Journal of Visualized Experiments

Visualization of Replisome Encounters with an Antigen Tagged Blocking Lesion

--Manuscript Draft--

Article Type:	Invited Methods Collection - JoVE Produced Video
Manuscript Number:	JoVE61689R1
Full Title:	Visualization of Replisome Encounters with an Antigen Tagged Blocking Lesion
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TITLE:

Visualization of Replisome Encounters with an Antigen Tagged Blocking Lesion

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KEYWORDS:

replisome, blocking lesion, PLA, proximity ligation assay, DNA interstrand crosslinks, antigen-tagged crosslinks

SUMMARY:

While replication fork collisions with DNA adducts can induce double strand breaks, less is known about the interaction between replisomes and blocking lesions. We have employed the proximity ligation assay to visualize these encounters and to characterize the consequences for replisome composition.

ABSTRACT:

Considerable insight is present into the cellular response to double strand breaks (DSBs), induced by nucleases, radiation, and other DNA breakers. In part, this reflects the availability of methods for the identification of break sites, and characterization of factors recruited to DSBs at those sequences. However, DSBs also appear as intermediates during the processing of DNA adducts formed by compounds that do not directly cause breaks, and do not react at specific sequence sites. Consequently, for most of these agents, technologies that permit the analysis of binding interactions with response factors and repair proteins is unknown. For example, DNA interstrand crosslinks (ICLs) can provoke breaks following replication fork encounters. Although formed by drugs widely used as cancer chemotherapeutics, there has been no methodology for monitoring their interactions of replication proteins.

Here, we describe our strategy for following the cellular response to fork collisions with these challenging adducts. We linked a steroid antigen to psoralen, which forms photoactivation dependent ICLs in nuclei of living cells. The ICLs were visualized by immunofluorescence against the antigen tag. The tag can also be a partner in the Proximity Ligation Assay (PLA) which reports the close association of two antigens. The PLA was exploited to distinguish proteins that were closely associated with the tagged ICLs from those that were not. It was possible to define replisome proteins that were retained after encounters with ICLs and identify others that were lost. This approach is applicable to any structure or DNA adduct that can be detected immunologically.

INTRODUCTION

The cellular response to double strand breaks is well documented owing to a succession of increasingly powerful methods for directing breaks to specific genomic sites[1-3]. The certainty of location enables unambiguous characterization of proteins and other factors that accumulate at the site and participate in the DNA Damage Response (DDR) thereby driving the Non-Homologous End Joining (NHEJ) and Homologous Recombination (HR) pathways that repair breaks. Of course, many breaks are introduced by agents such as radiation and chemical species that do not attack specific sequences[4]. However, for these there are procedures available that can convert the ends to structures amenable for tagging and localization[5,6]. Breaks are also introduced by biological processes, such as immunoglobulin rearrangement, and recent technology permits their localization, as well[7]. The relationship between responding factors and those sites can then be determined.

Breaks also appear as an indirect consequence of adducts formed by compounds that are not inherent breakers but disrupt DNA transactions such as transcription and replication. They may be formed as a feature of the cellular response to these obstructions, perhaps during repair or because they provoke a structure that is vulnerable to nuclease attack. Typically, the physical relationship between the adduct, the break, and the association with responding factors is inferential. For example, ICLs are formed by chemotherapeutics such as cisplatin and Mitomycin C[8] and as a reaction product of abasic sites[9]. ICLs are well known as potent blocks to replication forks[10], thereby stalling forks which can be cleaved by nucleases[11]. The covalent linkage between strands is often relieved by pathways that have obligate breaks as intermediates[12,13], necessitating homologous recombination to rebuild the replication fork[14]. In most experiments

the investigator follows the response of factors of interest to the breaks which are formed downstream of the collision of a replication fork with an ICL. However, because there has been no technology for the localization of a provocative lesion, the proximity of the replisome, and its component parts, to the ICL can only be assumed.

We have developed a strategy to enable the analysis of protein associations with non-sequence specific covalent adducts, illustrated here by ICLs. In our system these are introduced by psoralen, a photoactive natural product used for thousands of years as a therapeutic for skin disorders[15]. Our approach is based on two important features of psoralens. The first is their high frequency of crosslink formation, which can exceed 90% of adducts, in contrast to the less than 10% formed by popular compounds such as cisplatin or Mitomycin C[8,16]. The second is the accessibility of the compound to conjugation without loss of crosslinking capacity. We have covalently linked trimethyl psoralen to Digoxigenin (Dig), a long established immunotag. This enables detection of the psoralen adducts in genomic DNA by immunostaining of the Dig tag, and visualization by conventional immunofluorescence[17].

This reagent was applied, in our previous work, to the analysis of replication fork encounters with ICLs using a DNA fiber-based assay[16]. In that work we found that replication could continue past an intact ICL. This was dependent on the ATR kinase, which is activated by replication stress. The replication restart was unexpected given the structure of the CMG replicative helicase. This consists of the MCM hetero-hexamer (M) that forms an offset gapped ring around the template strand for leading strand synthesis which is locked by the proteins of the GINS complex (G, consisting of PSF1, 2, 3, and SLD5) and CDC45 (C)[18]. The proposal that replication could restart on the side of the ICL distal to the side of the replisome collision argued for a change in the structure of the replisome. To address the question of which components were in the replisome at the time of the encounter with an ICL we developed the approach described here. We exploited the Dig tag as a partner in Proximity Ligation Assays (PLA)[19] to interrogate the close association of the ICL with proteins of the replisome[20].

PROTOCOL

1. Cell preparation

1.1. Day 1

1.1.1. Pre-treat 35 mm glass-bottomed culture dishes with a cell adhesive solution.

1.1.2. Plate cells in the pre-treated dishes one day before treatment. Cell should be actively dividing and 50–70% confluent on the day of the experiment.

NOTE: HeLa cells were used in this experiment with Dulbecco Modified Eagle Medium DMEM, supplemented with 10% fetal bovine serum, 1x penicillin /streptomycin. There is no restriction for adherent cell lines. However, non-adherent cells must be centrifuged onto slides and fixed prior to the analysis by PLA.

1.2. Day 2

1.2.1. Prepare a stock solution of Digoxigenin Trimethyl psoralen (Dig-TMP) by resuspending a frozen aliquot of previously synthesized Dig-TMP in 1:1 EtOH:H_2O. Determine the concentration by measuring OD at 250 nm of a 100x dilution (in H_2O) of the dissolved Dig-TMP. The extinction coefficient of Dig-TMP is 25,000. Verify the concentration by measuring OD at 250 nm before each use and calculate stock concentration: Abs x 100 x 10^6/25,000 = Concentration (in µM). Generally, the stock solution is around 3 mM. The solution can be stored in –20 °C for about a month.

NOTE: Dig-TMP must be chemically synthesized in advance, following the procedure described here. Reflux 4'-chloromethyl-4,5',8-trimethylpsoralen with 4,7,10-trioxa-1,13-tridecanedi-amine in toluene under nitrogen for 12 h. Remove the solvent and recover the 4'-[N-(13-amino-4,7,10-trioxatrideca)] aminomethyl-4,5',8-trimethylpsoralen product by silica gel chromatography. Conjugate the product to digoxigenin NHS ester in dimethyl formamide and triethylamine at 50 °C for 18 h. Remove the solvent and purify the residue by preparative thin layer silica gel chromatography. Elute the product band chloroform: methanol: 28% ammonium hydroxide (8:1:0.1) mixture. Evaporate the solvents and dissolve the pellet in 50% EtOH:H_2O.

1.2.2. Add Dig-TMP stock in 50% EtOH:H_2O to the cell culture medium to a final concentration of 5 µM. Bring the medium to 37 °C. Aspirate the medium from the plates, add the pre-warmed Dig-TMP containing media, and place plates in an incubator (37 °C, 5% CO_2) for 30 min to allow the Dig-TMP to equilibrate.

1.2.3. While the cells are incubating, pre-warm the UV box (see **Table of Materials**) to 37 °C.

1.2.4. Place the plates in the pre-warmed UV box and expose the cells to a dose of 3 J/cm^2 of UVA light for 5 min for this experiment. Plates were placed on top of a thermo-block maintained at 37 °C, during irradiation. Calculate the time using the formula:

$$UV\ Dose\ =\ UV\ Intensity\ (\mu W/cm^2)\ x\ Exposure\ Time\ (seconds)\ ).$$

1.2.5. Aspirate the medium using a pipette, add fresh pre-warmed medium and place plates back in the incubator at 37 °C, 5% CO_2 for 1 h.

1.2.6. Remove media and wash dishes once gently with phosphate buffered saline (PBS).

1.2.7. Remove PBS and add 0.1% formaldehyde (FA) in PBS for 5 min at room temperature (RT). This prevents cell detachment during CSK-R (cytoskeleton extraction buffer containing RNase, described in 1.2.9.) pretreatment required to extract the cytoplasmic elements and reduce PLA background.

1.2.8. Aspirate off FA and wash dishes with PBS once.

1.2.9. Add CSK-R buffer and incubate for 5 min at RT to remove cytoplasm [CSK-R buffer: 10 mM PIPES, pH 7.0, 100 mM NaCl, 300 mM sucrose, 3 mM $MgCl_2$, 0.5%Triton X-100, 300 µg/mL RNase A]. Aspirate the buffer, add fresh CSK-R, and incubate for 5 min at RT.

NOTE: A stock of CSK buffer can be stored at 4 °C, and Triton X and RNaseA added right before use.

1.2.10. Wash with PBS thrice.

1.2.11. Fix cells with 4% formaldehyde in PBS for 10 min at RT.

1.2.12**.** Wash cells with PBS. Perform this step three times.

1.2.13. Add cold 100% methanol and incubate for 20 min at -20 °C.

1.2.14. Wash cells with PBS thrice. At this point cells can be stored in PBS in a humid chamber at 4 °C for up to a week.

1.2.15. Incubate cells in 80 µL of 5 mM TritonX-100 for 10 min at 4 °C.

1.2.16. Incubate cells with 100 µL of 5 mM EDTA in PBS supplemented with 1 µL of 100 mg/mL RNase A for 30 min at 37 °C.

1.2.17. Wash cells with PBS thrice.

1.2.18. Store cells in blocking buffer (5% BSA and 10% goat serum in PBS) in a humid chamber overnight at 4 °C.

2. Proximity ligation assay

NOTE: Perform proximity ligation assay on Day 3.

2.1. Antibody staining

2.1.1. Prepare 40 µL of the primary antibody solution per plate: Add the appropriate volume of primary antibodies to achieve desired dilution (mouse anti Digoxigenin and rabbit antibody against a replisome component such as MCM5, CDC45, PSF1 or pMCM2, dilutions specified in **Table of Materials**) into blocking buffer (to reach a final volume of 24 µL). Mix by tapping and let it stand for 20 min at RT. Prepare a master mix for multiple samples and mix by tapping before applying to the wells.

2.1.2. Add 40 mL of primary antibody solution to the center of the well and incubate in a humid chamber for 1 h at 37 °C. During staining, allow the PLA probes and blocking buffer to warm to the room temperature.

2.1.3. Wash cells with PBS-T [PBS-T: 0.05% Tween-20 in 1X PBS] at RT. Perform this step three times.

2.1.4. While washing, prepare 40 µL of PLA probe solution per dish (PLA probes consist of a secondary antibody recognizing either rabbit or mouse IgG, covalently linked to a PLUS or MINUS oligonucleotide): 8 µL of PLA probe anti mouse-PLUS + 8 µL of PLA probe anti rabbit-MINUS antibody + 24 µL of blocking buffer. Mix and let it stand for 20 min at RT. Prepare a master mix for multiple samples and mix well before applying to the wells. Place the solution in the middle of the well in the plate.

2.1.5. Remove last wash, add 40 mL of PLA probe solution to the center of the well, and incubate in a humid chamber for 1 h at 37 °C.

2.1.6. Wash in buffer A thrice, for 10 min each, on a tilting platform at RT. During washing, bring the ligation mix to RT.

2.2. Ligation and amplification

2.2.1. Prepare 40 µL of Ligation mix per plate: 8 µL (5x) of ligation stock + 31 µL of distilled water + 1 µL of ligase. Prepare master mix for multiple plates and mix well before applying to the wells.

2.2.2. Add 40 µL of ligation solution to each plate and incubate in a humid chamber for 30 min. at 37 °C.

2.2.3. Wash cells 3x with buffer A, each for 2 min, on a tilting platform at RT.

2.2.4. Prepare 40 µL of amplification solution per dish: 8 µL (5x) of amplification stock + 31.5 µL of distilled water + 0.5 µL of DNA Polymerase. Prepare a master mix for multiple samples and mix well before applying to the wells.

2.2.5. Add 40 µL of amplification solution to each plate and incubate in a humid chamber at 37 °C for 100 min.

2.2.6. Aspirate off the amplification solution and wash with buffer B. Perform 6 washes each for 10 min, on a tilting platform at RT.

2.2.7. Wash once with 0.01x buffer B for 1 min at RT.

2.2.8. Aspirate buffer B and incubate plates with secondary antibodies, Alexa Fluor 488 anti-mouse IgG and Alexa Fluor 568 anti-rabbit IgG in blocking solution at appropriate dilutions in blocking buffer, in a humid chamber, for 30 min at 37 °C or overnight at 4 °C .

2.2.9. Wash cells three times with PBS-T, for 10 min each on a tilting platform at RT.

2.2.10. Aspirate PBS-T and mount in mounting medium with DAPI. The mounted plates can be imaged immediately or stored at 4 °C in the dark for no more than 4 days before imaging.

3. Imaging and quantification

3.1. Perform imaging in an epifluorescent or confocal microscope (if 3D imaging is desirable, cover at least 3 µm in 15 stacks). Perform experiments in triplicates and image enough number of fields to make at least 100 observations per sample or condition. Image all fields and samples, including controls, using the same exposure settings.

3.2. Quantify with an appropriate image analysis software (see **Table of Materials** for open source and commercial software capable of performing this analysis in single or multiple plane-images).

3.2.1. Segment cell nuclei based on the DAPI staining. Perform detection of nuclear PLA dots. Assign PLA dots to their corresponding nucleus. Export PLA dots per nucleus results as a csv file (see **Supplementary File 1** and **Supplementary File 2**).

3.3. Statistical analysis (see **Table of Materials** for suggested open source and commercial software).

3.3.1. Verify if the samples follow a normal distribution with a Shapiro-Wilk test.

3.3.2. Determine whether there is a significant difference between two samples using a Student-t test (if normal distribution assumption met) or a Wilcoxon-Rank Sum test (if normal distribution assumption is violated for a sample).

3.4. Data visualization: Generate dot plots combined with box plots to visualize data distribution, median (Q_2), 25th (Q_1) and 75th percentile for the different samples (See **Table of Materials** for suggested open source and commercial software).

4. 3D display of pMCM2: ICL interactions

4.1. Image PLA plates on a spinning disk confocal microscope, using a Plan Fluor 60x/1.25 numerical aperture oil objective. Acquire 16 stacks covering 1.6 µm and generate the 3D reconstruction with the appropriate image analysis software (see **Table of Materials**).

REPRESENTATIVE RESULTS

PLA of Dig-TMP with replisome proteins

The structure of the Dig-TMP is shown in **Figure 1**. The details of the synthesis, in which trimethyl psoralen was conjugated through a glycol linker to digoxigenin, have been discussed previously[17,21]. Incubation of cells with the compound followed by exposure to 365 nm light (UVA) photoactivates the compound and drives the crosslinking reaction. Slightly more than 90% of adducts are ICLs[16]. The Dig tag can be visualized by immunofluorescence which reveals the presence of ICLs throughout the nucleus (**Figure 2**). Immunofluorescence of a replisome protein such as MCM2 also indicates a distribution throughout the nucleus, a distribution that is unaffected by the introduction of ICLs. These results demonstrated that the focal appearance of responding proteins, such as seen in the DNA Damage Response (DDR) to DSBs, is not a feature of replisome: ICL interactions.

In order to visualize the interaction of replisomes with ICLs in the experiment shown in Fig 2 we applied the PLA, which reports the proximity of two antigens (**Figure 3a**). We measured the frequency of association of MCM5 and the Dig tag 1 h after introduction of the ICLs (**Figure 3b**). The PLA signals demonstrated the proximity of replisomes to ICLs.

Replication stress, including that presented by ICLs, activates the ATR kinase[22]. Among the many substrates of ATR are MCM proteins, including MCM2 at serine 108[23]. A replisome encounter with the ICL would be expected to result in the phosphorylation of MCM2, among many other substrates. In accord with this expectation, the PLA between pMCM2Ser108 and the Dig tag was positive (**Figure 3c).** In other experiments we found that the plateau frequency was reached at 1 h[20]. We interpreted these results as indicating that replisomes variously located throughout the genome, and variably distant from an ICL, eventually encounter the block, triggering ATR activation, and MCM2 phosphorylation.

The PLA results in the preceding figures are presented as a compressed summation of multiple optical planes. However, the results from individual nuclei can also be presented in a three-dimensional reconstruction, as shown for the pMCM2: Dig-TMP PLA in **Video 1**. This analysis indicated that replisome encounters with the ICLs could be observed throughout the nucleus.

Our study of replication fork showed that ICL encounters revealed an unexpected replication restart phenomenon[16]. Considering the locked ring structure of a functional replisome, it was of considerable interest to ask if the composition of the replication apparatus changed on colliding with an ICL. Since less than 10% of replication forks makes contact with an ICL, simply assaying the protein composition of all replisomes would not have been productive. However, the PLA between Dig and various components allowed us to address this question. In contrast to the positive results with pMCM2, we found that the proteins of the GINS complex failed to give PLA signal with the ICLs. On the other hand, the assay with CDC45 was positive, indicating that the other locking protein was retained (**Figure 4a,b**). When cells were incubated with an inhibitor of ATR, the restart was completely suppressed and the GINS: Dig PLA was strongly positive (**Figure 4c**). Our interpretation of these results was that in the absence of ATR activity the GINS proteins were retained, the CMG helicase remained in a locked configuration, and there was no replication restart past the ICL[20].

FIGURE LEGENDS

Figure 1: Structure of trimethyl psoralen linked to the digoxigenin antigen tag.

Figure 2: Immunofluorescence of replisome protein MCM5 and DIG TMP does not show discrete foci. Cells were treated with Dig-TMP and UVA and after 1 h immunostained for MCM5 and Dig. The white bar represents 5 μm.

Figure 3: PLA between Dig-TMP and MCM5. (**a**) Schematic of the Proximity Ligation Assay applied to the interaction between the MCM5 replisome protein and the Dig tag on the ICL. The scheme is simplified. In practice primary antibodies were bound by secondary antibodies covalently coupled to the oligonucleotides. (**b**) PLA between MCM5 and Dig-TMP. Note the discrete signals indicating sites of interaction. Dot and box plots showing signal distribution (dot plot) as well as the median (box plot red bar), the 25^{th} and 75^{th} percentiles (box ends) and the highest and lowest values excluding outliers (extreme lines). Wilcoxon-Rank Sum test confirmed there is a significant difference between the two conditions ($p<0.001$). The white bars represent 5 μm. (**c**) PLA between pMCM2 and Dig-TMP. These signals represent the encounter of the replisome with the ICL, which triggers an ATR dependent phosphorylation of MCM2. The PLA reports the variability of the encounter frequency in different cells. The white bar represents 5 μm.

Figure 4: PLA between Dig-TMP and the replisome locking proteins. (**a**) CDC45: Dig-TMP. The white bar represents 5 μm. (**b**) PSF1: Dig-TMP. The minimal signal frequency is greatly increased by treatment with an ATR inhibitor, which blocks the traverse pathway and the release of the GINS complex which includes PSF1. The white bars represent 5 μm.

Video 1: Three-dimensional reconstruction of pMCM2: Dig PLA signals demonstrates the distribution throughout the nucleus. PCNA is stained in green, PLA in red, DAPI in blue.

Supplementary File 1: Cellprofiler pipeline for PLA quantification.

Supplementary File 2: IMARIS cell module batch parameters for PLA quantification.

DISCUSSION

Although the PLA is a very powerful technique, there are technical concerns that must be solved in order to obtain clear and reproducible results. The antibodies must be of high affinity and specificity. Furthermore, it is important to reduce the non-specific background signals as much as possible. We have found that membranes and cellular debris contribute to the background, and we have removed them as much as possible. The washes with detergent containing buffers prior to fixing, and the wash with methanol after fixing help reduce the non- specific binding. The caveat is that detergent treatment prior to fixation can result in cell detachment. We find that treating the plates with a cell adhesive and prefixing with 0.1% FA before the CSK treatment alleviates this problem.

It is also helpful to identify non-S phase cells when monitoring S phase specific events. This can be done by post PLA staining with cell cycle markers such as PCNA or NPAT[24,25]. Not only do these markers confirm S phase phenomena but they also provide an internal biological control for non-specific interactions. Positive signals in G1 phase cells, in assays that measure events that should be exclusive to S phase, are an indication that additional effort to reduce non-specific interactions is required.

Single cell imaging strategies have advantages not available with other approaches for monitoring molecular interactions. Homogenization techniques, such as employed in immunoprecipitation experiments eliminate any connection to events in individual cells. Consequently, insight as to the influence of cell cycle status, or the variability across a cell population is lost. However, since the PLA reports event frequencies in individual cells these insights can be recovered.

A frequent limitation of the PLA is the lack of immunologic detection reagents for targets of interest. This is a concern when addressing questions regarding the cellular response to DNA perturbations introduced by agents other than direct breakers. We have overcome that limitation by use of an immunotagged DNA reactive reagent. Although we have focused our studies on interstrand crosslinks, there are many genotoxic compounds, including chemotherapy drugs, that would lend themselves to this approach. Additionally, interactions between proteins and DNA structures, such as G quadruplexes, could also be examined with this strategy.

ACKNOWLEDGEMENTS

This research was supported, in part, by the Intramural Research Program of the NIH, National Institute on Aging, United States (Z01-AG000746–08). J.H. is supported by the National Natural Science Foundations of China (21708007 and 31871365).

DISCLOSURES

The authors have nothing to disclose

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Figure 1

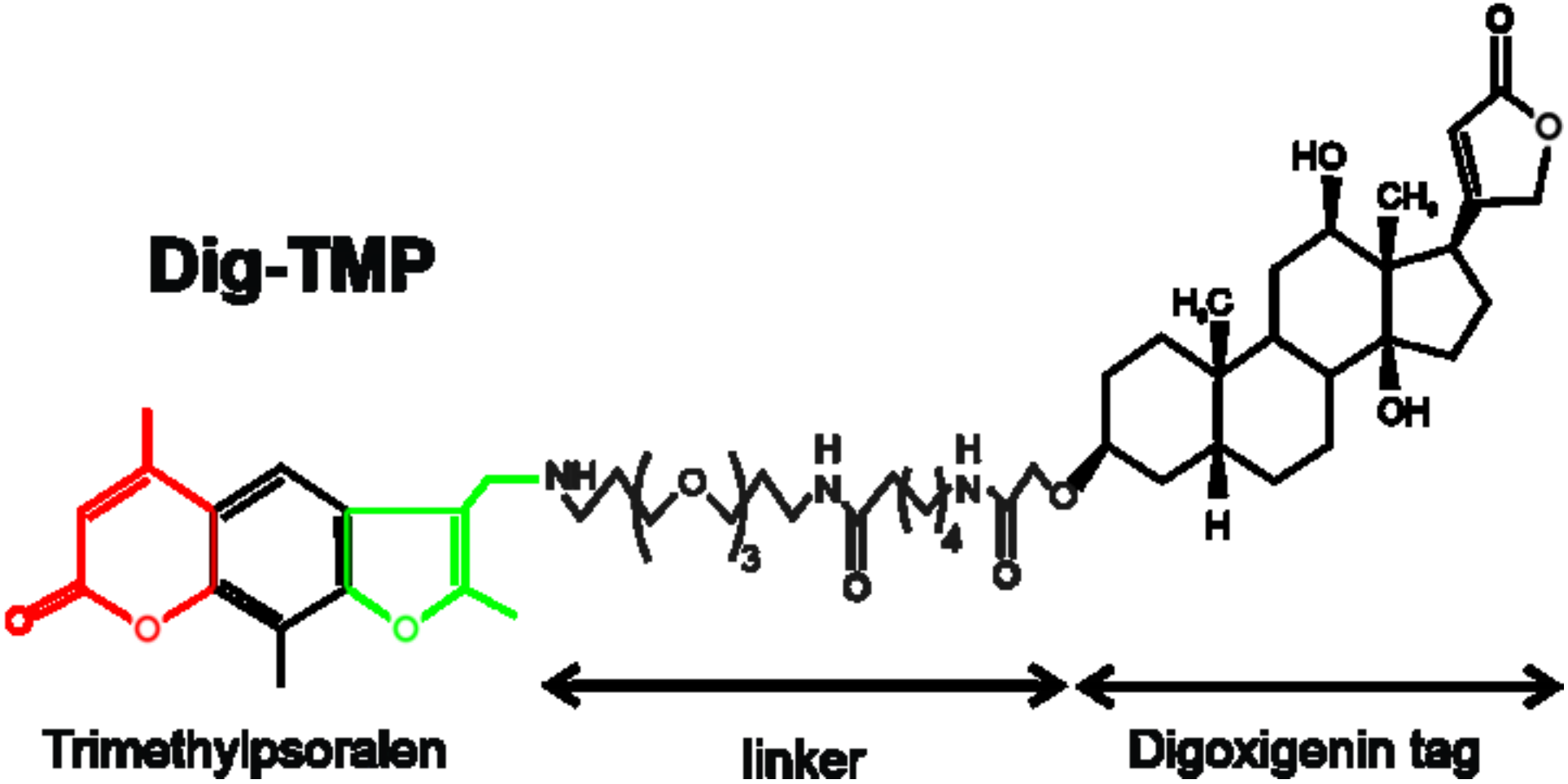
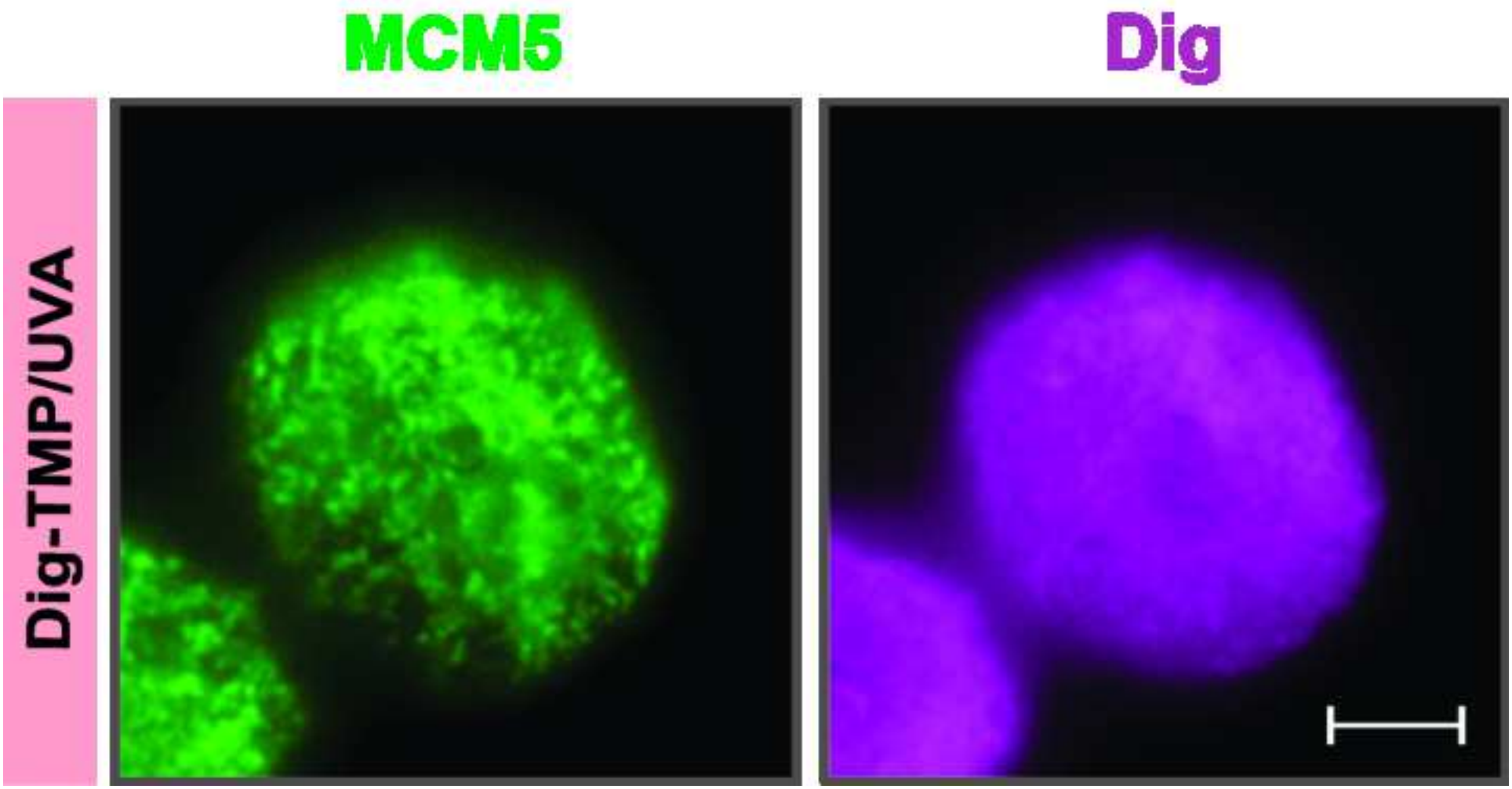


Figure 2



Figure

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

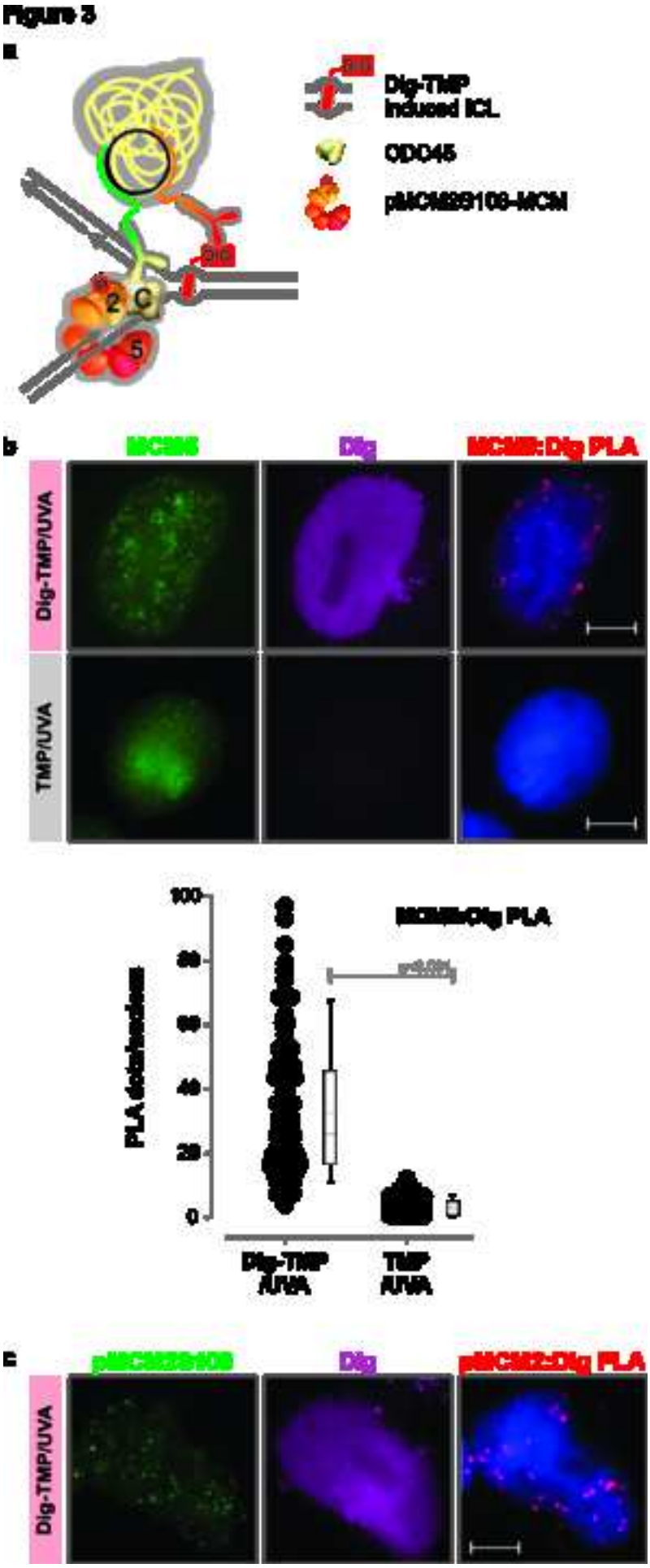
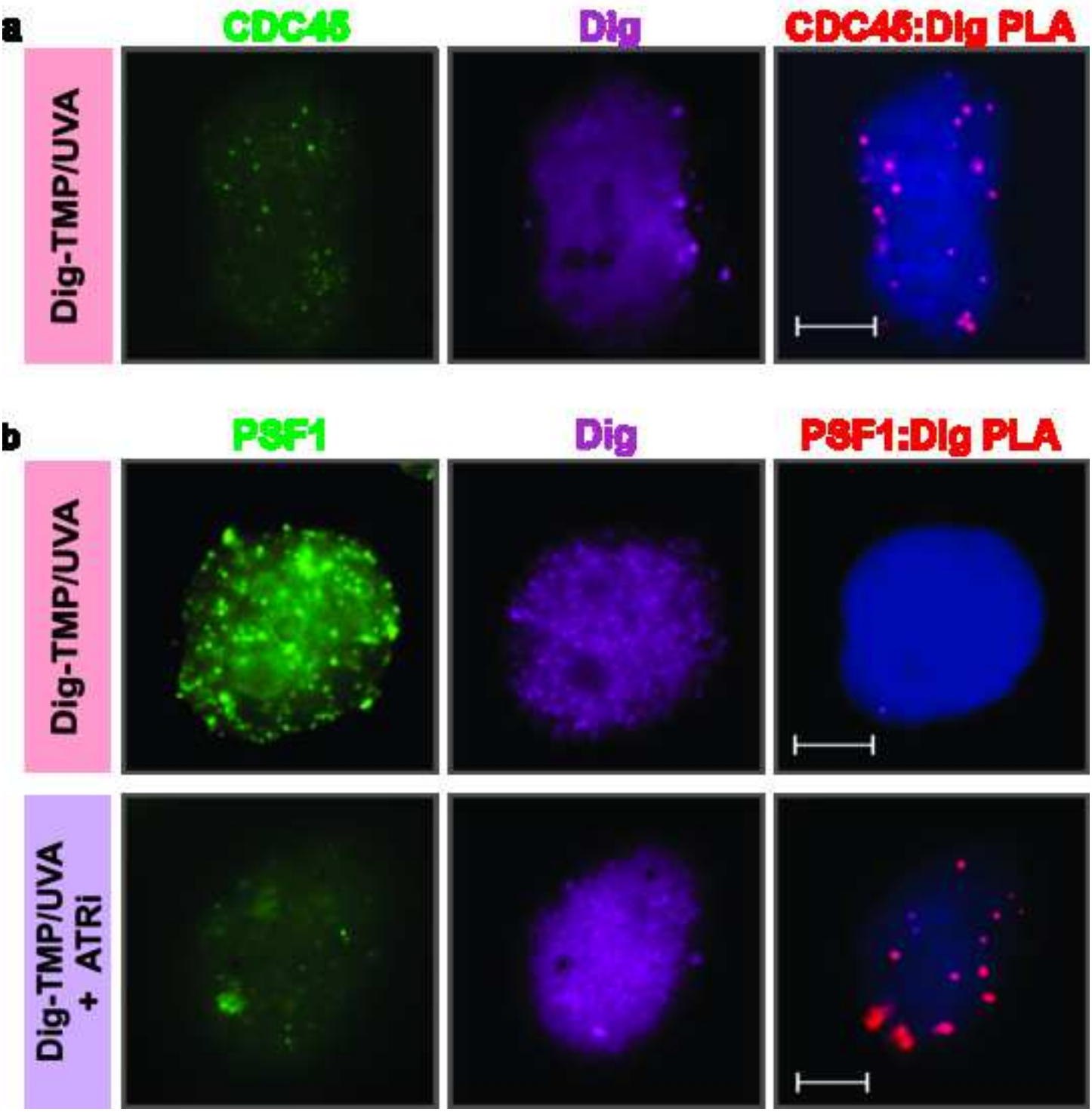


Figure 4



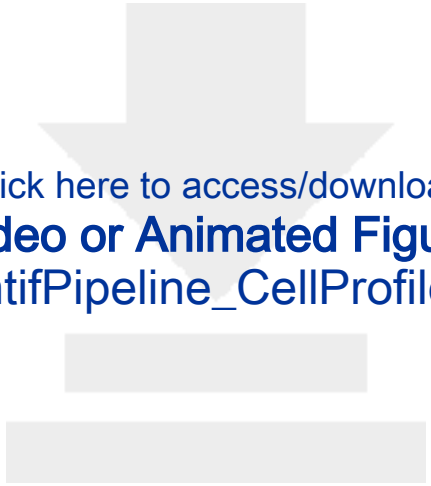
Video or Animated Figure

Click here to access/download

Video or Animated Figure

13_PLA2_PCNA pMCM2 Dig 12.mp4

Supplementary File 1

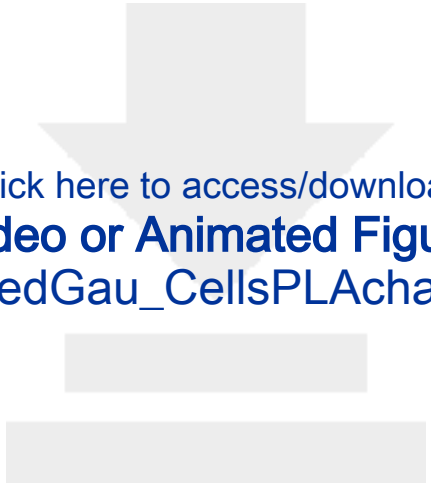


Click here to access/download

Video or Animated Figure

PLAquantifPipeline_CellProfiler.cppipe

Supplementary File 2



Click here to access/download

Video or Animated Figure

DAPIMedGau_CellsPLAchan2.icsx

Name of Material/ Equipment	Company
Alexa Fluor 568, Goat anti-Rabbit IgG (H+L) Cross-Adsorbed Secondary Antibody	Invitrogen
35 mm plates with glass 1.5 coverslip	MatTek
Alexa Fluor 488,Goat anti-Mouse IgG (H+L) Cross-Adsorbed Secondary Antibody	Invitrogen
Bovine serum albumin (BSA)	SeraCare
CDC45 antibody (rabbit)	Abcam
Cell adhesive	Life Science
Confocal microscope	Nikkon
Digoxigenin (Dig) antibody (mouse)	Abcam
Dig-TMP	synthesized in the Seidman Lab
Duolink Amplification reagents (5×)	Sigma-Aldrich
Duolink *in situ* detection reagents	Sigma-Aldrich
Duolink *in situ* oligonucleotide PLA probe MINUS	Sigma-Aldrich
Duolink *in situ* oligonucleotide PLA probe PLUS	Sigma-Aldrich
Duolink in situ wash buffer A	Sigma-Aldrich
Duolink in situ wash buffer B	Sigma-Aldrich
epifluorescent microscope	Zeiss
Formaldehyde 16%	Fisher Scientific
Goat serum	Thermo
Image analysis software	open source
Image analysis software-license required	Bitplane
Ligase (1 unit/µl)	Sigma-Aldrich
Ligation reagent (5×)	Sigma-Aldrich
MCM2 antibody (rabbit)	Abcam
MCM5 antibody (rabbit monoclonal)	Abcam
Methanol	Lab ALLEY
phosphoMCM2S108 antibody (rabbit)	Abcam
Polymerase (10 unit/µl)	Sigma-Aldrich
Prolong gold mounting media with DAPI	ThermoFisher Scientific
PSF1 antibody (rabbit)	Abcam
RNAse A 100 mg/ml	Qiagen

Statistical analysis and data visualization software	open source
Statistical analysis and data visualization software-license required	Systat Software
TMP (trioxalen)	Sigma-Aldrich
TritonX-100	Sigma-Aldrich
Tween 20	Sigma-Aldrich
UV box	Southern New England Ultraviolet
UV test Chamber	Opsytec
VE-821	Selleckchem

Catalog Number

A-10011

P35-1.5-14-C

A-10001

1900-0012

ab126762

354240

Nikon TE2000 spinning disk microscope

ab420

DUO82010

DUO92007

DUO92004

DUO92002

DUO82046

DUO82048

Axiovert 200M microscope

PI28906

31873

Cell profiler

Imaris

DUO82029

DUO82009

ab4461

Ab75975

A2076

ab109271

DUO82030

P36935

ab181112

19101

R studio
Sigmaplot V13
T6137_1G
T8787_250ML
P9416_100ML

UV TEST CHAMBER BS-04
S8007

Comments/Description
1 in 1000 Glass Bottom Microwell Dishes 35mm Petri Dish Microwell
1 in 1000 Blocking solution, reagents need to be stored at 4 °C
1 in 200 for cell-TAK solution
equiped with Volocity software
1 in 200
reagents need to be stored at -20 °C
reagents need to be stored at -20 °C
anti-mouse MINUS, reagents need to be stored at 4 °C
anti-rabbit PLUS, reagents need to be stored at 4 °C
Duolink Wash Buffers, reagents need to be stored at 4 °C
Duolink Wash Buffers, reagents need to be stored at 4 °C
Equipped with the Axio Vision software packages (Zeiss, Germany)
for fix solution
Blocking solution, reagents need to be stored at 4 °C
works for analysis of single plane images
Cell Biology module needed. Can quantify PLA dots/nuclei in image stacks (3D) and do 3D reconstructions
reagents need to be stored at -20 °C
reagents need to be stored at -20 °C
1 in 200
1 in 1000
pre-cold at -20°C before use
1 in 200
reagents need to be stored at -20 °C
1 in 200 reagents need to be stored at 4 °C

ggplot2 package for generation of dot plot and box plots

Discontinued. See Opsytec UV test chamber as a possible replacement

final concentrtion is 1µM

Editorial comments:

You will find Editorial comments and Peer-Review comments listed below. Please read this entire email before making edits to your manuscript.

NOTE: Please include a line-by-line response to each of the editorial and reviewer comments in the form of a letter along with the resubmission.

Editorial Comments:

• Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammatical errors.

• **Protocol Language:** Please ensure that all text in the protocol section is written in the imperative voice/tense as if you are telling someone how to do the technique (i.e. “Do this”, “Measure that” etc.) Any text that cannot be written in the imperative tense may be added as a “Note”, however, notes should be used sparingly and actions should be described in the imperative tense wherever possible. Some examples NOT in the imperative: “PLA plates were imaged…”

• **Protocol Detail:** Please note that your protocol will be used to generate the script for the video, and must contain everything that you would like shown in the video. **Please add more specific details (e.g. button clicks for software actions, numerical values for settings, etc) to your protocol steps.** There should be enough detail in each step to supplement the actions seen in the video so that viewers can easily replicate the protocol.

We have revised accordingly.

1) What is the cell culture medium composition?

We have added the cell culture medium composition.

• **Protocol Numbering:**
1) Provide numbering for “First day”, Second day”, Antibody staining etc.
2) Please adjust the numbering of your protocol section to follow JoVE’s instructions for authors, 1. should be followed by 1.1. and then 1.1.1. if necessary.
3) Please add a one-line space after each protocol step.

We have revised accordingly

• **Protocol Highlight:** Please highlight ~2.5 pages or less of text (which includes headings and spaces) in yellow, to identify which steps should be visualized to tell the most cohesive story of your protocol steps.
1) The highlighting must include all relevant details that are required to perform the step. For example, if step 2.5 is highlighted for filming and the details of how to perform the step are given in steps 2.5.1 and 2.5.2, then the sub-steps where the details are provided must be included in the highlighting.
2) The highlighted steps should form a cohesive narrative, that is, there must be a logical flow from one highlighted step to the next.
3) Please highlight complete sentences (not parts of sentences). Include sub-headings and spaces when calculating the final highlighted length.
4) Notes cannot be filmed and should be excluded from highlighting.

Done

• **Discussion:** JoVE articles are focused on the methods and the protocol, thus the discussion should be similarly focused. Please ensure that the discussion covers the following in detail and in paragraph form (3-6 paragraphs):
1) modifications and troubleshooting,
2) limitations of the technique,
3) significance with respect to existing methods,
4) future applications and
5) critical steps within the protocol.

Done

• **Figures:** Please provide each figure (if multiple panels are present per figure, keep them within 1 file) as an individual SVG, EPS, AI, TIFF, or PNG file.

Done

• **References:**
1) Please spell out journal names.

Done

• **Commercial Language:**JoVE is unable to publish manuscripts containing commercial sounding language, including trademark or registered trademark symbols (TM/R) and the mention of company brand names before an instrument or reagent. Examples of commercial sounding language in your manuscript are Cell-TAK, Nikon TE2000, Volocity, IMARIS (Bitplane).

We have adjusted the text accordingly

1) Please use MS Word's find function (Ctrl+F), to locate and replace all commercial sounding language in your manuscript with generic names that are not company-specific. All commercial products should be sufficiently referenced in the table of materials/reagents. You may use the generic term followed by "(see table of materials)" to draw the readers' attention to specific commercial names.

Done

• **Table of Materials:**
1) Please remove the registered trademark symbols TM/R from the table of reagents/materials.
2) Please sort in alphabetical order.

Done

• Please define all abbreviations at first use (e.g., Dig-TMP, CSK-R)

Abbreviations have been defined

• If your figures and tables are original and not published previously or you have already obtained figure permissions, please ignore this comment. If you are re-using figures from a previous publication, you must

obtain explicit permission to re-use the figure from the previous publisher (this can be in the form of a letter from an editor or a link to the editorial policies that allows you to re-publish the figure). Please upload the text of the re-print permission (may be copied and pasted from an email/website) as a Word document to the Editorial Manager site in the "Supplemental files (as requested by JoVE)" section. Please also cite the figure appropriately in the figure legend, i.e. "This figure has been modified from [citation]."

Comments from Peer-Reviewers:

Reviewers' comments:

Reviewer #1:

Manuscript Summary:
Dr. Seidman's laboratory is specialized in the visualization of DNA damage response at the site of interstrand crosslink (ICL) damage. In this manuscript, they are providing a detailed protocol to visualize replisome-ICL interactions using DIG-tagged TMP psolaren and a PLA kit. This method might be useful for researchers who are interested in ICL repair and replication. I would like to ask some questions so that we can perform the experiment in my lab.

Major Concerns:

1. Cell preparation. What kind of cell line are they using here? Do they have any recommendations for cells to use? What is the purpose of the inclusion of Cell TAK solution?

The examples presented were with HeLa cells. However, the approach is applicable to any cell type, although adherent cells are most amenable. Nonadherent cells can be used but must be centrifuged onto slides and fixed prior to the PLA. Cell Tak, a cell adhesion reagent, is helpful for keeping cells attached during the many wash steps.

2. I wonder how we can make a stock solution of Dig-TMP. Please provide a reasonable amount of the reagents and volume of the solvent when they make the stock solution.

We have described the methods of making the solution of Dig-TMP in the revised manuscript.

3. The UV-box is probably a UV irradiator. Please provide the identity of the machine they are using. Did they calibrate the machine?

The UV box is by Rayonet. Calibration is by a UV light meter. It has been discontinued by the company, but we have provided an alternative UV chamber by Opsytec (see Table of Materials for more details).

4. CSK-R buffer. Please provide a recipe for the buffer.

We have added the formula in the revised text.

5. Antibody staining. Please provide an actual volume of the primary antibodies they used here. I understand that the dilution is in the table.

We have added this information.

6. What is the concentration of Triton in their PBS-T?

The T refers to Tween-20. We have added the concentration of Tween-20 in PBS-T in the text.

7. Table of materials. Please provide the origin of anti-PSF1 antibody.

We have added this information.

8. What is the Rayonet chamber?

We have added the information for the UV chamber in the text and the Table of Materials.

Minor Concerns:

Figures. I see black spots in the photographs.

The photograph has been replaced

Reviewer #2:

In their paper, Zhang and colleagues devise and employ a new variation of the Proximity Ligation Assay to study the evolution of replication fork components in the presence of ICLs. Briefly, they use Dig tagged Psoralen to visualize the adducts created by the compound and then assess if MCMs and GINS protein could be detected in the proximity of the ICLs. Overall, I find the technique ingenious and potentially really powerful as it could be further modified to allow localisation of other types of damaging adducts. Given the finicky nature of PLA I suggest that the authors perform a couple of additional controls to further strengthen their results.

Major Concern:
Fig3b lacks negative controls. The authors should confirm that the PLA signal is dependent on the presence of ICLs. To solve this issue they should either perform the PLA reaction in cells treated with untagged psoralen or, even better, treat the cells with the tagged compound without the UVA activation (ie no ICLs should be formed). This would allow assessing any possible background and PLA false positive signal.

Fig 3b contains a control experiment in which cells were treated with TMP/UVA. This introduces psoralen crosslinks into the genome, but they are not Dig tagged. Consequently, the cells have experienced all the stress imposed by Dig-TMP/UVA treatment. As is apparent from the figure there is no signal with the antibody against Dig. Identical results (no signal) are obtained when cells are exposed to Dig-TMP without UVA.

Minor Concerns:

I really appreciate how the authors presented all the channels in Fig3b, showing the signals for each individual protein used in the assay. I think this presentation should be carried throughout all the other figures.

These have been added

I think it would be useful to add some quantitation to the experiments, showing the number of PLA foci in each experimental condition.

Quantitation has been added to Figure 3b. Quantification for PLAs described in Figures 3c and 4a-b please refer to Huang et al. Cell Reports. 27 (6), 1794-1808, (2019).

Reviewer #3:

Manuscript Summary:

The authors present a protocol for detection of replisome factors upon induction of interstrand crosslinks on DNA mediated by TMP. For this the authors took advantage of proximity ligation assay coupled to fluorescent imaging. The manuscript is quite comprehensive in its description of the protocol and the authors also show representative data to support their claims. There are a couple of minor issues the authors should address before publication of the manuscript.

Minor Concerns:

1.The authors should highlight the most critical steps of the protocol.

These have been highlighted

2. The most important aspect of this manuscript is the accessibility of DIG-TMP for the scientists who would like to adapt this technology in their labs. For this, the authors should mention the source of DIG-TMP or whether the compound was synthesized in their own lab.

This compound is not available commercially. We have described the synthesis in the revised text, and in previous publications, including in JoVE [DOI: 10.3791/55541]. We note that this is not a difficult synthesis and no specialized equipment is required.

3. I could not find the complete composition of the CSK-R buffer in the manuscript (as this could be critical step for the background reduction). The authors should show the complete composition of the CSK-R buffer in the manuscript.

We have added this information to the revised text.

Author biographies

Jing Zhang received her PhD from Nanjing University, China in 2015. She is currently a Visiting Fellow at the National Institutes on Aging, National Institutes of Health.

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