Protocol to analyze endogenous translesion DNA synthesis in single mammalian cells

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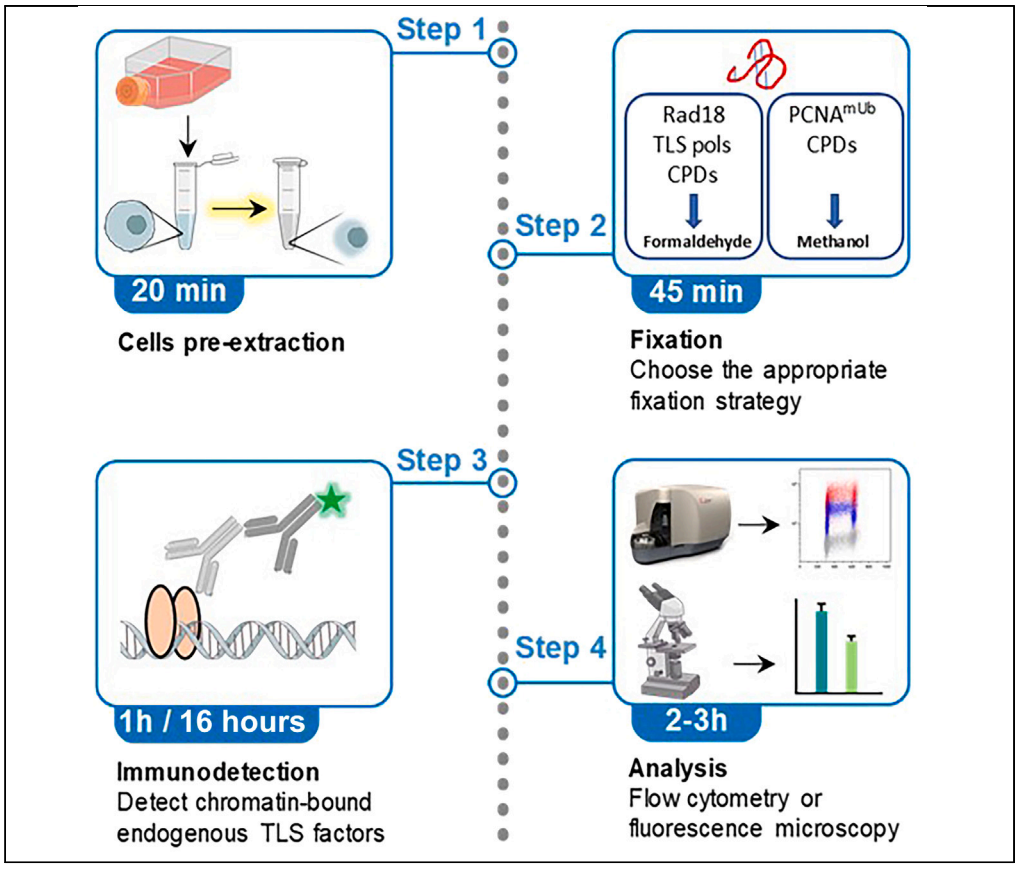

Translesion DNA synthesis (TLS) is an evolutionarily conserved branch of the cellular DNA damage tolerance pathway that is often exploited by cancer cells to overcome therapy resistance. Here, we present a protocol to analyze endogenous TLS in single mammalian cells in the absence or presence of DNA damage. We describe steps for detecting chromatin-bound TLS factors, such as monoubiquitinated PCNA(mUb) and TLS DNA polymerases (pols) by flow cytometry. We then detail a procedure to detect their nuclear localization using immunofluorescence.

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

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Highlights

Detection of endogenous TLS factors by flow cytometry and immunofluorescence

Monitoring chromatin-bound TLS factors in respect to cell cycle phases

Monitoring TLS factors in respect to DNA synthesis and DNA lesions

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Protocol to analyze endogenous translesion DNA synthesis in single mammalian cells

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SUMMARY

Translesion DNA synthesis (TLS) is an evolutionarily conserved branch of the cellular DNA damage tolerance pathway that is often exploited by cancer cells to overcome therapy resistance. Here, we present a protocol to analyze endogenous TLS in single mammalian cells in the absence or presence of DNA damage. We describe steps for detecting chromatin-bound TLS factors, such as monoubiquitinated PCNA(mUb) and TLS DNA polymerases (pols) by flow cytometry. We then detail a procedure to detect their nuclear localization using immunofluorescence.

For complete details on the use and execution of this protocol, please refer to Egger et al. (Cell Reports Methods, in press).[1]

BEFORE YOU BEGIN

Cell culture and media preparation

Timing: 30 min

The protocol below is inspired by a previous method to detect chromatin-bound proteins[2] and describes step-by-step procedures to detect chromatin-bound TLS factors in HCT116 cells.[1] However, we have also used this protocol with HEK293, T98G and H1299 cells. It may require adaptation for other cell lines requesting specific media (such as stem cells for instance).

HCT116 cells are cultured at 37°C, 5% CO_2 (saturated humidity) in DMEM supplemented with GlutaMAX and 10% Fetal Bovine Serum (FBS). Cells were passaged every 2–3 days and maintained in exponential growth phase. When microscopy immunofluorescence (IF) experiments are conducted, coverslips must be coated with Poly-D-Lysine prior to cell seeding, since the pre-extraction step is relatively harsh and may result in consequent material loss on uncoated glass. Subsequent analysis will require a flow cytometer and/or a microscope (at least ×63 magnification objectif with ApoTome or equivalent) equipped for the detection of the wavelengths to be used during the detection steps (see key resources table).

1. Prepare cell culture media.
   a. Add FBS to 10% of the final volume of DMEM + GlutaMAX.
   b. Pre-heat the media to 37°C before splitting or doing experiments with cells.
2. Split HCT116 every 2–3 days maintaining them in exponential growth phase (70%–90% confluence).

3. For IF, prepare coverslips by coating them with Poly-D-Lysine according to the manufacturer's instruction. These can be stored at 4°C for several weeks before use.

Seeding cells for TLS assays (by IF or flow cytometry)

Timing: 1–2 days

4. Cells are seeded in 6-well plates (containing coated-coverslips).
   a. Seed 1 × 10^6 cells per well if cells are to be analyzed the next day.
   b. Alternatively, if other steps are to be performed (i.e., siRNA knockdown), adapt the cell density accordingly (divide by 2 for each supplemental day of culture for HCT116).
   c. Allow cells to attach at least 16 h at 37°C, at 5% CO_2 (saturated humidity) before conducting any experiments.

⚠ CRITICAL: It is important to avoid that cells reach full confluence. The optimal confluence to perform experiments with this protocol is 70%–90%. The optimal cell seeding density must be determined for each cell line, taking into consideration the doubling time and size of cells, in order to reach 70%–90% confluence at the time of the experiment.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Ubiquityl-PCNA (Lys164)	Cell Signaling Technology	RRID:AB_2798219
PCNA	Millipore	RRID:AB_11203836
Pol η	Abcam	RRID:AB_2756352
Pol ι	Proteintech	RRID:AB_2167009
Rad18	Abcam	RRID:AB_1603946
CPDs	Cosmo Bio	RRID:AB_1962813
Goat Anti-Rabbit Alexa 488	Thermo Fisher Scientific	RRID:AB_2576217
Goat anti-Rabbit Alexa 568	Thermo Fisher Scientific	RRID:AB_10563566
Goat anti-Mouse Alexa 660	Thermo Fisher Scientific	RRID:AB_2535722
Chemicals, peptides, and recombinant proteins		
DMEM-GlutaMAX	Gibco	31966-021
Fetal bovine serum (FBS)	Eurobio	CVFSVF00-01
Bovine serum albumin (BSA)	Sigma-Aldrich	A2153
Hydrogen peroxyde (H_2O_2)	Sigma-Aldrich	216763
Triton X-100	Sigma-Aldrich	X-100
Formaldehyde (FA)	Pierce	28906
DAPI	Sigma-Aldrich	D9542
Perm/Wash buffer (10 x)	BD Biosciences	51-2091 KZ, sold as 554723
PBS	Gibco	14190-094
Methanol	Normapur	20847-295
Halt Proteases and Phosphatases inhibitors	Thermo Fisher Scientific	78440
Experimental models: Cell lines		
HCT116		RRID:CVCL_0291
Enzymes		
RNAse A	EMD Millipore	70856
Software and algorithms		
Cell Profiler 4.2.1	See source[3]	N/A
Kaluza	Beckman Coulter	N/A
Zen Blue	Zeiss	N/A
Gallios	Beckman Coulter	N/A

(Continued on next page)

Continued		
REAGENT or RESOURCE	SOURCE	IDENTIFIER
FlowJo	BD Biosciences	N/A
ImageJ	Open source –(NIH)	N/A
Other		
6-Well plates	Falcon	353224
Poly-D-Lysine	Gibco	A38904-01
Eppendorf safe-Lock microtubes	Eppendorf	0030 120.086
Coverslips	Marienfeld Superior	0117520
Prolong Gold AntiFade	Invitrogen	P36930

MATERIALS AND EQUIPMENT

- Cell Culture Media: Complete DMEM-GlutaMAX with 10% of FBS.

Store at 4°C and heat to 37°C before use.

- Pre-extraction buffer: PBS-0.2% Triton X-100: add 200 µL of Triton X-100 in 100 mL 1 × PBS.

Store at 4°C and keep on ice during the use for pre-extraction.

- PBS-1 mg/mL BSA: add 50 mg BSA in 50 mL 1 × PBS.

Store at 4°C and keep on ice during the use for pre-extraction.

- PBS-2% Formaldehyde (FA): add 1mL of 16% in 7 mL 1 × PBS **extemporaneously.**

Store at 4°C for a maximum of 1 week or make a new batch.

- Analysis buffer (Flow cytometry): add 1 µg/mL of DAPI and 100 µg/mL of RNAse A **extemporaneously** to the PBS-1 mg/mL BSA solution

Store at 4°C for a maximum of 1 week or make a new batch.

- 1 × Perm/Wash buffer (P/W): dilute the 10× stock solution to 1 × in dH_2O (store at 4°C for up to a month).

STEP-BY-STEP METHOD DETAILS

TLS induction by DNA damaging agents

Timing: 30 min–5 h

1. Include positive TLS controls, such as UV-C irradiation or treating cells with H_2O_2.
   a. For UV-C irradiation, expose cells to 15–30 J/m^2 of UV-C light using an irradiator and release them in normal culture conditions (37°C, 5% CO_2) for 5 h prior to harvesting.
   b. For H_2O_2 treatment, add a final concentration of 1 mM H_2O_2 to the cell culture media for 30 min (37°C, 5% CO_2) before harvesting.

Pre-extraction and fixation

Timing: 1 h

Table 1. Optimal pre-extraction and fixation for each target

Target	Pre-extraction buffer	Fixation	Dilution
Ubiquityl-PCNA (Lys164)	PBS-0,2% Triton X-100	PBS-90% Methanol, -20°C	IF:1/100; WB:1/1000
CPDs	PBS-0,2% Triton X-100	PBS-90% Methanol, -20°C	IF:1/100
Pol η	PBS-0,2% Triton X-100 containing 200 mM sucrose	PBS-2% FA, RT	IF:1/50; WB:1/1000
Pol ι	PBS-0,1% Triton X-100 containing 200 mM sucrose	PBS-2% FA, RT	IF:1/50; WB:1/1000
Rad18	PBS-0,2% Triton X-100	PBS-2% FA, RT	IF:1/100; WB:1/1000
PCNA	PBS-0,2% Triton X-100	PBS-90% Methanol, -20°C	IF:1/200; WB:1/2000
Goat Anti-Rabbit Alexa 488	N/A	N/A	IF:1/250
Goat anti-Rabbit Alexa 568	N/A	N/A	IF:1/250
Goat anti-Mouse Alexa 660	N/A	N/A	IF:1/250

Following TLS-inducing treatments, harvest cells (on coverslips for IF or trypsinization for flow cytometry).

Always include negative controls (untreated cells and treated cells that will not be incubated with primary antibodies).

2. Rinse cells with ice-cold PBS.
   a. Immunofluorescence.
      i. Gently transfer coverslips in a 12-well plate containing 1 mL PBS on ice.
      ii. Aspirate the PBS from the corner of the wells, keep on ice.
   b. Flow cytometry (from this point on, process the samples in 1.5 mL tubes).
      i. Centrifuge cells at 400 × *g* for 3 min at 4°C and discard the supernatant.
      ii. Resuspend cells in 1 mL ice-cold PBS.
      iii. Centrifuge cells 400 × *g* for 3 min at 4°C and discard the supernatant.
3. Pre-extraction of the cytosolic compartment using the appropriate buffer (see Table 1).
   a. Immunofluorescence.
      i. Very gently pipet 1 mL of ice-cold extraction buffer to the edge of wells.
      ii. Incubate coverslips for 3–5 min on ice (depending on the cell line used, see troubleshooting).
      iii. Discard extraction buffer and rinse cells once with PBS 1 mg/mL BSA solution.
      iv. Gently aspirate buffer from the edge of the wells on ice. Avoid moving the plate and proceed to the fixation (step 3.a.i/ii) within a minute.
   b. Flow cytometry.
      i. Resuspend cells in 100 µL of ice-cold extraction buffer by pipetting up and down 5–10 times.
      ii. Incubate cells on ice for 5–10 min (depending on cell line, see troubleshooting).
      iii. Add 1 mL of PBS-BSA 1 mg/mL to stop the extraction.
      iv. Centrifuge 500 × *g* for 3 min at 4°C and carefully remove the supernatant.
4. Fixation of the extracted nuclei using the appropriate fixative (see Table 1).
   a. Immunofluorescence (proceed with i. or ii here below).
      i. For FA fixation: gently pipet 1 mL of PBS-2% FA to the edge of wells and incubate (30 min, at 20°C–23°C).
      ii. For methanol fixation: gently pipet 1 mL of −20°C pre-chilled PBS 90% methanol to the edge of the wells, close the plate and incubate immediately at −20°C for 10 min. Avoid shaking the plate while moving it.
      iii. Remove the fixation buffer and **gently** rinse coverslips with 1 mL of 1× Perm/Wash (P/W) buffer.

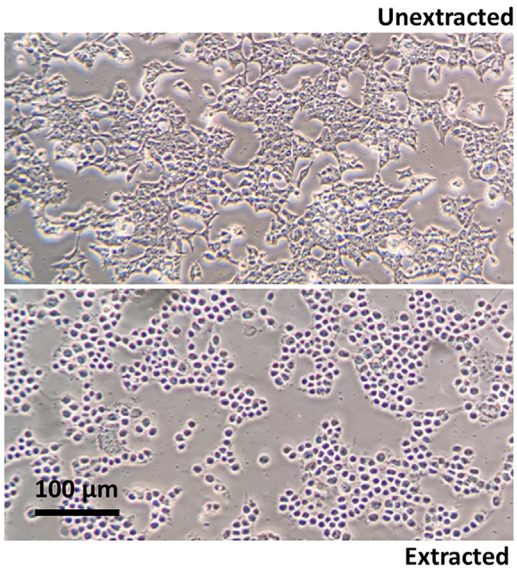

Figure 1. Representative bright field (100×) images of either non pre-extracted (top) or pre-extracted (bottom) HEK293T cells in PBS 0,2% Triton-X100 for 3min on ice

⚠ CRITICAL: For immunofluorescence, avoid shaking the plates before cells get fixed by FA or methanol. Determine the optimal duration of the pre-extraction step by monitoring cells under a microscope (100×) every minute. A too long incubation will result in cell dissociation from coverslips. Optimal extraction point is reached when only the nuclei remain visible under the microscope (100×). Optimal duration for HCT116 and HEK293 cells is 3–5min on ice. Representative images of unextracted *vs* extracted cells are shown in Figure 1.

b. Flow cytometry (proceed with i. or ii here below).
   i. For FA fixation: gently resuspend the extracted cell pellet in 100 µL of PBS-2% FA and incubate 30 min at 20°C–23°C.
   ii. For methanol fixation: add 100 µL of PBS-90% methanol (pre-chilled to −20°C) **dropwise** on the extracted pellet in a pre-chilled −20°C block, homogenize **very gently** the cell solution by pipetting up and down 3 times maximum and incubate for 10 min at −20°C.
   iii. Add 1 mL of ice-cold 1× P/W buffer, centrifuge the fixed nuclei 750× *g* for 3 min at 4°C. Carefully remove the supernatant.

Pause point: although a direct processing through the immunodetection should be preferred, cells can be stored for a maximum of 2 days in 1× P/W buffer at 4°C.

Note: The pre-extraction buffer can be completed with proteases and phosphates inhibitors and replace a classical "CSK buffer" to achieve a chromatin/soluble fractionation for western blot analysis in the same conditions than IF and flow cytometry. In this case, histones (chromatin) and alpha/beta-tubulin (cytoplasm) should be used as fractionation controls. Typically, we achieved even higher purity of fractions (chromatin/soluble proteins) while using this extraction buffer rather than classical, sucrose-based "CSK buffers".

Immunodetection of target proteins

Timing: 4 h–16 h

In this step, endogenous TLS components will be immunodetected using highly specific, validated, commercial antibodies. Although it is preferable to perform the primary antibody incubation 16 h at

4°C, it is possible to shorten this step to 1h at 20°C–23°C, therefore allowing a fast – single working day – processing of samples from cell culture to the analysis steps.

5. Primary antibodies.
   a. Immunofluorescence.
      i. Block samples with 1 mL of 1× P/W for 1 h at 20°C–23°C.
      ii. Put the coverslips (cells side up) onto a parafilm in a humid chamber and gently pipet a drop of 50 µL of 1× P/W buffer containing the appropriate dilution of primary antibody (see Table 1).
      iii. Incubate 16 hours at 4°C or 1 h at 20°C–23°C in a humid chamber.
   b. Flow cytometry
      i. Block samples in 100 µL of 1× P/W at 20°C–23°C.
      ii. Add 1 mL of 1× P/W and centrifuge at 750 × *g* for 5 min at 20°C–23°C.
      iii. Resuspend the cell pellet in 50 µL of 1× P/W buffer containing the appropriate dilution of primary antibody (see Table 1).
      iv. Incubate at 16 hours 4°C, or 1 h at 20°C–23°C on a rotating wheel.

Pause point: The incubation with primary antibodies being most efficient 16 hours at 4°C, this step represents a convenient pause point. However, it is still possible to shorten this step to 1h at 20°C–23°C to process through the whole workflow in a single working day.

6. Secondary antibodies.
   a. Immunofluorescence.
      i. Gently aspirate the drop of primary antibody and wash (3 × 5 min) with 100 µL of 1× P/W buffer.
      ii. Put the coverslips (cells side up) onto a parafilm in a humid chamber and gently pipet a drop of 50 µL of 1× P/W buffer containing the appropriate dilution of Alexa Fluor-conjugated secondary antibody (see Table 1).
      iii. Incubate 1 h at 20°C–23°C in a humid chamber, protected from light.
   b. Flow cytometry
      i. Add 1 mL of 1× P/W buffer, centrifuge at 750 × *g* for 5 min at 20°C–23°C and remove the supernatant.
      ii. Resuspend the cell pellet in 50 µL of 1× P/W buffer containing the appropriate dilution of Alexa Fluor-conjugated secondary antibody (see Table 1).
      iii. Incubate 1 h at 20°C–23°C on a rotating wheel, protected from light.
7. DNA staining and sample preparation.
   a. Immunofluorescence.
      i. Gently aspirate the drop containing the antibody and wash (3 × 5 min) with 100 µL of 1× P/W buffer.
      ii. Gently pipet 100 µL of PBS containing 2 µg/mL of DAPI onto the coverslips.
      iii. Incubate 10 min at 20°C–23°C, protected from light.
      iv. Gently aspirate the drop and rinse 3 times for 1 min with 100 µL of PBS.
      v. Rinse the coverslips once in dH_2O to remove the residual PBS salts.
      vi. Mount coverslips in 4–5 µL of Prolong Gold AntiFade mounting medium.
      vii. Let the mounting medium solidify 16 h at 20°C–23°C. Placing the slides at 37°C for 2–3 h will speed up the reaction without a noticeable impact on the subsequent imaging.
   b. Flow cytometry
      i. Add 1 mL of 1× P/W buffer, centrifuge at 750 × *g* for 5 min and remove the supernatant.
      ii. Resuspend the cell pellet in 300 µL of analysis buffer (PBS-1 mg/mL BSA containing 1 µg/mL DAPI and 100 µg/mL RNAse A).
      iii. Incubate 16 hours at 4°C or 30 min at 37°C before analyzing the samples.

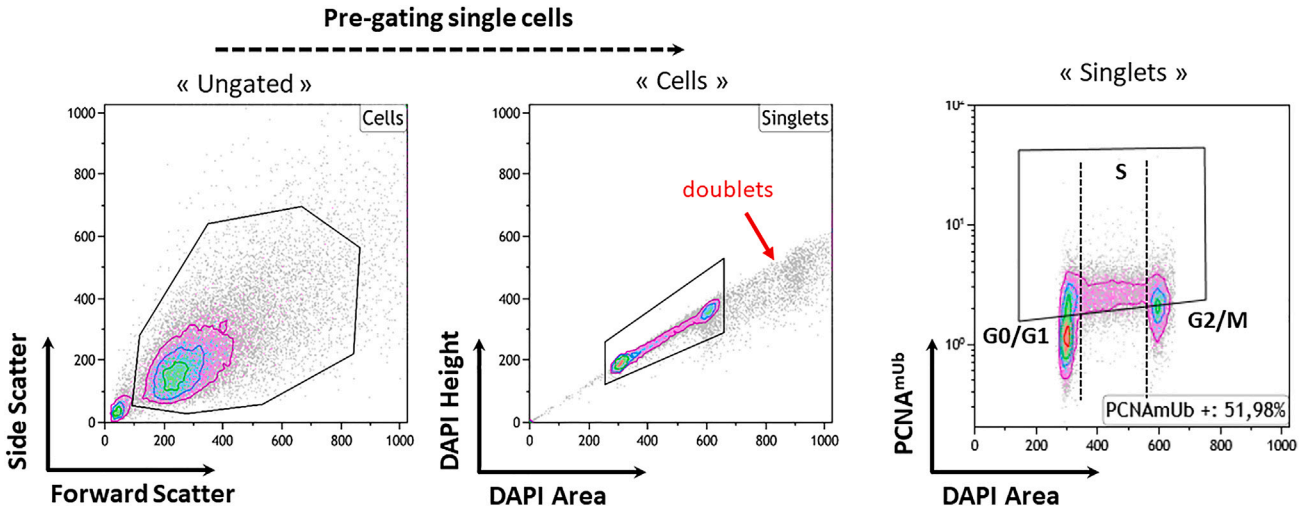

Figure 2. Pre-gating general workflow performed on Kaluza Acquisition Software for flow cytometry analysis (related to 7. b. II-V)

Sample analysis

8. Sample analysis.
   a. Immunofluorescence.
      i. Observe with a fluorescence microscope equipped with ApoTome or an equivalent option.
      ii. Set the exposure time by scanning through your positive control slide (i.e., UV-C-treated cells). The signal spectrum should never saturate in order to have a reliable quantification of the fluorescence intensities.
      iii. Save images and quantify using Image J or CellProfiler 4.2.1 software using the DAPI to make a nuclear mask. Pipelines for fully-automated nuclear foci counting, as well as intensities and colocalization of 2 or 3 targets are available upon request (and see Egger et al., Cell Reports methods, in press).
   b. Flow cytometry.
      i. Run the sample through a cytometer equipped with laser lines matching your antibodies and DNA counterstaining settings. In our experience, the best cytometer in terms of resolution and coefficient of variation (CV) for this kind of experiments is the Gallios.
      ii. Plot Forward Scatter (FSC) and Side Scatter (SSC) to gate in the cell population and gate out putative debris (see Figure 2).
      iii. Plot DAPI Height/DAPI Area to gate in the singlets and gate out putative doublets (see Figure 2).
      iv. Set the voltages for the TLS targets using your negative (no primary antibody) and positive controls (i.e., UV-C-treated cells). These settings should never be changed afterward, between the different experimental conditions.
      v. Analyze at least 20,000 events per sample to obtain robust statistics.
      vi. Process the data with Kaluza Analysis or Flow Jow software.

EXPECTED OUTCOMES

This protocol allows an accurate quantification of chromatin-bound, endogenous TLS factors in single cells, in respect to the cell cycle, as well as their nuclear localization in untreated cells, or upon exposure to DNA damaging agents. By combining labeling of ongoing DNA synthesis with detectable nucleotides analogs, this protocol will also allow to study TLS dynamics at DNA replication forks stalled by DNA damage. For the endogenous $PCNA^{mUb}$ labeling in HCT116, you can expect an heterogeneous labeling of untreated cells (S-phase cells, which can be detected by labeling with EdU, display a higher level of signal, with a few foci per nucleus, whereas non replicating cells will display a

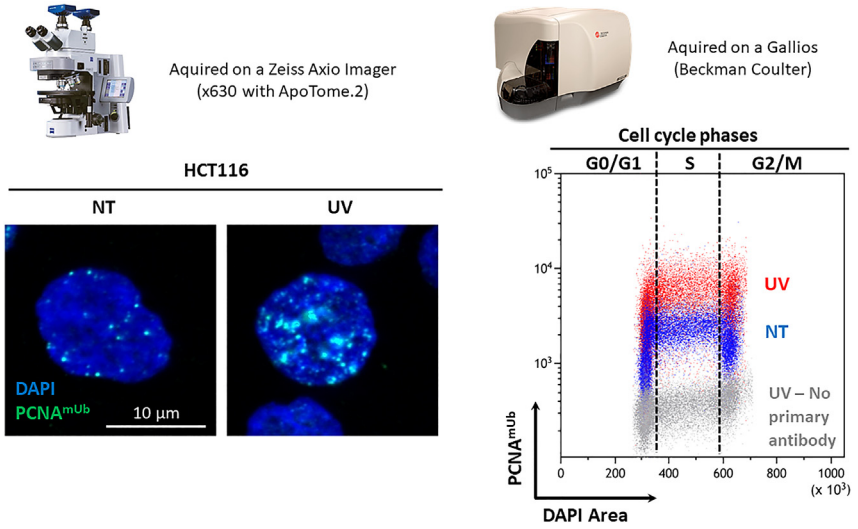

Figure 3. Typical localization of endogenous PCNA^{mUb} bound to chromatin by immunofluorescence (left panel) in either untreated cells (NT) or upon exposure to 20 J/m^2 and its detection by flow cytometry (right panel) in pre-extracted HCT116 cells

lower signal). The signal is expected to rise (in terms of both intensity and number of foci) after cell exposure to a TLS inducing treatment such as UV-C or H_2O_2 (see Figure 3). In flow cytometry, as the DNA is counterstained, you can expect a horseshoe pattern with replicating cells (Intermediate DNA content, see Figure 3) displaying a higher signal than non-replicating cells (i.e., G1 and G2/M cells). These kinds of results are also expected to a lesser extent with TLS polymerases that are less expressed and less tightly bound to chromatin than PCNA.

QUANTIFICATION AND STATISTICAL ANALYSIS

For experiments involving flow cytometry, 20,000 events (nuclei) should be analyzed per condition to obtain robust statistics.

For the microscopy immunofluorescence, at least 10 fields of each condition (500–1000 cells) should be captured to ensure robustness of the statistical analysis. We encourage you to quantify results using Cell Profiler 4.2.1, which is a fast and robust way to generate a variety of different measurements such as:

- The number of foci per nucleus.
- The mean intensity of each nucleus (do not use the integrated intensity, as it will also depend on the nuclei area).
- The putative colocalization with other markers (i.e., EdU, CPDs etc.).

LIMITATIONS

Although this protocol can, in principle, be applied to detect TLS factors in any cell, their relative cellular abundance, and the degree of endogenous replication stress may be limiting parameters for detection. A critical limitation is clearly the availability of a suitable antibody to detect TLS polymerases, whose specificity must be first tested in cells depleted of the desired target. Generation of transgenic cell lines in which a tag is inserted into the endogenous gene of interest (for instance by CRISPR/CAS9 technology) may help to circumvent this problem. In this latter situation, one must take into account that the tag might change the affinity of the protein for chromatin.

TROUBLESHOOTING

Problem 1

Cell detachment during pre-extractions for microscopy analysis.

Potential solution (related to step 2.a)

- Seed more cells to perform the experiment at a higher confluence.
- Use freshly-coated Poly-D-Lysine coverslips.
- Reduce the percentage of Triton X-100 to 0.1–0.05% during the on-ice pre-extraction.
- Reduce the time of the on-ice pre-extraction, closely monitoring the cells under a microscope.

Problem 2

Low, or no increased signal in the positive controls (IF/Flow cytometry).

Potential solution (related to step 2.a and step 2.b)

- Reduce the percentage of Triton X-100 to 0.1%–0.05% during the on-ice pre-extraction.
- Add a final concentration of 200 mM of sucrose to the extraction buffer to be less stringent in the pre-extraction conditions. Proteins less tightly bound to chromatin will be retained. This procedure is specifically suitable for the detection of TLS polymerases.
- Reduce the time of the on-ice pre-extraction.

Problem 3

Loss of biological material during the processing of flow cytometry samples and flow rates issues.

Potential solution

- Use Eppendorf tubes of higher plastic quality. Avoid the use of autoclaved Eppendorfs which can affect plastic quality and lead material loss during the repeated centrifugation steps.
- Verify that post-extraction and post-fixation centrifugation speeds are set to 500 × *g* and 750 × *g* as described in the protocol, as the extraction and fixation steps result in lighter events (i.e., nuclei rather than full sized cells) therefore requiring higher centrifugation speeds.
- Adapt the volume of Analysis buffer (step 7.b.2) in order to achieve a flow rate of 500–1000 events/sec during the acquisition.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Domenico Maiorano (domenico.maiorano@igh.cnrs.fr).

Materials availability

Any materials generated in this study are being made available. This study did not generate new unique reagents.

Data and code availability

This study does not report sequencing data, nor original code.

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AUTHOR CONTRIBUTIONS

Conceptualization, T.E.; writing of the first draft, T.E., D.M. Revision, T.E., D.M., A.A.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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