repair is not yet well understood. Hence, there is a great deal of interest in this

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field and consequently in the development of novel methodological tools that can further aid studies on R-loops in DNA repair.

In situ Proximity Ligation Assay (PLA) is a molecular biology technique first developed to detect the proximity of two proteins within ~40 nm of each other and visualize their location within the cell by observation of discrete fluorescent foci [8, 9]. The technique uses specific antibodies from two different species recognizing the proteins of interest. Secondary antibodies with oligonucleotide conjugates, known as PLA probes, are then added, and if these are in close proximity, i.e., the two proteins of interest are within ~40 nm of one another, the connector oligonucleotides will be ligated together after the addition of a ligase. A DNA polymerase is added, and the ligated oligonucleotides can act as primers for amplification, which can then be bound by complementary fluorescently labeled oligonucleotides. The result is a distinct PLA signal which can be visualized by microscopy and quantified using various software such as cell profiler (Fig. 1) [8–10].

Since its conception over a decade ago [11, 12], PLA has been optimized and adapted for use in a wide variety of applications, including but not limited to; the detection of protein–protein

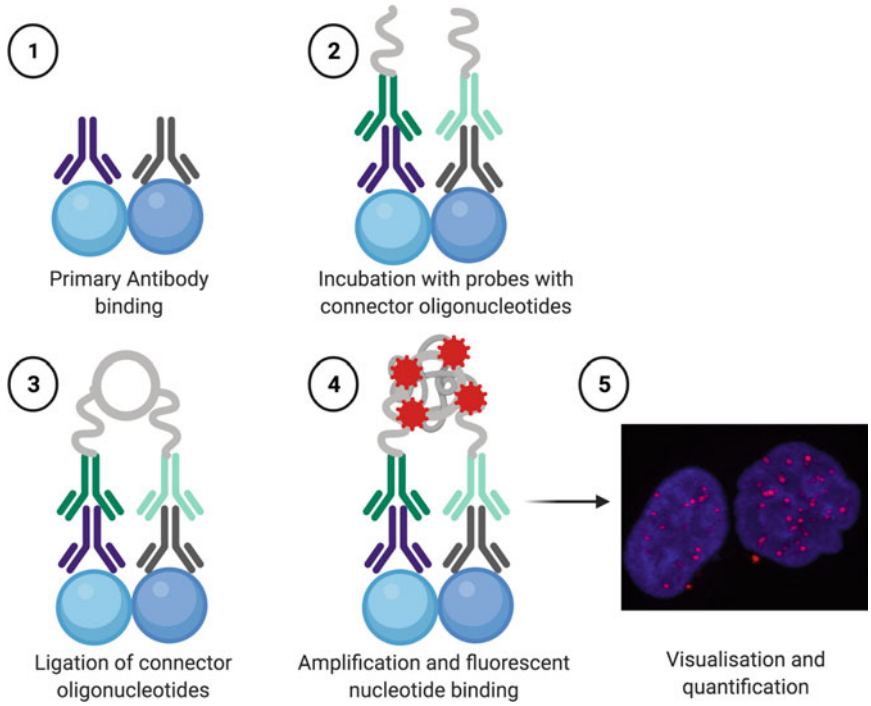

Fig. 1 Principles of in situ Proximity Ligation Assay. Cells seeded on coverslips are incubated with primary antibody, followed by incubation with secondary antibodies (PLA probes) with connector oligonucleotides. The oligonucleotides will ligate together upon addition of a ligase enzyme if in close proximity. This acts as a template for a DNA polymerase and rolling circle amplification (RCA) will take place, with binding of complementary fluorescent nucleotides resulting in a PLA foci visualised by confocal microscope

interactions [13]; monitoring post-translational modification of a protein of interest [14]; RNA–protein interactions [15]; RNA–DNA association [16]; and detection of proteins at DSBs [17].

We present here a method using the PLA Duolink® In Situ Red Fluorescent kit (https://www.sigmaaldrich.com/GB/en/products/protein-biology/duolink-proximity-ligation-assay) to detect both proteins and RNA modifications in close proximity to RNA:DNA hybrids using the S9.6 antibody. Additionally, we perform this in biologically relevant IR-induced DNA damage conditions. We show the specificity of this method by expressing a V5-tagged RNaseH1 enzyme, which binds the RNA:DNA hybrid and degrades the RNA strand, and catalytically dead (D210N) and binding (WKKD) RNaseH1 mutants [18]. As expected, performing PLA with V5 and S9.6 antibodies results in visible PLA signals in the presence of the D210N RNaseH1 mutant, which binds but does not cleave the RNA:DNA hybrids. These PLA signals are reduced in the presence of WT RNaseH1 which will bind and degrade the RNA:DNA hybrids. The PLA foci observed with the WKKD mutant are on the level of background, as this mutant will not bind the RNA:DNA hybrids (Fig. 2). This method can be applied to a wide variety of RNA:DNA hybrid interacting proteins (Fig. 3) and RNA modifications (Fig. 4) in different cellular contexts. As an example, for proof of concept, we chose to apply PLA to detect the interaction between DHX9, a known R-loop interactor [19], and R-loops. We observed the generation of PLA foci using antibodies for DHX9 and S9.6 (Fig. 3), and we validate the specificity of these PLA signals by expressing the D210N RNaseH1 and WKKD RNaseH1 mutants, where PLA signals are observed, and the WT RNaseH1, where the PLA signals are reduced due to degradation of the RNA:DNA hybrid. Furthermore, we performed this experiment in the context of DNA damage, where R-loops are enriched, and detected significantly increased numbers of PLA foci upon IR treatment (Fig. 3). Next, we wished to test the application of the same experimental principle for the detection of R-loop modifications. In order to do this, we employed PLA using an antibody for the RNA modification m^5C in combination with the S9.6 antibody in normal and IR treated samples, as R-loops have been shown to be methylated upon DNA damage. Indeed, we observed a significant increase in PLA signals in the presence of D210N and WKKD RNaseH1 expression in irradiated samples. These foci were significantly reduced by expression of RNaseH1 WT, further confirming the specificity of the PLA assay in the context of DNA damage (Fig. 4).

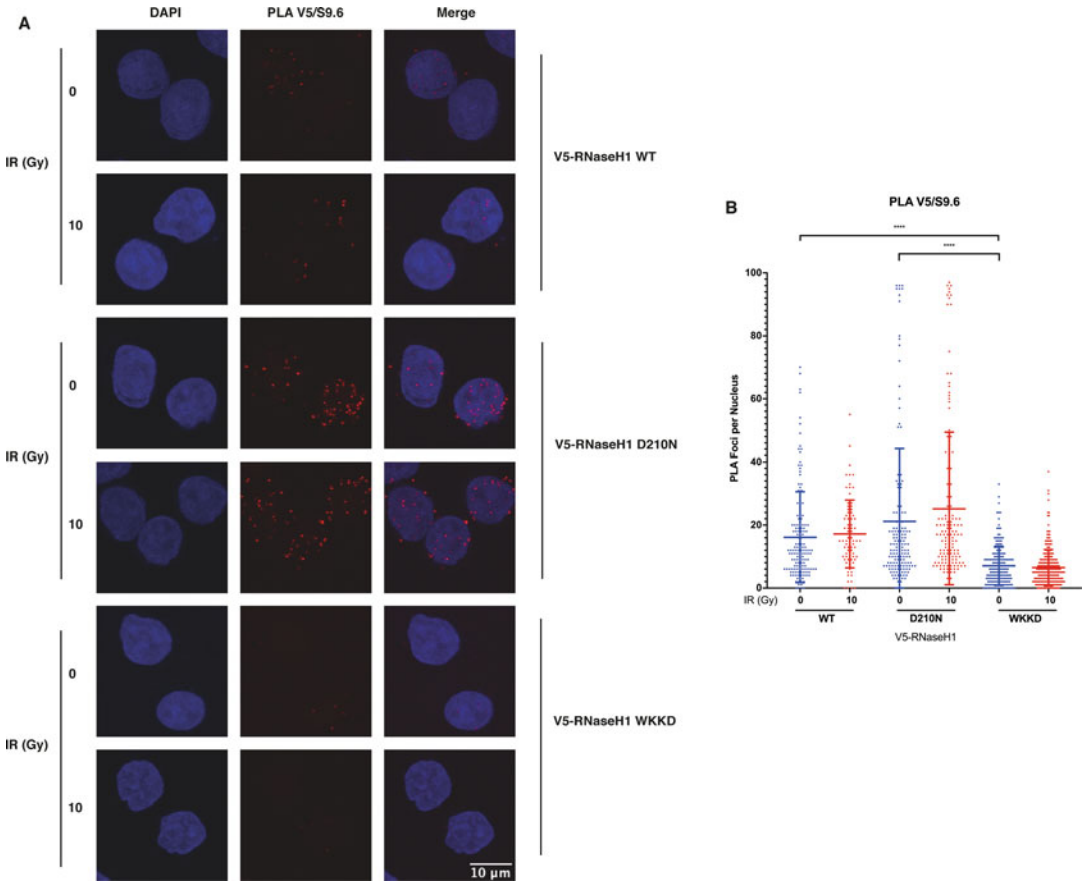

Fig. 2 Specific detection of RNA:DNA hybrids in close proximity to the RNA:DNA hybrid binding enzyme RNaseH1. (**a**) V5-tagged RNaseH1 wild type (WT) (top panel), RNaseH1 catalytically dead D210N mutant (middle panel), and RNaseH1 non-binding catalytically dead WKKD mutant (bottom panel) were expressed in cells. Antibodies for V5 and S9.6 were used for PLA to detect the RNaseH1 in close proximity to the RNA:DNA hybrids. Cells were exposed to IR (10 Gy) or not (0 Gy) before carrying out the PLA protocol. (**b**) Quantification of the results shown in panel **A**. Error bars show mean and standard deviation. The Mann–Whitney Statistical test was used as a test for significance

2 Materials

2.1 Equipment

1. Microscope slides.
2. Round coverslips (22 mm).
3. Humidity chamber (*see* **Note 3**).
4. Tissue culture plates (6-well).
5. Tissue culture dishes (35 mm).
6. Rocker.
7. Fume hood.
8. Tissue culture hood.

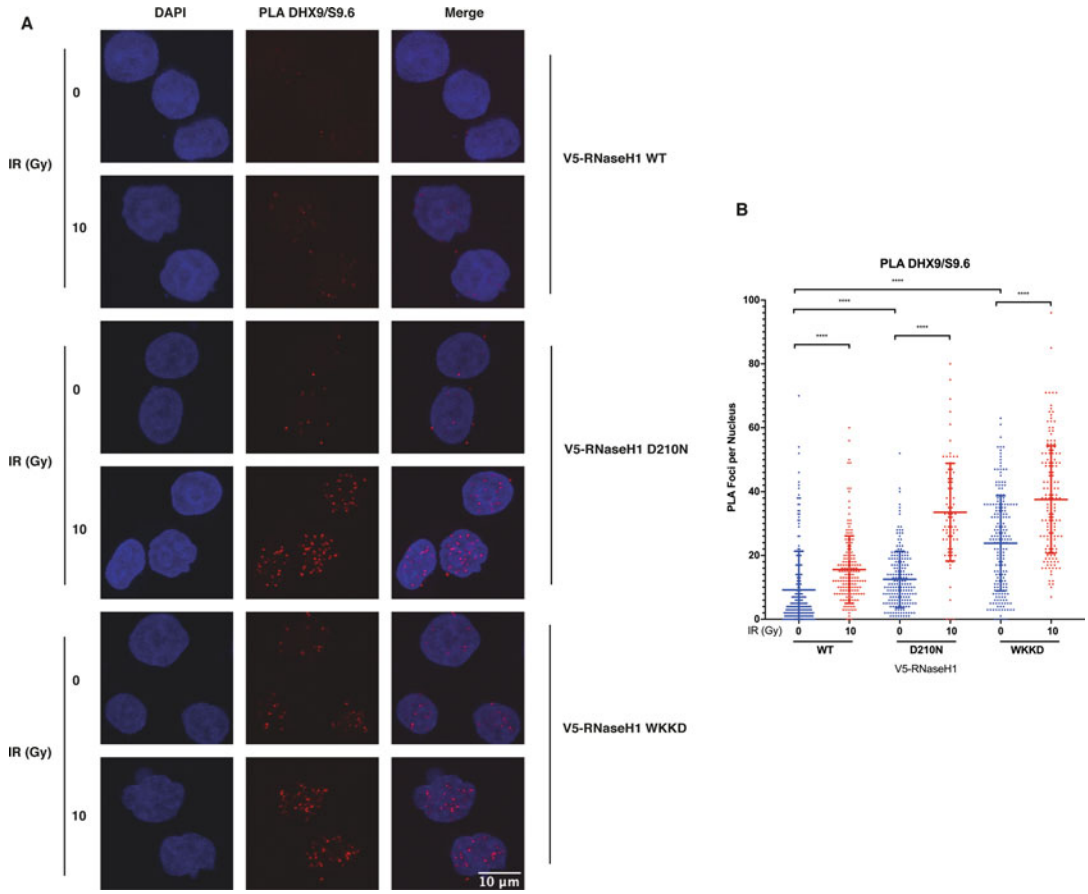

Fig. 3 Detection of DHX9/S9.6 PLA foci. (**a**) An antibody for endogenous DHX9, a known R-loop interactor, was used in combination with the S9.6 antibody for PLA. Cells were transfected with either WT (top), D210N mutant (middle), or WKKD mutant (bottom) RNaseH1. Cells were exposed to IR (10 Gy) or not (0 Gy) before carrying out the PLA protocol. (**b**) Quantification of the results shown in panel **A**. Error bars show mean and standard deviation. The Mann–Whitney Statistical test was used as a test for significance

9. Freeze block for enzymes.
10. 37 °C incubator.
11. Water bath.
12. Fluorescence microscope.
13. Analysis software (ImageJ and Cell profiler).

2.2 Reagents

1. Protease inhibitor cocktail tablets (Roche).
2. RNAse T1 (Thermo).
3. Shortcut RNAse III (New England Biolabs).
4. Duolink® In Situ Red Starter Kit Mouse/Rabbit (Sigma) containing:

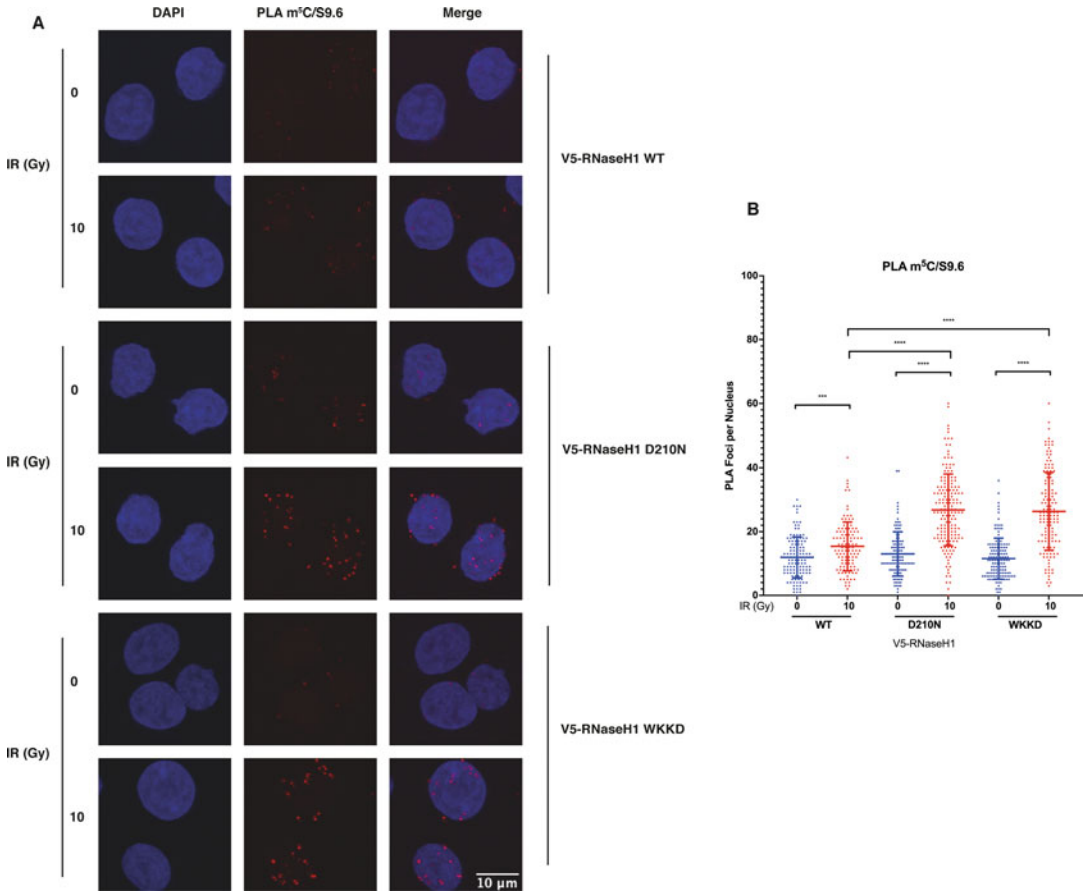

Fig. 4 Detection of m^5C/S9.6 PLA foci. (**a**) m^5C was detected in close proximity to RNA:DNA hybrids using antibodies for m^5C and S9.6. Cells were transfected with either WT (top), D210N mutant (middle), or WKKD mutant RNaseH1 (bottom). Cells were exposed to IR (10 Gy) or not (0 Gy) before carrying out the PLA protocol. (**b**) Quantification of the results shown in panel **A**. Error bars show mean and standard deviation. The Mann–Whitney Statistical test was used as a test for significance

(a) Duolink® In Situ Mounting Medium with DAPI.
(b) Duolink® In Situ Wash Buffers (Buffer A and B).
(c) Duolink® In Situ PLA® Probe Anti-Rabbit PLUS.
(d) Duolink® In Situ PLA® Probe Anti-Mouse MINUS.
(e) Duolink® In Situ Detection Reagents Red.

5. Primary antibodies: Anti-DNA–RNA hybrid antibody (clone S9.6) mouse monoclonal (MABE1095, Sigma-Aldrich), Anti-DNA–RNA hybrid antibody (clone S9.6) rabbit monoclonal (Ab01137-23.0, Absolute Antibody), Anti-V5 tag antibody rabbit monoclonal (ab206566, Abcam), Anti-RNA Helicase A antibody rabbit monoclonal (ab183731, Abcam), Anti-5-methylcytosine (5-mC) antibody (ab214727, Abcam).

6. Hela cells (CCL-2 ATCC).
7. Fetal bovine serum (FBS), heat inactivated.
8. Cell dissociation enzyme (e.g. TrypLE).
9. Dulbecco's Modified Eagle's Medium high glucose (DMEM).
10. Penicillin/Streptomycin solution (P/S).
11. Opti-MEM™ I Reduced Serum Medium.
12. Lipofectamine 3000 (Thermo).
13. Plasmids: ppYCAG_RNAseH1_wt (#111906, Addgene), ppY-CAG_RNAseH1_D210N (#111904, Addgene), and ppY-CAG_RNASEH1_WKKD (#111905, Addgene).
14. Clear nail varnish.

2.3 Solutions

Prepare all solution using ultra-pure water and DEPC-treated and analytical grade reagents. Autoclave or 0.22 μm filter to sterility. Store all reagents at room temperature (unless indicated otherwise).

1. Diethylpyrocarbonate (DEPC)-treated and sterile filtered H2O.
2. Phosphate-buffered saline without $CaCl_2$ and $MgCl_2$ (PBS).
3. 0.1% Poly-D-lysine solution (w/v).
4. 4% paraformaldehyde (PFA) solution in PBS (4% PFA).
5. 70% Ethanol solution in DEPC-H_2O.
6. 10% Triton X-100 in DEPC-H_2O.
7. 1 M Sucrose in DEPC-H_2O.
8. 1 M Pipes in DEPC-H_2O, store at 4 °C.
9. 5 M NaCl in DEPC-H_2O.
10. 1 M $MgCl_2$ in DEPC-H_2O.
11. 10% BSA in DEPC-H_2O store in dark at 4 °C.
12. 1 M ethylene glycol-bis(β-aminoethyl ether)-*N,N,N′,N′*-tetra-acetic acid (EGTA) in DEPC-H_2O.
13. 1 M Glycine in PBS.
14. Coating solution: 0.01% Poly-D-lysine in PBS, (store at 4 °C).
15. CSK buffer: 10 mM PIPES, 100 mM NaCl, 300 mM Sucrose, 3 mM $MgCl_2$, 1 mM EGTA, 0.7% Triton X-100, 1× protease inhibitor cocktail, (make fresh and store at 4 °C).
16. Quenching solution: 200 mM Glycine in PBS.
17. RNAse treatment solution: 0.1% BSA, 3 mM $MgCl_2$, 1:200 (v/v) RNAse T1, 1:200 (v/v) ShortCut RNAse III in PBS, (make fresh and store at 4 °C).

3 Methods

Day 1

3.1 Reverse Transfection with RNaseH1 Plasmids

1. Remove media from a 70% confluent flask of Hela cells and wash once with PBS.
2. Add tryPLE to the flask until detachment of cells has occurred and resuspend in DMEM to obtain a single cell suspension.
3. Count cells and resuspend in DMEM supplemented with 10% FBS without P/S.
4. Prepare RNaseH1 plasmids for transfection using Lipofectamine 3000 transfection reagents according to manufactures instructions. For each well of a 6-well plate the following volumes are used in Opti-MEM™ Reduced Serum Medium:
   (a) 3 μg of plasmid.
   (b) 4.5 μL of Lipofectamine 3000.
   (c) 6 μL of reagent 3000.
5. Vortex mixture for 10 s and incubate at room temperature (RT) for 20 min.
6. Add the plasmid-lipofectamine complexes at the bottom of each well (total 250 μL of Opti-MEM™).
7. Seed Hela cells to reach a confluency of 90% 16–18 h later.
8. Add DMEM supplemented with FBS 10% without P/S up to 2 mL.
9. Incubate cells at 37 °C for 16–18 h.

Day 2

3.2 Cover Glass Preparation

1. Place one microscope coverslip into a 35 mm dish.
2. Add 2 mL 70% ethanol to each dish and incubate at RT for 10 min.
3. Remove the 70% ethanol and wash twice with 2 mL PBS.
4. Add 2 mL of coating solution to each dish and incubate 20 min at 37 °C.
5. Remove the coating solution and wash twice with PBS.
6. Seed RNaseH1-tranfected HeLa cells to reach ~60% confluency after 24 h.

Day 3

3.3 Irradiation and Proximity Ligation Assay

1. Irradiate Hela Cells with a 10 Gy dose of IR (*see* **Note 1**).
2. Incubate cells for 15 min at 37 °C (*see* **Note 1**).

3. Remove media from the cells and wash twice with 2 mL ice cold PBS.
4. Add 2 mL of CSK buffer to each 35 mm dish and place on a rocker with gentle rocking for 15 min at 4 °C.
5. Remove the CSK buffer and wash three times with 2 mL ice cold PBS.
6. In a fume hood, add 2 mL of 4% PFA in PBS to each 35 mm dish and incubate for 10 min at 37 °C.
7. Remove the PFA and add 2 mL quenching solution, and incubate for 5 min at RT on the rocker with gentle rocking.
8. Prepare fresh RNase treatment solution (*see* **Note 2**).
9. Add 1 mL of RNase treatment solution to each 35 mm dish and incubate for 1 h at RT on a rocker with gentle rocking.
10. Wash three times with 2 mL PBS and incubate for an additional 5 min with rocking.
11. In prewarmed humidity chamber (*see* **Note 3**), add 100 μL of Duolink® blocking solution from the PLA kit on top of the parafilm for each coverslip sample.
12. Take out the coverslip from the 35 mm dish, tap off excess solution and place it cell side down on the blocking solution avoiding bubbles (*see* **Notes 4** and **5**).
13. Incubate for 1 h at 37 °C.
14. Prepare the primary antibody solution by diluting mouse and rabbit antibodies in 60 μL per sample of Duolink® Antibody Dilutant from the PLA kit (*see* **Notes 6–8**).
15. Add 60 μL of the diluted antibody solution to the parafilm surface of the humidity chamber.
16. Tap off excess buffer from the coverslip and place cell side down onto the antibody solution.
17. Incubate overnight at 4 °C in the humidity chamber.

Day 4

3.4 Proximity Ligation Assay

1. Prewarm PLA washing buffer A and B to RT in a water bath (*see* **Note 9**).
2. Place coverslips cell side up in 6-well plate with 2 mL of PLA washing buffer A and wash twice for 5 min with gently rocking at RT (*see* **Note 10**).
3. Pre-warm the humidity chamber to 37 °C.
4. Dilute the PLUS and MINUS PLA probes 1:5 in Duolink® Antibody Diluent.
5. Add 60 μL of probe solution on to the parafilm of the humidity chamber.

6. Tap off the excess of buffer from the coverslips and place coverslips cell side down onto the probe solution in the humidity chamber.
7. Incubate at 37 °C for 1 h.
8. Tap off the excess of probe solution and wash twice with 2 mL of washing buffer A for 5 min at RT.
9. Thaw Duolink ligase buffer and dilute ligase buffer at 1:5 ratio in DEPC-H_2O (*see* **Note 11**).
10. Add 12 μL of ligase enzyme (*see* **Note 12**) provided by the Duolink® Red Starter Kit to 48 μL diluted ligase buffer solution for a total of 60 μL for each coverslip.
11. Add 60 μL of Ligation reaction solution to the parafilm in the humidity chamber.
12. Incubate at 37 °C for 30 min.
13. Tap off the excess of ligation solution and wash twice with 2 mL of washing buffer A for 5 min at RT.
14. Thaw Duolink amplification buffer, dilute 1:5 in DEPC-H_2O and protect from light (*see* **Note 13**).
15. Add 6 μL Polymerase enzyme provided by the Duolink® Red Starter Kit to 54 μL diluted amplification buffer for a total of 60 μL for each coverslip.
16. Add 60 μL of Amplification reaction solution to the parafilm in the humidity chamber.
17. Place coverslips cell side down onto the amplification reaction solution.
18. Incubate for 1 h and 40 min at 37 °C.
19. Tap off the excess of amplification solution from the coverslip.
20. Wash twice with 2 mL of washing buffer B for 10 min at RT, protecting from light.
21. Perform the last wash with 0.01× washing buffer B for 1 min at RT, protecting from light.
22. Add 20 μL of Duolink in situ mounting medium with DAPI on a microscope slide.
23. Tap off the excess of washing solution and mount the coverslip on the microscope slide.
24. Seal the edges of coverslip with clear nail varnish (*see* **Note 14**).
25. Wait at least 15 min before acquiring images at fluorescent microscope using a 60× objective lens.
26. Slides can be stored in the dark at 4 °C (*see* **Note 15**).
27. Images can be processed using ImageJ, and PLA foci quantified using software such as Cell Profiler (*see* **Note 16**).

4 Notes

1. Ionizing radiation is delivered at a dose of 10 Gy and cells are fixed 15 min after exposure. The most appropriate time post-IR and IR dosage may differ depending on your experimental question and multiple different doses and time points can be tested.
2. It has been demonstrated that the S9.6 antibody can bind other RNA species distinct from RNA:DNA hybrids in immunofluorescence experiments [20]. Therefore, RNase treatment is crucial for the specific identification of the RNA:DNA hybrids (*see* Fig. 3 (with treatment) and Fig. 5 (without treatment) for comparison).
3. The humidity chamber can be set up according to Fig. 6.
4. Excess solution can be gently tapped off the slide on paper towel between wash and incubation steps.
5. Slides should not be allowed to dry out, always keep the coverslips moist. Drying out of the coverslips can cause excess background signals.
6. Some antibodies are not appropriate for use in PLA. Generally, if the antibody is suitable for immunofluorescence (IF) microscopy, it may be suitable for use in PLA. It is a good idea to have a single antibody control (where addition of a second antibody is omitted) (Fig. 7), in order to determine the background level of PLA signals (and therefore non-specific signals) generated by the antibody alone. Some antibodies will generate a very high background level of PLA signals and are, therefore, unsuitable.
7. A titration of each antibody can also be performed to determine the optimum concentration using at least three different concentrations, such as 1:250, 1:1000, and 1:2000.
8. If possible, it is good to test different antibodies raised for the same protein to determine the optimum (specific signal, minimal background) in your experiment. A successful PLA experiment can depend on the primary antibody used.
9. Wash buffer A and B can be warmed in a 37 °C water bath to RT prior to beginning the PLA protocol.
10. For washing steps, slides can be placed back in a 6-well plate cell side facing upwards and approximately 2 mL of wash solution used per well.
11. Ligase buffer should be thawed fully and mixed well before use. It may take some vortexing and pipetting to get the ligase buffer fully in solution. It can help to take the ligase buffer out of the −20 °C approximately half an hour in advance of

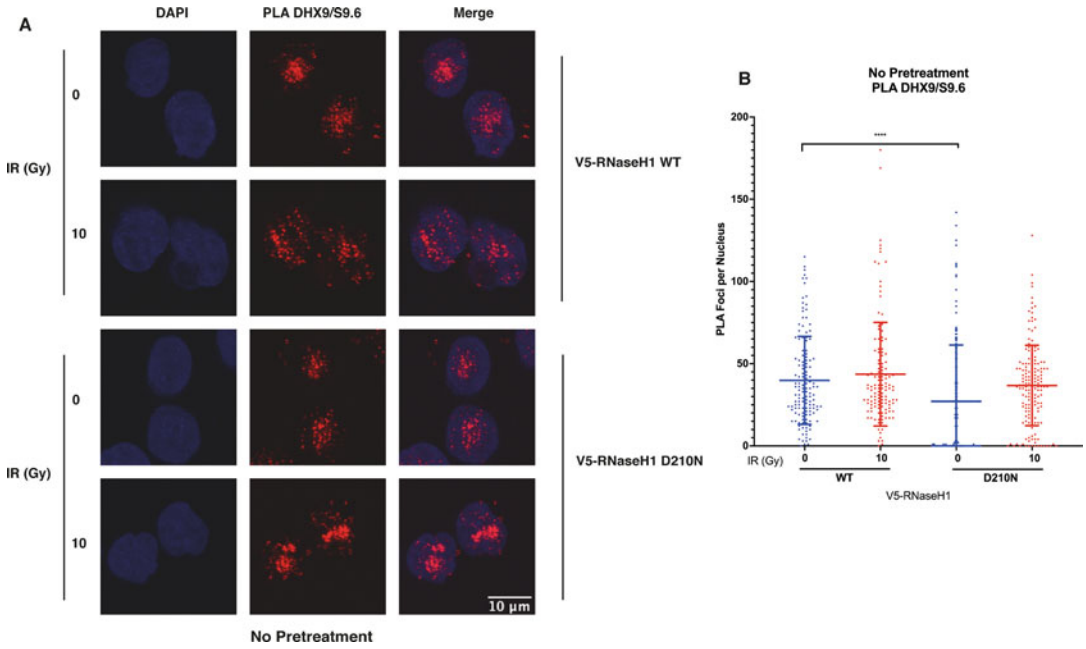

Fig. 5 The importance of the RNase pre-treatment step (*see* **Note 2**). The S9.6 antibody has been suggested to give non-specific signals which are insensitive to RNaseH1 treatment in immunofluorescence (IF) [20]. To overcome this, a pre-treatment step using both RNase III and RNase T1 was developed to ensure the S9.6 signals are specific for RNA:DNA hybrids in IF [20]. We employ this methodology in our set up for Proximity Ligation Assay with the S9.6 antibody in combination with the DHX9 antibody and show here the result without this pre-treatment step. The signals generated with DHX9 and S9.6 were insensitive to RNaseH1 WT expression and therefore likely unspecific. This is not the case when the RNase pre-treatment step is used (Fig. 3). (**b**) Quantification of the results shown in panel A. Error bars show mean and standard deviation. The Mann–Whitney Statistical test was used as a test for significance

use. Incomplete mixing of the ligase buffer can result in the generation of excess background signals.

12. All enzymes (ligase and polymerase) should be kept at −20 °C until just before use and kept in a freezer block or on ice when out of the −20 °C. Do not prepare the ligase or polymerase solutions in advance of use.
13. Protect the amplification buffer from light. Slides and reagents should be kept in the dark during and after the polymerase amplification step.
14. Coverslips can be mounted to slides and secured using clear nail varnish around the perimeter of the coverslip, taking care to seal the edges but covering as little of the coverslip surface as possible.
15. It is better to image the slides the same day or following day after mounting with DAPI. However, we have used slides which are 3–4 days old with similar quality to slides imaged the same day after mounting when stored at 4 °C.

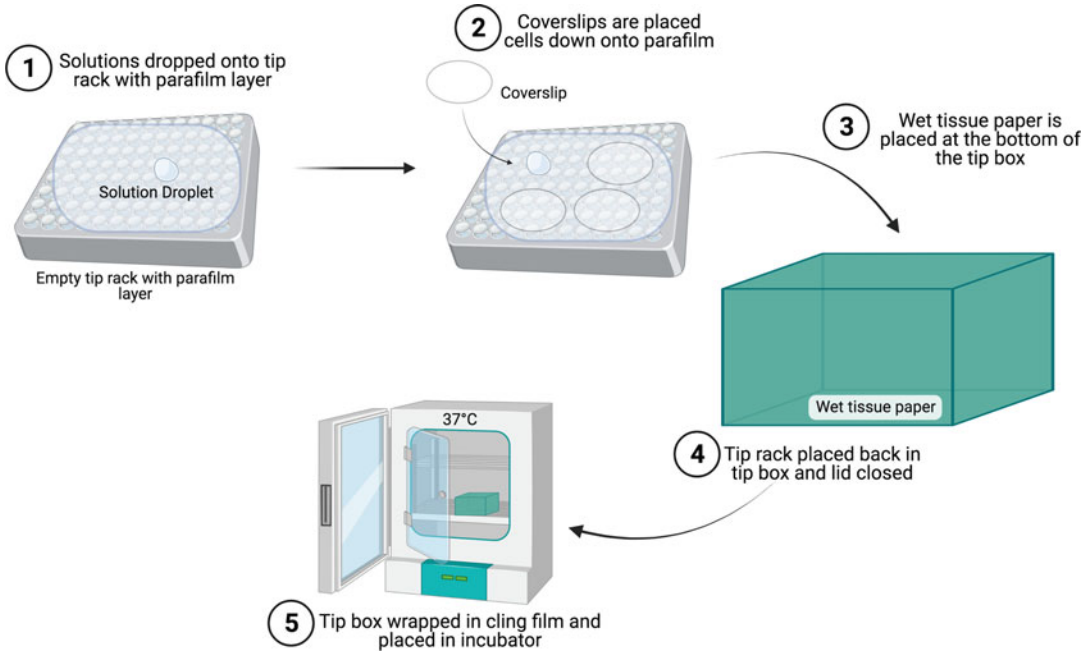

Fig. 6 PLA humidity chamber set up. Schematic of the preparation of the humidity chamber for Proximity Ligation Assay. An empty tip box was used to set up a DIY humidity chamber for coverslip incubation steps. Parafilm is placed on the tip box rack (at least 2 layers is best to give an even surface) and the edges taped down to secure this in place. Between reactions the parafilm surface can be wiped with 70% ethanol and dried to clean it. Reaction mixtures are pipetted onto the parafilm surface, and coverslips are placed cells down on the drops of solution. The rack is then placed in the tip box with wet paper towel placed at the bottom. The tip box lid is closed and the whole box wrapped in cling film to ensure the humid environment is maintained. During the polymerase reaction incubation step, which is light sensitive, the cling film-wrapped box can then be additionally wrapped in tin foil. The box can then be placed in a 37 °C incubator

16. The cell profiler speckle counter pipeline is used to quantify PLA foci. This can be downloaded from: https://cellprofiler.org/examples

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Figures 1 and 6 were created using BioRender.com.

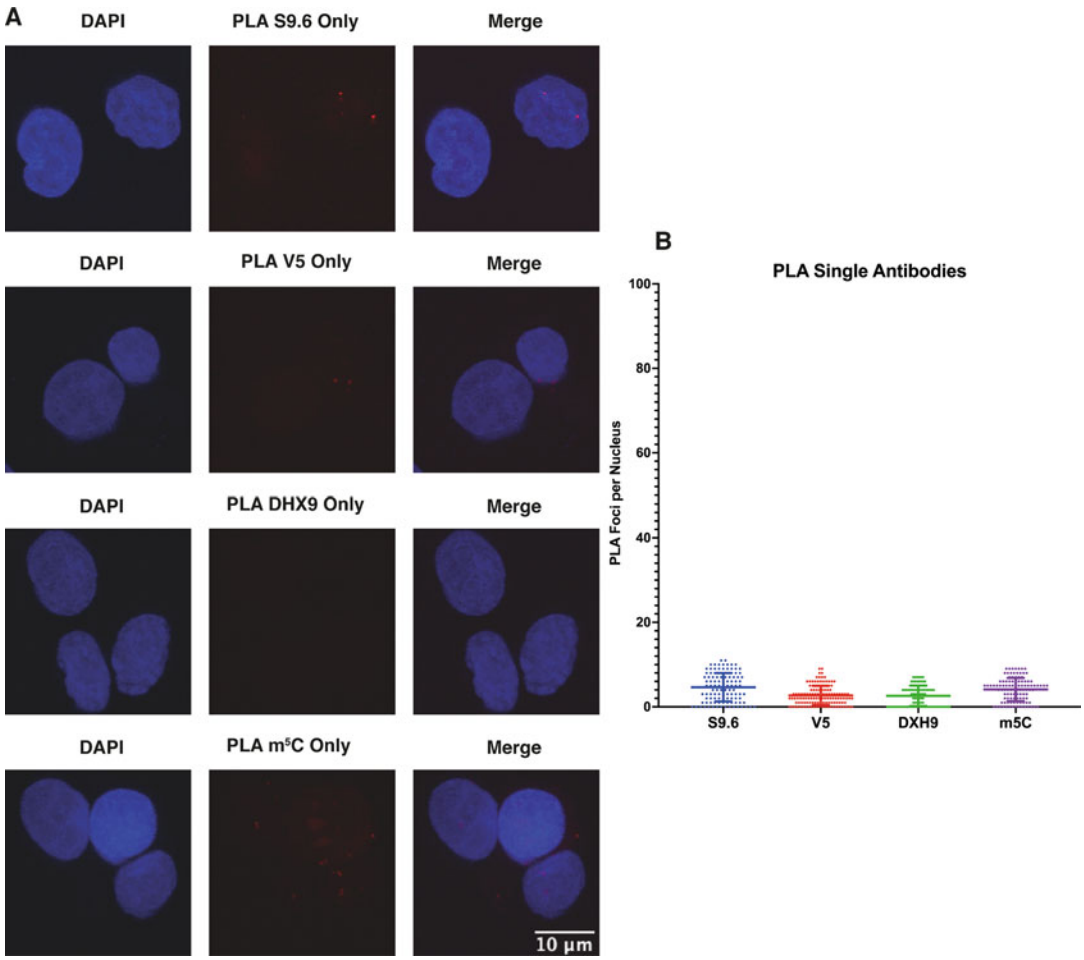

Fig. 7 Absence of PLA signals when individual antibodies are used. PLA was carried out with only a single antibody as indicated to verify the absence of background signal generated by the antibody alone. Error bars show mean and standard deviation

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