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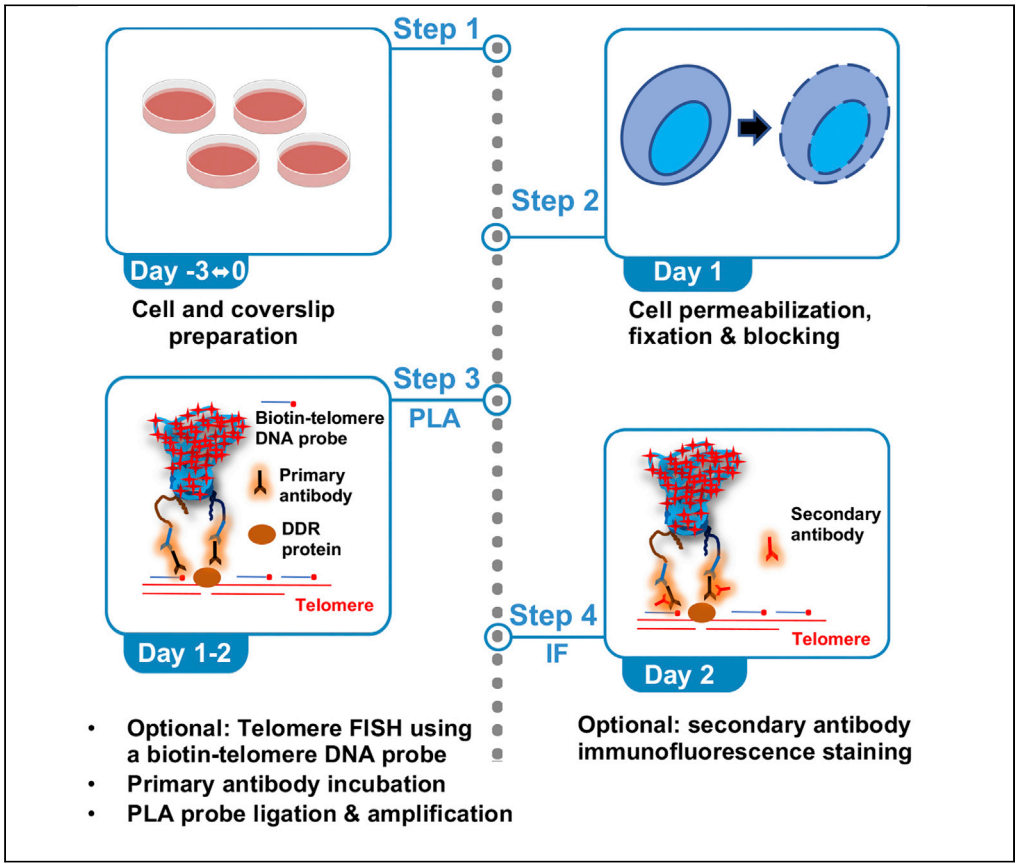
An optimized proximity ligation assay to detect telomere dysfunction induced foci in human and mouse cells



Telomere dysfunction-induced foci (TIF) can be measured by immunofluorescence, combined with telomere-fluorescent *in situ* hybridization. We modified this approach by combining the proximity ligation assay (PLA), which detects colocalization of two molecules in proximity through a signal amplification step and improves the fidelity and sensitivity of TIF detection in human and mouse cells. The protocol includes cell preparation, permeabilization, fixation, and blocking PLA detection of DNA damage response proteins within proximity with telomeres and optional PLA verification by immunofluorescence-based technique.

Publisher's note: Undertaking any experimental protocol requires adherence to local institutional guidelines for laboratory safety and ethics.

Yajun Wang, Luigi Ferrucci, Michael M. Seidman, Yie Liu

liuyie@mail.nih.gov

Highlights

PLA detecting DNA damage response proteins within proximity with telomeres

Optional PLA verification by immunofluorescence-based technique

Increases the fidelity and sensitivity of TIF detection in human and mouse cells

Suitable for detection of telomere dysfunction at single-cell level

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An optimized proximity ligation assay to detect telomere dysfunction induced foci in human and mouse cells

Yajun Wang,[1,4] Luigi Ferrucci,[2] Michael M. Seidman,[3] and Yie Liu[1,5,*]

[1]Laboratory of Genetics and Genomics, National Institute on Aging, NIH, 251 Bayview Blvd, Baltimore, MD 21224, USA

[2]Translational Gerontology Branch, National Institute on Aging, NIH, 251 Bayview Blvd, Baltimore, MD 21224, USA

[3]Laboratory of Molecular Biology and Immunology, National Institute on Aging, NIH, 251 Bayview Blvd, Baltimore, MD 21224, USA

[4]Technical contact: yajun.wang@nih.gov

[5]Lead contact

*Correspondence: liuyie@mail.nih.gov
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SUMMARY

Telomere dysfunction-induced foci (TIF) can be measured by immunofluorescence, combined with telomere-fluorescent *in situ* hybridization. We modified this approach by combining the proximity ligation assay (PLA), which detects colocalization of two molecules in proximity through a signal amplification step and improves the fidelity and sensitivity of TIF detection in human and mouse cells. The protocol includes cell preparation, permeabilization, fixation, and blocking PLA detection of DNA damage response proteins within proximity with telomeres and optional PLA verification by immunofluorescence-based technique.

BEFORE YOU BEGIN

Telomeres are chromosome termini-capping structures consisting of tandem TTAGGG repeats and a six-protein shelterin complex, which includes the key telomere repeat binding factors 1 and 2 (TRF1 and TRF2) (de Lange, 2018). Loss of telomere repeats or loss of protection by the shelterin complex can evoke the DNA damage response (DDR) that results in telomere dysfunction-induced foci (TIF) formation (d'Adda di Fagagna et al., 2003; Takai et al., 2003), which is measured by the colocalization of DNA damage response proteins, such as γ-H2AX and the p53 binding protein 1 (53BP1), and telomere markers, such as telomeric proteins and telomeric DNA using indirect immunofluorescence (IF) (Takai et al., 2003) or IF in combination with telomere-fluorescent in situ hybridization (IF-Telomere FISH) (Hockemeyer et al., 2006). Here, we added the proximity ligation assay (PLA) in the IF and IF-Telomere FISH assays, hereafter namely TIF-PLA, to quantify TIFs in single cells. In the PLA step, we employed antibodies against telomeric proteins or biotinylated telomeric DNA probe to detect the telomere, and the antibodies against DNA damage proteins, such as 53BP1 or γ-H2AX to detect the DNA strand breaks/damage response. PLA yields positive signal only when the two targets (for example, telomere and DDR marks) are in proximity. In addition, PLA involves signal amplification steps, increasing the detection sensitivity (Gustafsdottir et al., 2005). After the PLA step, another set of secondary antibodies were utilized to detect DDR and telomeric markers by the IF step, which served to validate PLA signals. We validated the sensitivity and reliability of our protocol in a human osteosarcoma epithelial (U2OS) cell line expressing the TRF1-FOK1 fusion protein that specifically generates DNA double-strand breaks at the telomere and induces TIFs (Cho et al., 2014;

Tang et al., 2013) and in a mouse embryonic fibroblast cell line with Trf2 deletion and telomere uncapping (Celli and de Lange, 2005).

The protocol below describes the specific steps for detecting TIFs in U2OS cells and is suitable for human and mouse cells in general.

Reagent setup

Timing: 2 h

1. Prepare cell culture medium, containing 10% Bovine Calf Serum, 1% Penicillin-Streptomycin, and 2 mM Glutamine in DMEM. The culture medium and the concentrations of the supplements may vary according to the cell type. For slowly growing cells, cell culture medium containing 10% or 20% Fetal Bovine Serum, 1% Penicillin-Streptomycin, 2 mM Glutamine in DMEM, and 1% Non-Essential Amino Acids Solution may be used.
2. Prepare 1× PBS buffer from 10× PBS stock. Store it at room temperature (20°C–25°C) for up to 6 months.
3. Prepare CSK solution on the day of the experiment. Filter by 0.2 μm filter.
4. Prepare $MgCl_2$ buffer, if telomere *in situ* hybridization step is performed. Store at 20°C–25°C for up to 6 months.
5. Prepare hybridization mixture on the day of the experiment, if telomere *in situ* hybridization step is performed.
6. Prepare hybridization wash buffer on the day of the experiment, if telomere *in situ* hybridization step is performed.
7. Make 1× PBS-T washing buffer. Mix 1× PBS (pH 7.4) with 0.05% (vol/vol) Tween-20. Store it at 20°C–25°C for up to 6 months.
8. Prepare Duolink*InSitu* Wash Buffers A and B. One bag of powder is dissolved in 1 L ultrapure water. The stock can be stored for months at 4°C or at 23°C–28°C for 1 week. These specific buffers are used throughout the washing steps.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Anti-TRF1 antibody (Mouse monoclonal, 1: 500) PRID: AB_2201461	Abcam	Cat#ab10579
Anti-TRF2 antibody (Rabbit monoclonal, 1: 500) PRID: AB_10866674	Abcam	Cat#ab108997
Anti-FLAG antibody (Mouse monoclonal, 1: 500) PRID: AB_262044	Sigma-Aldrich	Cat#F1804
Anti-53BP1 antibody (Rabbit polyclonal, 1: 1000) PRID: AB_10003037	Novus Biologicals	Cat#NB100-304
Anti-p-Histone H2A.X (Ser 139) antibody (Mouse monoclonal, 1: 1000) PRID: AB__2783871	Santa Cruz	Cat#sc-517348
Anti-Biotin antibody (Mouse monoclonal, 1: 500) PRID: AB_258625	Sigma-Aldrich	Cat#B7653
Anti-Biotin antibody (Rabbit polyclonal, 1: 500) PRID: AB_867860	Abcam	Cat#ab53494
Goat Anti-Rabbit IgG H&L (Alexa Fluor® 488) (against Rabbit TRF2 and 53BP1, 1: 500) PRID: AB_2630356	Abcam	Cat#ab150077
Goat Anti-Mouse IgG H&L (Alexa Fluor® 488) [against Mouse H2A.X (Ser 139), 1: 500] PRID: AB_2576208	Abcam	Cat#ab150113

(*Continued on next page*)

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Donkey Anti-Rabbit IgG H&L (Alexa Fluor® 568) (against Rabbit Biotin, 1: 500) PRID: AB_2783823	Abcam	Cat#ab175470
Goat Anti-Mouse IgG H&L (Alexa Fluor® 568) (against Mouse TRF1, FLAG, and Biotin, 1: 500) PRID: AB_2895153	Abcam	Cat#ab175473
Experimental models: Cell lines		
U2OS	ATCC	ATCCHTB-96™
Critical commercial assays		
Duolink® *In Situ* Detection Reagents FarRed	Sigma-Aldrich	Cat#DUO92013-100RXN
Duolink® *In Situ* PLA® Probe Anti-Rabbit PLUS	Sigma-Aldrich	Cat#DUO92002-100RXN
Duolink® *In Situ* PLA® Probe Anti-Mouse MINUS	Sigma-Aldrich	Cat#DUO92004-100RXN
Duolink® *In Situ* Wash Buffers, Fluorescence	Sigma-Aldrich	Cat#DUO82049-4L
Telomere—Biotin probe $(CCCTAA)_4$	Integrated DNA Technologies	Custom synthesis
Chemicals and peptides		
1 M Tris HCl, pH 7.5	Thermo Fisher Scientific	Cat#15567027
1 M $MgCl_2$	Quality Biological	Cat#351-033-721
Citric Acid	Sigma-Aldrich	Cat#27487
Na_2HPO_4	Sigma-Aldrich	Cat#S9763
Blocking Reagent	Sigma-Aldrich	Cat#11096176001
Formamide	Thermo Fisher Scientific	Cat#BP228-100
Trypsin-EDTA	Thermo Fisher Scientific	Cat#25200056
Trypsin neutralization solution	Thermo Fisher Scientific	Cat#R002100
10× PBS buffer	Sigma-Aldrich	Cat#P5493
Tween-20	Sigma-Aldrich	Cat#P1379
MeOH	Sigma-Aldrich	Cat#67-56-1
NaCl	Sigma-Aldrich	Cat#S9888
Sucrose	Sigma-Aldrich	Cat#S0389
PIPES	Sigma-Aldrich	Cat#P6757
Triton X-100	MilliporeSigma	Cat#X100
block	MilliporeSigma	Cat#A9418
Deionized formamide, pH 7.0	Ambion	Cat#AM9344
Glutamine	Thermo Fisher Scientific	Cat#P36931
Other		
0.2 μm filter	Thermo Fisher Scientific	Cat#5963320
Bovine Calf Serum	Thermo Fisher Scientific	Cat#A3520502
Penicillin-Streptomycin	Thermo Fisher Scientific	Cat#15140148
DMEM	Thermo Fisher Scientific	Cat#11971025
ProLong™ Gold Antifade Mountant with DAPI	Thermo Fisher Scientific	Cat#P36931
Poly-Lysine coated Slide	Masterflex	Cat#ZY-75955-45
35 mm Dish | No. 1.5 Coverslip | 10 mm Glass Diameter | Collagen Coated	MatTek	Cat#P35GCOL-1.5-10-C
Refrigerated incubator	N/A	N/A
Oven for hybridization	N/A	N/A
Benchtop centrifuge	N/A	N/A
Cytospin centrifuge*	Shandon	Cat#4010121 GB
Confocal Fluorescent Microscope*	ZEISS	Cat#LSM 710
Incubator for cell culture	N/A	N/A
cell counter	N/A	N/A
Orbital shaker	N/A	N/A

Alternatives: We list the suppliers of standard molecular biological reagents and buffers. Similar products from other suppliers can be utilized.

MATERIALS AND EQUIPMENT

CSK solution		
Reagent	Final concentration	Amount
NaCl (1 M)	100 mM	1 mL
PIPES pH6.8 (1 M)	10 mM	100 μL
Sucrose (1 M)	300 mM	3 mL
$MgCl_2$ (1 M)	3 mM	30 μL
Triton X-100	0.5%	50 μL
ddH_2O	N/A	5.82 mL
Total	**N/A**	**10 mL**
Make fresh and mix by vortexing.		

$MgCl_2$ solution		
Reagent	Final concentration	Amount
$MgCl_2$ (1 M)	25 mM	250 μL
Citric Acid	9 mM	0.02 g
Na_2HPO_4	82 mM	0.12 g
Total	**N/A**	**10 mL**
Mix by vortexing. Store at 20°C–25°C for up to 6 months.		

Hybridization Mixture		
Reagent	Final concentration	Amount
Formamide (ultrapure, pH 7–7.5)	60%	150 μL
Blocking Reagent in Formamide 2.5%	0.25%	25 μL
Telomere—Biotin $(CCCTAA)_4$ probe	0.5 μL/mL	2.5 μL
$MgCl_2$ Buffer	5%	21.4 μL
Tris, pH7.5 (1 M)	10 mM	2.5 μL
ddH_2O	N/A	48.6 μL
Total	**N/A**	**250 μL**
Make fresh on the day of the experiment. Store on ice. Mix thoroughly by gentle inversion before application. Make 50 μg/mL telomere probe stock in formamide. Store the probe stock at −80°C for up to 12 months.		

Note: Dissolve blocking reagent in formamide (2.5%), vortex the mixture, then heat the mixture in heating block for 10 min.

Hybridization wash buffer		
Reagent	Final concentration	Amount
Tris-HCl pH 7.5 (1 M)	10 mM	0.1 mL
Formamide	70%	7 mL
Bovine serum albumin	0.1%	0.01 g
ddH_2O	N/A	2.9 mL
Total	**N/A**	**10 mL**
Make fresh on the day of the experiment. Mix thoroughly by gentle inversion.		

⚠ CRITICAL: Formamide is a hazardous substance and a known teratogen. It must be disposed properly. Working with it in a fume hood is critical for the health of the experimenter.

STEP-BY-STEP METHOD DETAILS

Cell and coverslip preparation – Day 0

Timing: variable; 6 h to several days

1. Wash cells with 1× PBS at 20°C–25°C. Dissociate cultured cells with Trypsin-EDTA for 5 min.

   Alternatives: Any cell dissociation method may also work in this step.

2. Neutralize cells trypsin neutralization solution or with an equal volume of cell culture medium supplemented with 10% or 20% Bovine Calf Serum or Fetal Bovine Serum.
3. Suspend cells by pipetting the cell culture medium several times.
4. Spin at 300 g for 3 min at 20°C–25°C using a benchtop centrifuge to pellet cells. Meanwhile, count cells using a hemocytometer manually or a cell counter.
5. Pour out the remaining medium and resuspend cells in a 35 mm plate with a 10 mm collagen-coated coverslip, using 2 mL fresh medium to obtain approximately 150,000 cells/mL.

   Note: The plating density may vary according to the cell type, culture time, and following steps. We suggest conducting preliminary experiments with several seeding densities to reach the best plating density, i.e., 80% confluency for optimal imaging.

   Optional: Sterilized collagen-coated coverslip(s) could be placed at the bottom of a culture dish (e.g., 24-well, 12-well, and 6-well plate). Leave the plate at 20°C–22°C for 30 min which may slow down the speed of adhesion and is helpful if cells tend to clump (troubleshooting 1).

6. Incubate cells at 37°C for 6 h to several days, until cells reach to the desired confluency.
7. When using suspension cells, for example, peripheral blood mononuclear cells, spin cells (72.26 g for 3 min) onto the poly-lysine coated slide using a cytospin centrifuge, before permeabilization and fixation steps.

Cell permeabilization, fixation, and blocking (Day 1)

Cell permeabilization

Timing: 30 min

This step removes non-specific background due to signal from membranes, extracellular matrix, etc., Any non-specific loose associations are typically disrupted by the mild detergent, while true tight binding interactions are not affected.

8. Wash cells with 1× PBS for 3 × 5 min at 20°C–25°C.
9. Treat cells with 100 μL cytoskeletal (CSK) solution for 5 min on ice.
10. Remove the CSK solution gently with pipet.
11. Treat cells with the CSK solution for another 5 min on ice.

Fixation

Timing: 50 min

This step preserves cells from decay and stabilizes cellular structure.

12. Wash cells with 1× PBS for 3 × 5 min at 20°C–25°C.
13. Treat cells with 100 μL pre-cooled 100% MeOH for 20 min at −20°C.

The fixation time may vary according to the cell type. For cell types with high cytoplasmic background, reducing the fixation time to 10–15 min.

Pause point: Fixed cells can be stored in 1× PBS at 4°C for 24 h or transferred to 70% ethanol and stored at −20°C for up to a week. The plates should be sealed with parafilm to prevent drying out during storage, and should be warmed to 20°C–25°C before proceeding the blocking step.

Blocking

Timing: 1 h 20 min

This step minimizes non-specific binding of primary antibodies within the cell.

14. Wash cells with 1× PBS for 3 × 5 min at 20°C–25°C.
15. Block cells in 100 μL Blocking solution (provided by Duolink® *In Situ* PLA® Probe kit) for 1 h at 37°C. Alternatively, block cells in 10% BSA in PBS-T.

Telomere *in situ* hybridization (Day 1–2)

Timing: 2 h 40 min

This step enables the stable hybridization of the probe to telomere DNA and washes away any excess probe.

16. Prepare hybridization mixture containing the telomere—biotin probe (Integrated DNA Technologies).

Note: Biotin DNA probe may be synthesized by other venders, such as Eurofins Scientific.

17. Tap off blocking solution, add hybridization mix to the central coverslip, and place the plate inside a pre-heated humidified chamber.
18. Preheat hybridization oven till reaching to 80°C. Incubate the plate in a humidified chamber (approximately 85% humidity) in preheated oven at 80°C for 3 min to denature DNA.

Note: Time to denature DNA in 80°C oven can be extended from 3 min to 10 min when using mouse cells with extralong telomeres, such as mouse embryonic fibroblasts derived from C57BL/6 strain.

19. Incubate the plate in a humidified chamber for 2 h at 20°C–25°C.
20. Wash cells with the hybridization wash buffer for 3 × 5 min, and with 1× PBS for 3 × 5 min at 20°C–25°C.

Note: This step is only applied for detecting telomeric DNA by Telomere-FISH using the telomere—biotin probe, if not, skip to "Primary antibodies".

Proximity ligation assay (Day 1–2)

All reagents besides enzymes should be thawed at 20°C–25°C and vortex before use. Enzymes should be kept at −20°C and added to the reaction mix before use. The 5× stock solutions should be diluted with high purity water before use. All incubations should be performed in a humidity chamber. A 40 μL reaction volume can cover 1 cm^2 sample area on the coverslip. An open droplet reaction is used. The entire reaction area must always be covered by reagents/solutions.

Table 1. Primary and secondary antibodies for PLA, IF, and IF-Telomere FISH

PLA	Primary antibodies	Secondary antibodies	IF	Telomere FISH
TRF1 and TRF2	Anti-TRF1 antibody (Mouse) Anti-TRF2 antibody (Rabbit)	Goat Anti-Mouse IgG H&L (Alexa Fluor® 568) Goat Anti-Rabbit IgG H&L (Alexa Fluor® 488)	√	
FLAG and 53BP1	Anti-FLAG antibody (Mouse) Anti-53BP1 antibody (Rabbit)	Goat Anti-Mouse IgG H&L (Alexa Fluor® 568) Goat Anti-Rabbit IgG H&L (Alexa Fluor® 488)	√	
Telomere DNA and 53BP1	Anti-Biotin antibody (Mouse) Anti-53BP1 antibody (Rabbit)	Goat Anti-Mouse IgG H&L (Alexa Fluor® 568) Goat Anti-Rabbit IgG H&L (Alexa Fluor® 488)	√	√
Telomere DNA and γ-H2AX	Anti-Biotin antibody (Rabbit) Anti-γ-H2AX antibody (Mouse)	Donkey Anti-Rabbit IgG H&L (Alexa Fluor® 568) Goat Anti-Mouse IgG H&L (Alexa Fluor® 488)	√	√

Primary antibodies

Timing: variable; 1 h to 12 h

Two primary antibodies specifically recognize each of the two proximity targets (e.g., DDR proteins and telomere DNA/proteins) in the cell. Primary antibodies must be raised in different species.

21. Discard the blocking solution, and wash cells with 1× PBS for 3 × 5 min at 20°C–25°C, if telomere *in situ* hybridization step is skipped. Dilute the primary antibodies (Table 1) to suitable concentrations (e.g., anti-TRF1, anti-TRF2 or anti-Biotin at 1:500 for detecting telomeres; anti-53BP1 or anti-γ-H2AX at 1:1000 for detecting DDR) in Antibody diluent (provided by Duolink® *In Situ* PLA® Probe kit) and add solutions to each sample (50 μL/coverslip).
22. Incubate the plate in a humidity chamber ~12 h (overnight) at 4°C or 1 h at 37°C.

PLA probes

Timing: 1 h 40 min

Secondary antibody-coupled PLA MINUS and PLA PLUS oligonucleotides (or probes) recognize and bind two individual primary antibodies according to their host species.

23. Preparation of PLA probe solution: mix and dilute the two PLA probes 1:5 in Antibody diluent, e.g., for a 40 μL reaction, take 8 μL of PLA probe MINUS stock, 8 μL of PLA probe PLUS stock, and 24 μL of the antibody buffer (provided by Duolink® *In Situ* PLA® Probe kit), and mix the solution by pipetting up and down. Allow the mixture to sit for 20 min at 20°C–25°C.
24. Tap off the primary antibody solution from the coverslip at the bottom of the plate.
25. Wash the plate with 2 mL 1× Wash Buffer A on a shaker with gentle orbital shaking for 3 × 5 min at 20°C–25°C. Tap off the excess wash buffer at the last wash.
26. Add 40 μL PLA probe solution.
27. Preheat a humidity chamber till 37°C. Incubate the plate in pre-heated humidity chamber for 1 h at 37°C.

Ligation

Timing: 1 h 10 min

Connecting oligonucleotides in the Ligation solution hybridize the PLA PLUS and MINUS probes, if both probes are in proximity, which prompts the PLA probes to ligate and form a DNA circle.

28. Dilute the Ligation solution 1:5 in ddH_2O, e.g., for a 40 μL reaction, mix 8 μL of 5× Ligation solution (provided by Duolink® *In Situ* Detection Reagents FarRed kit) and 31 μL of ddH_2O.
29. Tap off the PLA probe solution from the coverslip at the bottom of the plate.

30. Wash the plate with 2 mL 1× Duolink *In Situ* Wash Buffer A for 3 × 10 min under gentle agitation on a shaker. Discard the excess wash buffer A at the last wash step.
31. Add the Ligase (provided by Duolink® *In Situ* Detection Reagents FarRed kit) to the Ligation solution at a 1:40 dilution (e.g., for a 40 μL reaction add 1 μL of Ligase to 39 μL of Ligation solution) and vortex.
32. Add 40 μL Ligation-Ligase solution to each coverslip.
33. Incubate the plate in a pre-heated humidity chamber for 30 min at 37°C.

Amplification

Timing: 1 h 50 min

The DNA polymerase Phi29 pol generates many complement copies of the DNA circle, followed by hybridization to fluorophore-conjugated nucleotides to produce the signals, which can be detected by standard fluorescence microscopy.

34. Preparation of Amplification solution: dilute the Amplification stock (provided by Duolink® *In Situ* Detection Reagents FarRed kit) 1:5 in ddH_2O and mix (e.g., for a 40 μL reaction, mix 8 μL of 5× Amplification stock and 31.5 μL of ddH_2O).
35. Tap off the Ligation-Ligase solution from the coverslip at the bottom of the plate.
36. Wash the plate in 2 mL 1× Duolink *InSitu* Wash Buffer A for 2 × 3 min under gentle agitation on a shaker. Tap off all wash solution after the last wash.
37. Add the Polymerase (provided by Duolink® *In Situ* Detection Reagents FarRed kit) to the Amplification solution at a 1:80 dilution and vortex (e.g., for a 40 μL reaction, mix 0.5 μL of Polymerase and 39.5 μL of Amplification solution).
38. Add the Amplification-Polymerase solution to each coverslip.
39. Incubate the plate in a pre-heated humidity chamber for 100 min at 37°C, covered with foil.

Note: Let the coverslip dry briefly at 20°C–25°C in the dark. Mount the coverslip onto the slide using ProLong™ Gold Antifade mounting solution with DAPI, if secondary antibody immunofluorescence staining step is skipped. Seal the edges with nail polish.

⚠ CRITICAL: Containing light-sensitive reagents: Work in reduced-light environment from this time forth.

Secondary antibody immunofluorescence staining (Day 2)

Timing: variable; 2 h to 12 h

This step detects the DDR proteins and the telomeric proteins by IF or the telomeric DNA by Telomere FISH, which helps verify PLA and could be omitted after the PLA procedure is successfully established (See details in "expected outcomes").

40. Tap off the Amplification-Polymerase solution from the coverslip at the bottom of the plate.
41. Wash the plate in 2 mL 1× Duolink*InSitu* Wash Buffer B for 4 × 10 min and then in 0.01 × Wash Buffer B for 5 min.
42. Wash the plate in 2 mL 1× PBS for 1 × 5 min at 20°C–25°C.
43. Dilute the secondary antibodies conjugated with fluorophores (Table 1) to suitable concentrations, e.g., 1:500 in the Antibody Diluent and add 40 μL secondary antibody solution to each coverslip. Incubate the plate in a humidity chamber ~12 h at 4°C or 1 h at 37°C.
44. Tap off the secondary antibody solution from the plate. Wash the plate with 2 mL 1× PBS-T on a shaker with gentle orbital shaking for 3 × 5 min at 20°C–25°C.

45. Let the coverslip dry briefly at 20°C–25°C in the dark. Mount the coverslip onto the slide using the ProLong™ Gold Antifade mounting solution with DAPI (0.01 mg/mL). Seal the edges with nail polish.

Pause point: Slides can be stored at 4°C for up to 2 months or −20°C for extended time before microscope image acquisition.

Image acquisition of PLA, IF, telomere-FISH signals by fluorescent microscope

Typically, images from 20–30 fields per slide are captured in z-stacks in four fluorescent channels, using a confocal fluorescent microscope with 100× (oil) objective. Capture images using a DAPI filter for nuclear staining, Cy5 filter (when DuolinkFarRed detection kit is used) for detection of PLA signals, FITC, Cy3, or Texas Red filters for detection of different targets (telomeric markers, e.g., TRF1, TRF2, or Biotin. DDR proteins, e.g., 53BP1 or γ-H2AX. To remove background from the image, adjust the brightness and contrast of the captured raw images using software, such as ImageJ or Adobe photoshop. Of note, the adjustment should be applied to all the images of the experiment.

Note: Capture all the signals in z-stacks. Each channel should have the same z-stacks.

Note: Acquisition of each channel should avoid binning.

Note: Exposure time should be adjusted to keep all channels with a good signal-to-noise ratio (such as >3-fold) and prevent the signals to be oversaturated. DAPI signal intensity versus IF, Telomere FISH, and PLA signals is supposed to be no less than 5:1 ratio. IF, Telomere FISH, and PLA signals should be easy to discriminate.

Note: Count TIF numbers per nucleus in PLA images, such as ≥5 TIFs/cell, followed by quantification of the percentage of cells with ≥5 TIFs. TIF signals by IF or IF-Telomere FISH are determined by colocalization of DDR proteins (such as 53BP1 and γ-H2AX) and telomeric proteins (such as TRF1 and TRF2) IF signals or by colocalization of 53BP1 or γ-H2AX IF signals and Telomere-FISH signals. Successful PLA procedure is indicated by the colocalization of most TIF signals by PLA with those by IF or IF-Telomere FISH. The IF (or IF-Telomere FISH) approaches serve to verify PLA, which can be omitted. See details in "expected outcomes".

Note: The representation of the data can be performed in any graph making software, e.g., GraphPad Prism to display TIF numbers per cell by column graph or a box-and-whisker plot.

⚠ CRITICAL: If slides are stored at 4°C or −20°C before imaging, warm slides at 20°C–25°C for 1 h or 37°C for 10 min to prevent possible condensation on the surface of slides. Use 70% ethanol to clean slides.

EXPECTED OUTCOMES

TIF formation is routinely determined by IF or IF-Telomere FISH (Hockemeyer et al., 2006; Takai et al., 2003). However, these assays have limited sensitivity in identifying DDR proteins and telomere markers. In addition, some DDR proteins, such as γ-H2AX are well known for spreading over mega bases across the site of DNA damage (Iacovoni et al., 2010) and thus colocalization of γ-H2AX foci with a telomeric marker measured by IF or IF-Telomere FISH may not be positively indicative of its telomeric origin. To overcome these pitfalls, we included the PLA step in the IF or IF-Telomere FISH assays. Of note, the IF or IF-Telomere FISH steps could be omitted after the PLA procedure is successfully established.

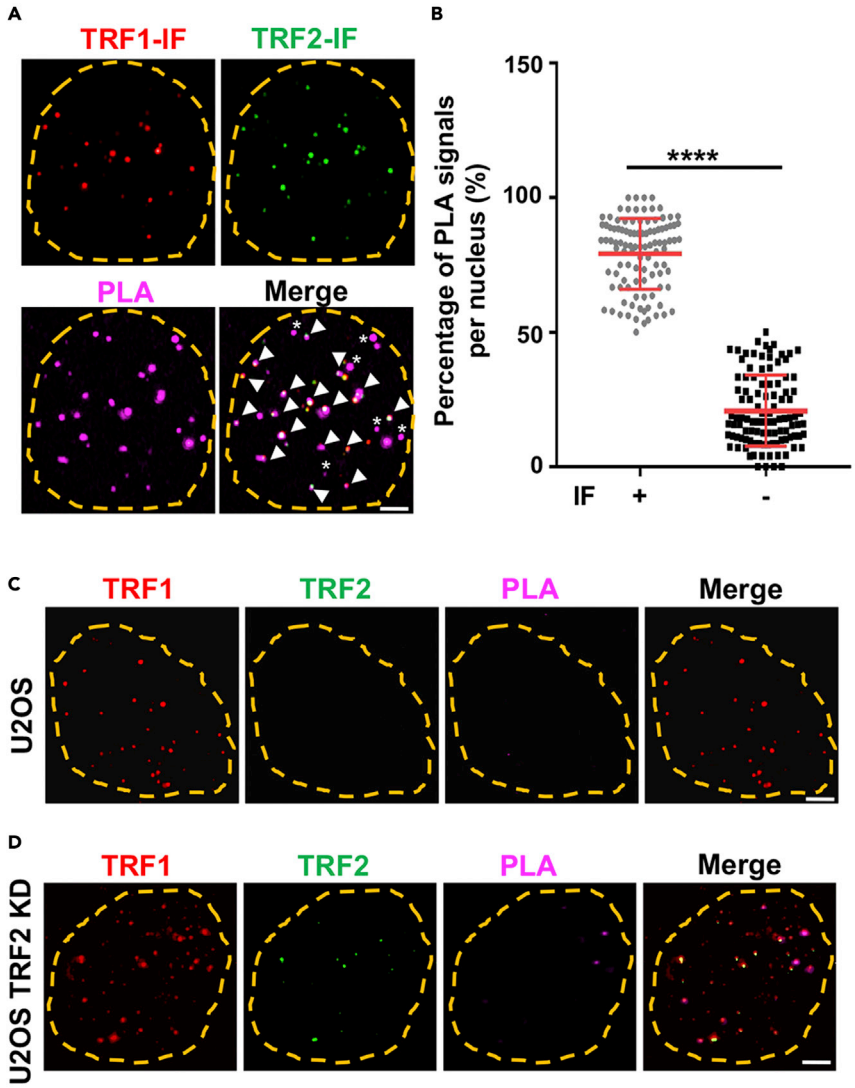

Figure 1. TIF-PLA detects telomeres in U2OS cells

(A) PLA detects telomeres using TRF1 and TRF2 as proximity ligation targets. PLA signals colocalize with TRF1 and TRF2 immunofluorescence (IF) signals (representative arrows). Of note, PLA also yields telomere signals that are not shown by TRF1 and TRF2 IF analysis (representative asterisks).

(B) Quantifications of PLA signals that colocalize with TRF1 and TRF2 IF signals (IF +) and PLA signals where TRF1 and TRF2 IF signals are not detected (IF -).

(C) PLA without the antibody against TRF2.

(D) PLA was performed between TRF1 and TRF2 in TRF2 knockdown U2OS cells. Scale bars: 5 microns.

****$p < 0.0001$, which was determined by Student's unpaired t tests. Data are mean ± SEM.

To test the feasibility of PLA in detecting telomeres, we performed PLA between the two telomeric proteins, TRF1 and TRF2, both of which bind directly to telomeric DNA and are the components of the shelterin protein complex (de Lange, 2018). For TRF1 and TRF2 IF step, fluorophore-conjugated secondary antibodies (steps 40–45) were used to detect the primary antibodies against TRF1 or TRF2 (steps 21 and 22). Majority of PLA signals colocalized with the TRF1 and TRF2 IF signals (Figure 1A, arrows, Figure 1B), demonstrating that PLA is able to detect telomeres and that the PLA and IF approaches serves to verify each other. Moreover, a fraction of PLA signals is not visualized by the conventional IF (Figure 1A, asterisks, Figure 1B), indicating that PLA is more sensitive in detecting telomeres, compared to the IF assay. As negative controls, PLA did not yield any signals, when

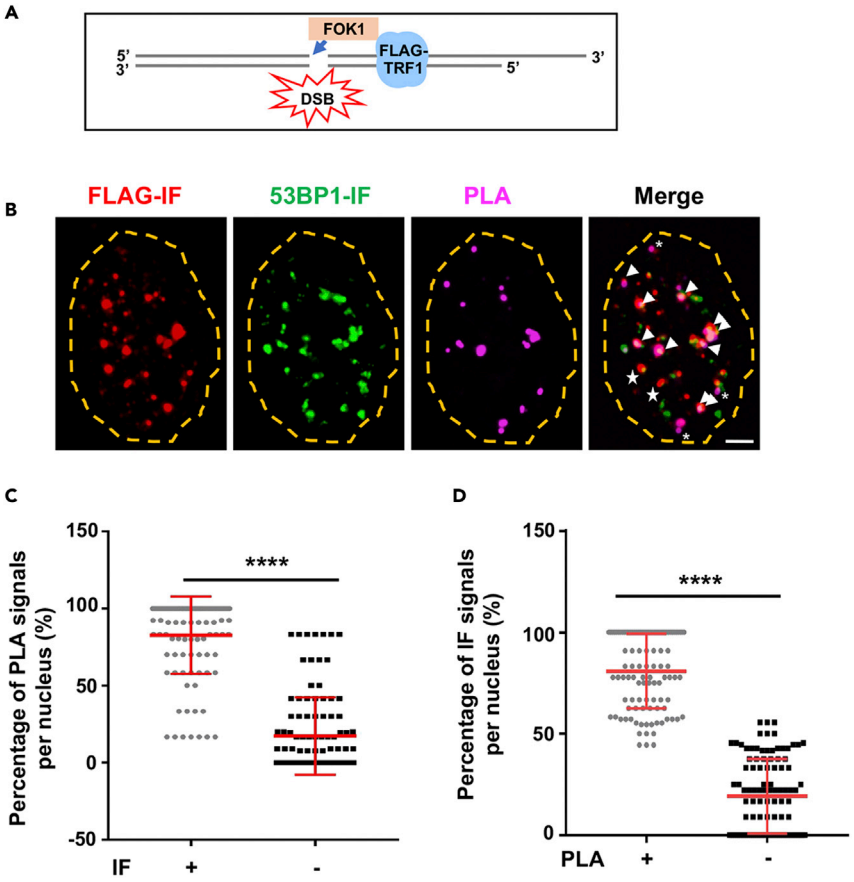

Figure 2. TIF-PLA detects TIFs in U2OS cells expressing the FLAG-TRF1-FOKI fusion protein

(A) Schematic of telomere-specific double strand break (DSB) induction by the FLAG-TRF1-FOKI fusion protein.

(B) PLA detects TIFs using 53BP1 and FLAG proximity ligation targets (arrows). PLA also detect TIF signals that are not shown by 53BP1 or FLAG IF analysis (asterisks). Notably, some 53BP1 and FLAG IF signals were not detected by PLA (stars).

(C) Quantifications of PLA signals that colocalize with FLAG and 53BP1 IF signals (IF +) and PLA signals where FLAG and 53BP1 IF signals are not detected (IF -).

(D) Quantification of IF signals that colocalize with PLA signals (PLA +) and IF signals where PLA signals are not detected (PLA -). Scale bars: 5 microns.

****$p < 0.0001$, which was determined by Student's unpaired t tests. Data are mean ± SEM.

PLA was performed in the absence of one or both of the primary antibodies against TRF1 and TRF2 (Figure 1C) or in the TRF2 knockdown cells (Figure 1D). These negative controls further confirmed the accuracy of PLA in detecting telomeres as well as the specificity of the antibodies utilized in PLA.

Next, we tested the feasibility of PLA in detecting TIFs using U2OS cells expressing the FLAG-TRF1-FOK1 fusion protein that specifically generates DNA double-strand breaks at telomeres and thereby induces TIFs (Cho et al., 2014; Tang et al., 2013) (Figure 2A). We performed PLA between 53BP1 and FLAG. For 53BP1 and FLAG IF step, fluorophore-conjugated secondary antibodies (steps 40–45) were added to detect the primary antibodies against 53BP1 and FLAG (steps 21 and 22). PLA between 53BP1 and FLAG yielded signals, which also colocalized with the 53BP1 and FLAG IF signals (Figure 2B, arrows). In addition, some PLA signals were not detectable by the 53BP1 or FLAG IF assays (Figure 2B, asterisks, Figure 2C), illustrating that PLA is more sensitive in detecting TIFs. Notably, some "TIF" signals, visualized by colocalization of the 53BP1 and FLAG IF signals, are negative for PLA (Figure 2B, stars, Figure 2D), indicating that 53BP1 is not within proximity with telomeres and likely has a non-telomeric origin. We also observed similar results using PLA between 53BP1 and

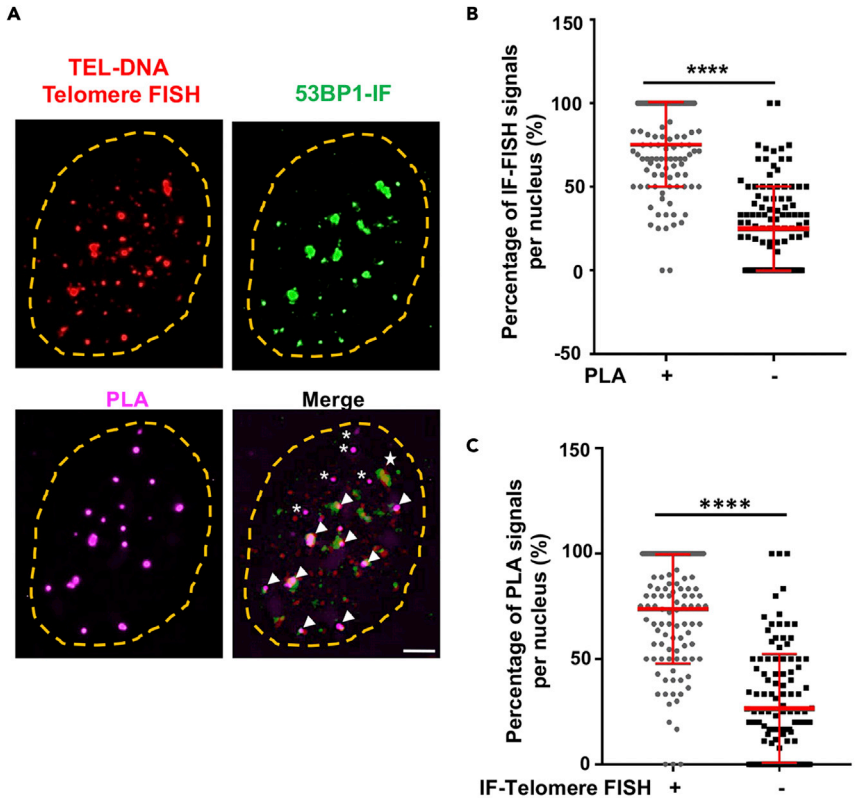

Figure 3. PLA detects TIFs using 53BP1 and biotin-labeled telomeres as proximity ligation targets in U2OS cells expressing the FLAG-TRF1-FOKI fusion protein

(A) PLA signals colocalize with 53BP1 IF signals and telomeric DNA signals (TEL-DNA) by Telomere FISH using the telomere—Biotin $(CCCTAA)_4$ probe (arrows). PLA also detects signals that are not shown by IF-Telomere FISH analysis of telomeric DNA and 53BP1 (asterisks). Some IF-Telomere FISH signals were not detected by PLA (stars).

(B) Quantification of IF-Telomere FISH signals that colocalize with PLA signals (PLA +) and IF-Telomere FISH signals where PLA signals are not detected (PLA -).

(C) Quantification of PLA signals that colocalize with IF-Telomere FISH signals (+) and PLA signals where IF-Telomere FISH signals are not detected (-). Scale bars: 5 microns.

****$p < 0.0001$, which was determined by Student's unpaired t tests. Data are mean ± SEM.

telomeric DNA, combined with IF-Telomere FISH that utilized fluorophore-conjugated secondary antibodies (steps 40–45) to detect the primary antibodies against 53BP1 and Biotin (steps 16–22) (Figure 3). Our data support the notion that addition of the PLA step to the conventional IF and IF-Telomere FISH improves accuracy and sensitivity in TIF detection.

To test the workability of this protocol in mouse cells, we performed PLA between γ-H2AX and telomere DNA in combination with the γ-H2AX IF and Telomere-FISH in *Trf2* null mouse embryonic fibroblasts (Celli and de Lange, 2005). *Trf2* deletion leads to telomere uncapping and generates TIFs in these cells (Celli and de Lange, 2005). TIF formation was verified by TIF-PLA (Figure 4A, arrows). PLA can detect more TIF signals than conventional γ-H2AX IF and Telomere FISH (Figure 4A, asterisks, Figure 4B). Thus, our protocol is also suitable for TIF detection in mouse cells.

LIMITATIONS

This protocol includes multiple washing steps, likely stripping cells off coverslips. Seeding cells at a higher density helps overcome this limitation (See troubleshooting Problem 1). Improper permeabilization, fixation, or hybridization may cause a low signal-to-noise ratio. The fixation step may destroy

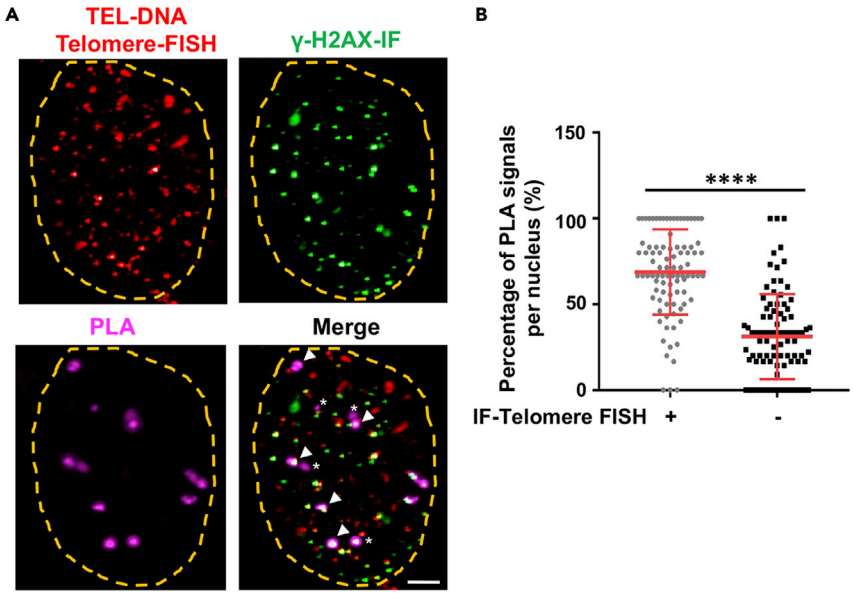

Figure 4. TIF-PLA detects TIFs in *Trf2* null MEFs

(A) PLA detects TIFs using γ-H2AX and biotin-labeled telomeres as proximity ligation targets. PLA signals colocalize with telomeric DNA (TEL-DNA) and γ-H2AX signals by IF-Telomere FISH (arrows). PLA also detects signals that are not shown by IF-Telomere FISH analysis of telomeric DNA and γ-H2AX (asterisks).

(B) Quantifications of PLA signals that colocalize with IF-Telomere FISH signals (+) and PLA signals where IF-Telomere FISH signals are not detected (-). Scale bars: 5 microns.

****$p < 0.0001$, which was determined by Student's unpaired t tests. Data are mean ± SEM.

antibody binding sites and reduce cell staining intensity. Therefore, choosing optional antibodies for PLA helps achieve satisfactory results.

TROUBLESHOOTING

Problem 1

Refer to steps 1–7 (Cell and coverslip preparation).

Cells are too sparse (Figure 5A) or too dense/clumped (Figure 5B) to produce high quality image. Optimal confluence of cells is 50%–70%, according to the manufacturer's instructions (https://www.abcam.com/ps/pdf/protocols/cell_culture.pdf). The ideal cell density used for imaging in this protocol is about 80% (Figure 5C).

Potential solution

Proper cell seeding density should be determined using various cell concentrations.

Cells could be lost during washing steps. Therefore, it is recommended to check cell density by a phase-contrast microscope after permeabilization, fixation, and final wash steps. It is helpful to seed cells at a higher density, reduce the number of washes, and increase the washing temperature (e.g., from 25°C to 30°C). To avoid cells from clumping, seed cells onto the plates at 20°C–25°C for 30 min, before placing the plates in the incubator.

Problem 2

Refer to steps 8–20 (cell permeabilization and Telomere *in situ* hybridization (Day 1–2)).

DAPI signals fail to reveal clear nuclear boundary.

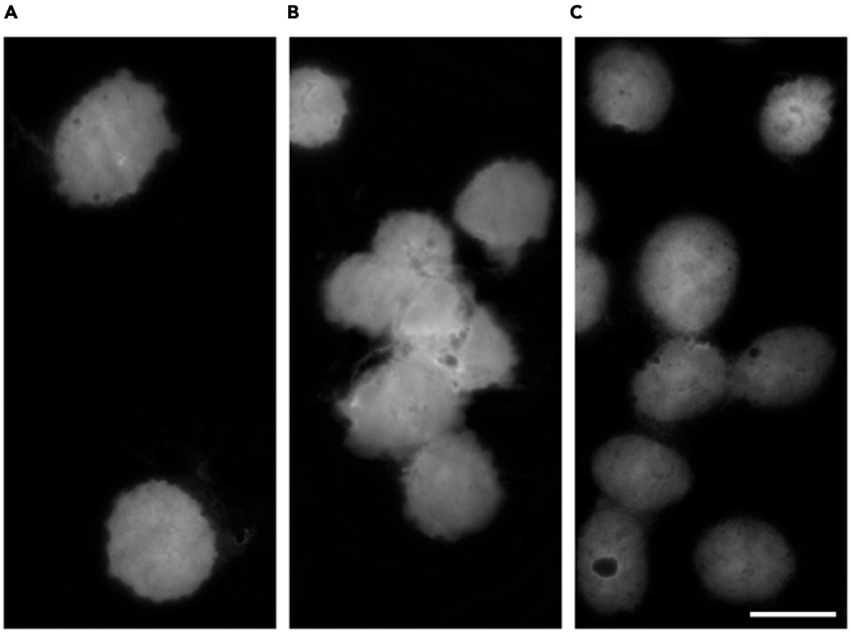

Figure 5. DAPI staining verifies proper cell density

(A) Sparse cells may require more imaging time to be fully sampled.

(B) Cells in clump are indistinguishable from one and another by imaging.

(C) Ideal density of adherent cells for imaging is usually about 80%. Scale bars: 25 microns.

Potential solution

DAPI specifically stains nucleus, thereby helping identify DDR proteins and telomere markers. Excessive permeabilization and too hot/long denaturation can damage the nuclear membrane and destroy the nuclear edges that would affect DAPI nuclear staining pattern and thereby downstream analysis. If this problem occurs, consider shortening the CSK Triton solution treatment to 3 min, reducing the concentration of the Triton solution to 0.2%, and replacing Triton with ethanol for gentler permeabilization (Related to steps 8–11 cell permeabilization). For gentler denaturation, the denaturation temperature and time can be reduced to 75°C and 2 min, respectively (Related to steps 16–20 Telomere *in situ* hybridization (Day 1–2)).

Problem 3

Refer to steps 16–20 (Telomere *in situ* hybridization (Day 1–2)).

Images with high background signals (or low signal to noise ratio).

Potential solution

Excess amount of primary antibody, insufficient blocking, drying of samples, precipitates in buffers, natural autofluorescence, fixation, or hybridization problems may cause high background signals. Selecting optimized primary antibody amount and blocking time are critical. Drying of samples may result in nonspecific labeling, *i.e.*, antibodies may attach nonspecifically to dry cells. During any washing steps, cells should not be allowed to dry out. Precipitates in buffers should be filtered before use.

Usually, permeabilization and fixation time, cell types, and hybridization solution impact background signals. Reducing the MeOH concentration and/or the permeabilization or fixation time could help reduce cytoplasmic background caused by over-fixation. Natural autofluorescence may be cell-type specific. The quality of hybridization solution and reagents, especially the probe

may cause low hybridization signals with high nuclear background (Related to steps 16–20 Telomere *in situ* hybridization (Day 1–2)). Selecting optimized probes are critical, which would prevent any hybridization to non-specific targets. In addition, increasing hybridization temperature and wash time, or adding formamide to the hybridization washing solution help reduce background signals.

Problem 4

Refer to steps 8–20 (cell permeabilization and Telomere *in situ* hybridization (Day 1–2)).

The PLA signal is too weak. This could reflect suboptional conditions, e.g., insufficient permeabilization or suboptiional DNA denaturation/hybridization.

Potential solution

Cells may be insufficiently permeabilized (Related to steps 8–11 cell permeabilization). This can be solved by increasing the concentration of the Triton X-100 solution to 1%, extending the permeabilization time to 10 min, or incubating samples in 0.05–0.5 mg/mL Pepsin.

Telomeric DNA may not be sufficiently denatured or the hybridization time may be too short (Related to steps 16–20 Telomere *in situ* hybridization (Day 1–2)). This problem can be solved by extending the denaturation step to 5 min, increasing temperature in the denaturation step to 85°C, or prolonging hybridization time (for example, ~12 h at 37°C).

Wash step may not be proper. Bring PLA wash buffer to 20°C–25°C before use. After each wash, make sure to completely remove the wash buffer before adding the ligation and amplification reagents. Adjust the amplification time for low abundance interactions (up to 37°C for ~12 h). Reduce the wash time.

Incubation temperature may not be proper. Perform all steps, especially blocking and enzymatic steps (PLA ligation and amplification) at the appropriate temperature.

Reagent storage and activity may not be proper. Ensure the ligase and polymerase are active and stored at –20°C.

Filter used for acquisition may not be proper. Depending on the kit used, ensure that images are acquired through a flurorescent microscope using the appropriate filters.

The amount of PLA probes may not be proper. In such case, adjust the amount of the PLA probes (Minus and Plus) to get a more pronounced signal.

Problem 5

Refer to steps 21–22 (primary antibodies), steps 34–39 (amplification), and image acquisition of PLA, IF, Telomere-FISH signals by fluorescent microscope.

Signal coalescence or poor imaging.

Potential solution

Primary antibody concentration is too high, which could induce signal coalescence. Choose the optional antibody concentration for PLA to achieve satisfactory results.

Amplification duration may not be proper. Use the recommended amplification times by the manufactures, since extended amplification durations could cause signal coalescence.

Image capture may not be proper. Overexposure during image capture may result in signal coalescence. Use appropriate settings during image capture.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Dr. Yie Liu (liuyie@mail.nih.gov).

Materials availability

This research did not produce any new unique reagents.

Data and code availability

Original data for figures are available in the figure legends.

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AUTHOR CONTRIBUTIONS

Y.L., M.M.S., and L.F. conceived the project. Y.W. performed the experiments and analyzed data. Y.L. and Y.W. wrote the manuscript. All authors read, reviewed, and/or edited the manuscript.

DECLARATION OF INTERESTS

The authors declare that they have no conflict of interest.

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