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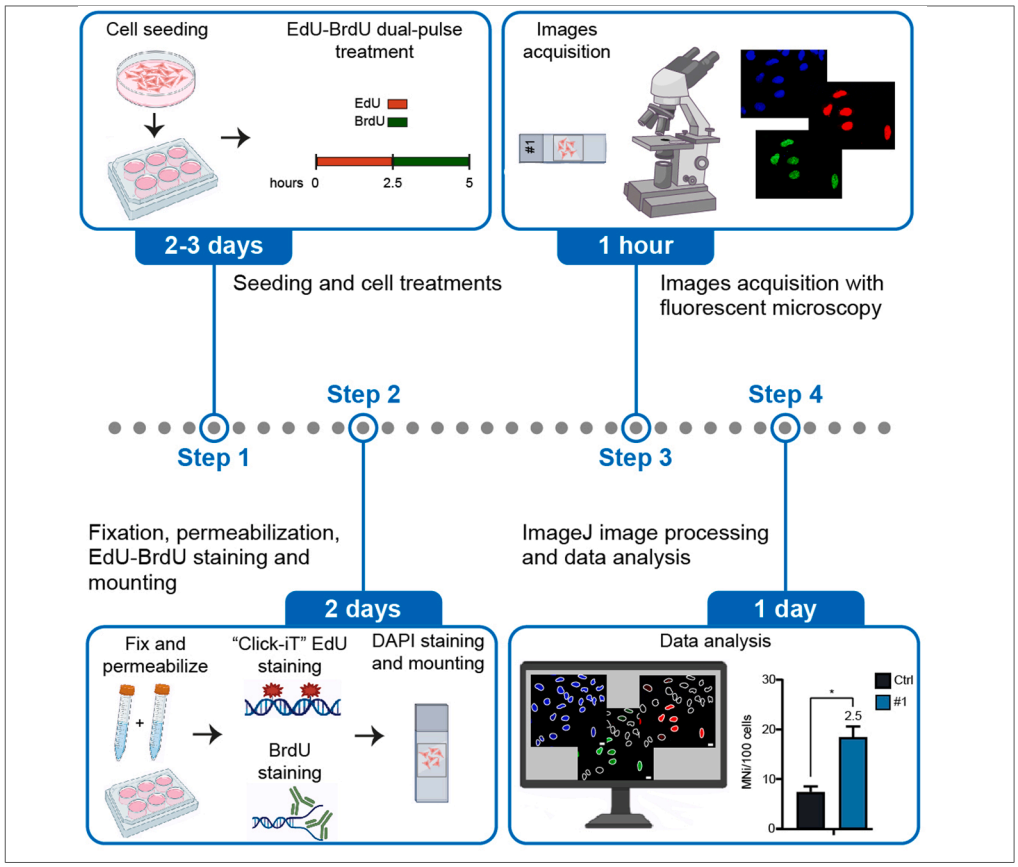
Protocol to investigate cell cycle-dependent modes of micronucleus formation using dual-pulse nascent DNA labeling



Here, we present a protocol to study the contribution of individual cell-cycle stages to genome instability mechanisms using dual-pulse nascent DNA labeling. We describe steps for sample treatment, immunofluorescence staining, imaging, and image processing. This protocol enables classification of cells according to their cell-cycle phase at the time of drug treatment of interest. It is valuable for investigating cell cycle-dependent treatment effects and is compatible with immunofluorescence-based analyses.

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

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Highlights

Instruction for seeding of cultured cells on coverslips into 6-well plates

Steps for performing dual-pulse EdU/BrdU labeling in cultured cells

Procedures for fixation, permeabilization, and immunofluorescence staining

Guidance on imaging and analysis of cell-cycle phases and damage markers

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Protocol to investigate cell cycle-dependent modes of micronucleus formation using dual-pulse nascent DNA labeling

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SUMMARY

Here, we present a protocol to study the contribution of individual cell-cycle stages to genome instability mechanisms using dual-pulse nascent DNA labeling. We describe steps for sample treatment, immunofluorescence staining, imaging, and image processing. This protocol enables classification of cells according to their cell-cycle phase at the time of drug treatment of interest. It is valuable for investigating cell cycle-dependent treatment effects and is compatible with immunofluorescence-based analyses.

For complete details on the use and execution of this protocol, please refer to Pepe et al.[1] and Duardo et al.[2]

BEFORE YOU BEGIN

The dual-pulse labeling technique with EdU (5-ethynyl-2′-deoxyuridine) and BrdU (5-bromo-2′-deoxyuridine) thymidine analogs is a powerful method for monitoring cell proliferation and precisely tracking cell cycle progression, key processes in assessing cell health, genotoxicity and drug efficacy.[3,4] Traditionally, cell proliferation has been evaluated using a single-pulse labeling approach with a nucleoside analog, which offers limited temporal resolution. Here, we present a dual-pulse labeling protocol based on the sequential incorporation of EdU and BrdU into newly synthesized DNA, enabling precise classification of cells according to their cell cycle phase at the time of labeling. This approach not only allows identification of the specific phase in which key cellular events occur but also provides a robust framework for analyzing cell cycle-dependent responses to treatments. In the studies by Pepe et al.[1] and Duardo et al.[2] HeLa and U2OS cells were treated with the Topoisomerase I poison camptothecin (CPT) and the G4 stabilizer pyridostatin (PDS) for a short period (1 hour), overlapping with the mid phase of EdU labeling and immediately preceding BrdU incorporation. This workflow enables precise classification of cells according to their cell cycle phase at the time of drug treatment and allows evaluation of cell cycle phase-specific responses to the compound, thereby advancing understanding of micronuclei formation mechanisms associated to Topoisomerase I poisoning and G4 stabilization. Moreover, we show that this protocol is highly compatible with immunofluorescence-based analyses, enabling the simultaneous detection of damage-associated proteins (e.g., γH2AX) and other markers of genomic instability, such as micronuclei and chromosomal aberrations. Unlike traditional synchronization methods, dual-pulse labeling does not require artificial cell cycle arrest, preserving natural cell cycle dynamics. While EdU has been reported to exhibit cytotoxic effects

under prolonged exposure (24 hour),[5,6] brief pulses are well tolerated. In this protocol, EdU is applied for a much shorter duration (2.5 h) followed by a recovery period, minimizing cytotoxic effects while enabling reliable tracking of cell cycle progression. Additionally, dual-pulse labeling provides a more precise and detailed classification of cells across different cycle phases compared to methods based solely on EdU or BrdU incorporation patterns.[7] Compared to earlier methods using CldU and IdU, EdU/BrdU dual-pulse labeling offers enhanced specificity and speed: EdU allows rapid and specific detection via click chemistry without DNA denaturation, preserving sample integrity, while BrdU remains highly reliable thanks to well-established antibodies and protocols.[8–11] Importantly, there is no antibody cross-reactivity between BrdU and EdU, ensuring clean signals and facilitating precise interpretation.[8,12] Emerging analogs further expand the toolkit: 5-vinyl-2′-deoxyuridine (VdU) allows copper-free labeling with minimal structural perturbation and reduced cytotoxicity, thought it generally produces weaker signals, and certain probes can induce DNA damage.[8,13,14] 2′-fluoro-arabinosyl-EdU (f-ara-EdU) incorporates efficiently with lower toxicity, making it suitable for long-term studies, although detection methods are currently less developed than for EdU.[5,8] In conclusion, by enabling high-resolution analysis of cell cycle progression and the effects of specific drug treatment, dual-pulse labeling stands out as a superior technique for investigating cell cycle regulation and genomic instability mechanisms.

Note: The protocol below describes the specific steps for using HeLa cells. However, we have also used this protocol in U2OS, HCT116 and HEK 293 cell lines and therefore can be adapted to different cell types.

Innovation

This protocol presents an optimized dual-pulse labeling method using EdU and BrdU thymidine analogs for high-resolution monitoring of cell cycle progression and phase-specific responses to treatment. Unlike traditional single-pulse assays, which provide limited temporal resolution, sequential EdU/BrdU incorporation allows precise classification of cells according to their cell cycle phase at the time of labeling. By performing a short drug treatment during EdU/BrdU incorporation, this protocol enables precise classification of cells according to their cell cycle phase at the time of drug treatment and allows evaluation of cell cycle phase-specific responses to the compound. The combined use of click chemistry–based EdU detection and antibody-based BrdU staining ensures rapid, specific, and non-overlapping signal acquisition. Compared with earlier analog combinations (e.g., CldU/IdU), this approach offers greater specificity, faster processing, and improved sample preservation. Importantly, the workflow avoids artificial synchronization, maintaining natural cell cycle dynamics and allowing physiologically relevant analysis. It is also fully compatible with immunofluorescence detection of DNA damage and genomic instability markers (e.g., γH2AX, micronuclei). Overall, this EdU/BrdU dual-pulse protocol provides a rapid, non-invasive, and versatile approach for dissecting cell cycle–dependent mechanisms of drug response and genomic instability, representing a significant methodological advancement over existing single-labeling or synchronization-based methods.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
BrdU monoclonal antibody (MoBU-1), Alexa Fluor 488 (used 200 times diluted)	Thermo Fisher Scientific	Cat# B35130
Anti-phospho-Histone H2A.X (Ser139) antibody, clone JBW301 (used 1,000 times diluted)	Merck	Cat# 05-636
Goat anti-mouse IgG (H+L) cross-adsorbed secondary antibody, Alexa Fluor 488 (used 500 times diluted)	Thermo Fisher Scientific	Cat# A11001

(*Continued on next page*)

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Goat anti-rabbit IgG (H+L) cross-adsorbed secondary antibody, Alexa Fluor 594 (used 500 times diluted)	Thermo Fisher Scientific	Cat# A11012
Chemicals, peptides, and recombinant proteins		
5-bromo-2′-deossiuridina (BrdU)	Sigma-Aldrich	Cat# 23151
Camptothecin (CPT)	Merck	Cat# C9911
Pyridostatin (PDS)	Selleckchem	Cat# S7444
4′,6-diamidino-2-fenilindolo (DAPI)	Sigma-Aldrich	Cat#D9542
Bovine serum albumin (BSA)	Sigma-Aldrich	Cat#A4503
DMEM medium	Thermo Fisher Scientific	Cat#21969035
Citric acid	Sigma-Aldrich	Cat#C1909
Ethanol 99.99%	Sigma-Aldrich	Cat#51976
Fetal bovine serum (FBS)	Thermo Fisher Scientific	Cat#A5256701
Formaldehyde (FA) 37%	Sigma-Aldrich	Cat#47608
Hydrochloric acid (HCL) 37%	Sigma-Aldrich	Cat#320331
Mowiol	Merck	Cat# 81381
Poly-L-lysine hydrobromide	Sigma-Aldrich	Cat#P2636
Sodium phosphate dibasic (Na_2HPO_4)	Sigma-Aldrich	Cat#106559
Sodium chloride (NaCl)	Sigma-Aldrich	Cat#S9625
Potassium chloride (KCl)	Sigma-Aldrich	Cat#P9541
Potassium phosphate monobasic (KH_2PO_4)	Sigma-Aldrich	Cat#P5379
Trypsin	Thermo Fisher Scientific	Cat#15090046
Triton X-100	Sigma-Aldrich	Cat#T8787
Critical commercial assays		
Click-iT EdU cell proliferation kit for imaging, Alexa Fluor 594 dye	Thermo Fisher Scientific	Cat# C10339
Click-iT EdU cell proliferation kit for imaging, Alexa Fluor 647 dye	Thermo Fisher Scientific	Cat# C10340
Experimental models: Cell lines		
HeLa	ATCC	N/A
HCT116	ATCC	N/A
HEK293-TFIISm	Gift by J. Q. Svejstrup (CGEN, University of Copenhagen, Denmark)	Previously characterized and validated in Zatreanu et al.[15]
Software and algorithms		
ImageJ (Fiji)	GitHub	https://imagej.net/software/fiji//
Prism 9	GraphPad Software	N/A
Other		
Cell culture 6-well plates	Corning	Cat#10578911
Cell culture dishes-10 cm	Corning	Cat#430167
Glass coverslip (24 × 24 mm, 0.13–0.16 mm thickness)	Heinz Herenz	Cat#1052450
Microscope slides	Knittel	N/A
Parafilm	Sigma-Aldrich	Cat#HS234526B

MATERIALS AND EQUIPMENT

Reagents and solutions for seeding of cultured cells

1X PBS solution		
Reagent	Final concentration	Amount
NaCl	137 mM	8 g
KCl	2.68 mM	0.2 g
Na_2HPO_4 x 7 H_2O	10 mM	2.7 g
KH_2PO_4	1.76 mM	0.24 g
ddH_2O	N/A	1 L
Autoclave and store at 4°C for up to one month.		

Reagents and solutions for dual-pulse labeling treatment

2X EdU working solution: Prepare a 10 mM EdU stock solution by dissolving EdU powder in DMSO according to the manufacturer's instructions provided with the Click-IT EdU Imaging Kit. Store at − 20°C protected from light. The stock solution is stable up to one year under these conditions. To prepare 10 mL of 2X EdU working solution (20 μM), add 20 μL of 10 mM EdU stock to 10 mL culture medium. Prepare immediately before use.

2X BrdU working solution: Prepare a 10 mM BrdU stock solution by dissolving 100 mg of BrdU (molar mass: 307.10 g/mol) in 32.5 mL of DMSO. Store at −20°C protected from light. The stock solution is stable up to one year under these conditions. To prepare 10 mL of 2X BrdU working solution (20 μM), add 20 μL of 10 mM BrdU stock to 10 mL culture medium. Prepare immediately before use.

> *Note:* Both solutions are light sensitive. Therefore, it is recommended to protect them from light during preparation, storage and throughout the experiment, for example, by wrapping them in aluminum foil.

Reagents and solutions for fixation and permeabilization

3.7% FA-PBS solution: Mix 1 mL of 37% formaldehyde (FA) in 10 mL of 1X PBS to prepare 10 mL solution.

> ⚠ CRITICAL: Formaldehyde is a toxic chemical. Handle in a fume hood and wear appropriate PPE.

0.5% Triton-PBS solution: Add 50 μL of 100% Triton X-100 in 10 mL of 1X PBS.

> *Note:* The 100% Triton X-100 solution is highly viscous; therefore, use a wide-bore tip to facilitate accurate and complete volume aspiration.

> *Note:* These solutions should be freshly prepared each time prior to use.

Reagents and solutions for denaturation

4 N HCl solution: Mix 4 mL of 10 N HCl in 10 mL of ddH_2O.

> ⚠ CRITICAL: HCl is a strong acid that can cause serious burns and irritation. Always wear appropriate PPE.

0.2 M sodium phosphate solution: Dissolve 1.2 gr of sodium phosphate dibasic (Na_2HPO_4, molar mass: 141.96) in 40 mL of ddH2O.

> *Note:* It is not necessary to prepare this solution fresh each time and can be stored at room temperature; however, check for the presence of any precipitate before use. If a precipitate is present, which may consist of sodium phosphate crystals formed during storage at low temperature, prepare a fresh solution.

0.1 M citric acid solution: Dissolve 0.2 gr of citric acid (molar mass: 210.14 g/mol) in 10 mL of ddH2O.

> ⚠ CRITICAL: Citric acid is harmful and may cause significant irritation. Always wear the appropriate PPE.

> *Note:* Heat the solution on a magnetic stirrer plate at 40°C to ensure complete dissolution.

Sodium phosphate-citric acid pH 7.4 solution (91% sodium phosphate solution-9% citric acid solution): Mix 9.1 mL of phosphate solution 0.2 M with 900 μL of 0.1 M citric acid solution to achieve a total volume of 10 mL. If performed correctly, the pH of the solution should be 7.4. Verify the pH for accuracy.

Note: All these solutions can be stored at room temperature up to one month.

3% BSA-PBS solution: Dissolve 0.3 gr of bovine serum albumin (molar mass: 66 g/mol) in 10 mL of 1X PBS.

Reagents and solutions for EdU and BrdU staining

EdU staining solution		
Reagent	Final concentration	Amount
1X Click-IT reaction buffer	0.875%	87.5 μL
$CuSO_4$	0.2%	2 μL
Alexa Fluor Azide 594 or 647	0.05%	0.5 μL
1X Click-IT EdU buffer additive	0.1%	10 μL
Total	**N/A**	**100 μL**

Add the reagents in the order listed at room temperature (RT) and, to use the solution within 15 minutes of preparation.

Blocking solution: Dissolve 0.3 gr of bovine serum albumin (BSA, molar mass: 66 g/mol) in 10 mL of 1X PBS.

Note: Blocking solution can be stored at 4°C. Use within one week of preparation is recommended.

BrdU staining solution: To obtain a 1:200 antibody dilution, add 5 μL of BrdU primary antibody to 1 mL of 3% BSA-0.1% Triton-PBS solution.

Reagent	Final concentration	Amount
BSA	3%	0.3 g
Triton X-100	0.01%	10 μL
PBS	N/A	10 mL

Note: To ensure optimal results, the 3% BSA-0.1% Triton-PBS solution should be cold at the time of BrdU antibody addition. Therefore, prepare the solution 10–15 minutes prior to adding the antibody and keep it at 4°C until use.

Secondary antibody solution: To obtain a 1:500 dilution, add 2 μL of BrdU primary antibody to 1 mL of the 3% BSA-PBS solution.

DNA staining solution: Prepare a 0.1 mg/mL DAPI stock solution by dissolving 1 mg of DAPI (molar mass: 350.25 g/mol) in 10 mL of ddH_2O. Aliquot and store at −20°C. To prepare a 3 μg/mL DAPI working solution, mix 33 μL of the 0.1 mg/mL DAPI stock solution with 1 mL of ddH_2O.

STEP-BY-STEP METHOD DETAILS

Seeding of cultured cells into 6-well plates

Timing: 1 h

Seed cells at low density on glass coverslips and allow them to adhere. Optimize the seeding density for imaging. Maintaining proper cell density is essential, as excessive confluency can make image analysis challenging by preventing clear distinction between individual nuclei. Ensure precise and consistent seeding to achieve uniform growth rates and confluency across replicates. This protocol uses HeLa cells as a model for adherent cell types.

1. Seeding HeLa cells in a 6-well plate.
   a. Culture HeLa cells in DMEM supplemented with 10% heat-inactivated FBS in a 10 cm dish, in a humidified incubator at 37°C with 5% CO_2, until they reach approximately 80% confluence.
   b. In a biosafety cabinet, place a sterile glass coverslip into the desired wells of a 6 well plate.
   c. Aspirate the old media from the 10 cm dish of HeLa cells and wash the cells with 5 mL of 1X PBS.
   d. Add 1 mL of 0.25% trypsin to the cells and incubate at 37°C for 5 min, or as needed, to detach the cells. Check under a microscope to ensure that cells are detached.
   e. Add 4-5 mL of 10% FBS-completed media to the flask to neutralize the trypsin. Pipette media up and down 1-3 times, dispensing media across the surface area of the flask to collect all the cells on suspension.
   f. Transfer cell suspension to a 15 mL tube and count cells using an automated cell counter or a Burker chamber.
   g. Seed 2×10^5 cells per well in 2 mL of media into the desired wells of a 6-well plate containing a sterile glass coverslip. Ensure the medium fully covers the coverslip and that it is properly seated at the bottom of each well by gently applying pressure with a pipette tip or sterile tweezers.
   h. Incubate cells for 24 hours in a humidified incubator at 37°C with 5% CO2.

Note: Coverslips are typically provided pre-sterilized and do not require further sterilization unless the packaging has been exposed to a non-sterile environment. If the sterility of the coverslips is uncertain, wash them first with H_2O and then with ethanol, then sterilize them in autoclave before use.

Dual-pulse labeling treatment

Timing: 5 h

Live cells are sequentially labeled with 10 μM EdU and 10 μM BrdU for 2.5 hours each, then cultured in drug-free medium until the next mitosis, which, for HeLa cells, occurs after approximately 24 hours (Figure 1A).

2. Labeling cells with EdU and BrdU.
   a. Prepare a 2X EdU working solution (20 μM) by diluting the 10 mM EdU stock in culture medium. This 2X solution will give a final concentration of 10 μM when added to the cells.
   b. Aspirate the old media from the 6-well plate containing HeLa cells.
   c. Add 1 mL of DMEM supplemented with 10% heat-inactivated FBS without EdU, then add an equal volume of the 2X EdU solution to obtain a 1X EdU solution.

Note: According to the manufacturer's instructions for the Click-IT EdU Imaging Kit, a direct addition of a 10 μM EdU solution is not recommended, as it may interfere with the proliferation rate. For additional details, please refer to Click-IT EdU Imaging Kit datasheet.

   d. Incubate in a humidified incubator for 30 minutes to 2.5 hours, depending on the experimental protocol.

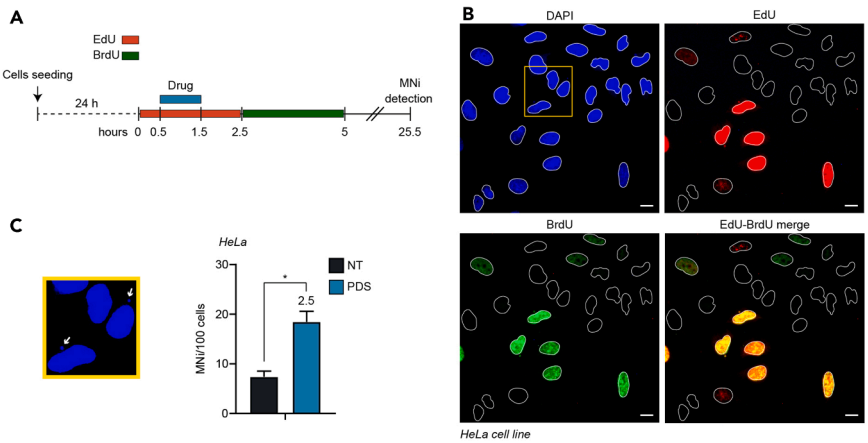

Figure 1. Experimental setup and immunofluorescence detection of EdU-BrdU labeled HeLa cells

(A) Schematic representation of the experimental design for micronuclei detection in EdU-BrdU-labeled HeLa cells.

(B) Immunofluorescence (IF) images of HeLa cells labeled with EdU and BrdU. EdU incorporation was detected using the Click-IT EdU Imaging Kit-Alexa Fluor 594, while BrdU was visualized using specific primary and secondary antibodies. Images were acquired using a 40X objective (planApo 40X/0.95). Scale bar 10 μm.

(C) IF image showing DAPI-stained HeLa cells with micronuclei (white arrows, left panel). The magnified area corresponds to the region highlighted in panel B. Quantitation of micronuclei in HeLa cells treated with the G4 binder pyridostatin (PDS) for 1 hour during the EdU period (right panel). Graph shows mean ± SEM, n=3. Average analyzed cells: 1400. *$p < 0.05$ (t test).

Note: The duration of EdU exposure allows an accurate measurement of cells actively synthesizing DNA. Therefore, the exposure time may vary depending on the cell growth rate and the specific experimental conditions used. For example, in Pepe et al.,[1] to assess the cell cycle phase of HeLa cells during treatment with the G4 binder Pyridostatin (PDS), EdU was administered for 2.5 hours, with PDS added during the final hour of EdU labeling. BrdU was then administered immediately after EdU for an additional 2.5 hours. Additionally, using different incubation times than those specified here may require adjusting the final concentration of EdU/BrdU.

e. At the end of EdU treatment, wash the cells with 2 mL of 1X PBS.
f. Prepare a 2X BrdU working solution (20 μM) by diluting the 10 mM BrdU stock in culture medium. This 2X solution will give a final concentration of 10 μM when added to the cells.
g. Add 1mL of DMEM supplemented with 10% heat-inactivated FBS without BrdU, then add an equal volume of the 2X BrdU solution to obtain a 1X BrdU solution (see note in step c).
h. Incubate in a humidified incubator for 30 minutes to 2.5 hours, depending on the experimental protocol (see note in step d).
i. At the end of BrdU treatment, wash the cells with 2 mL of 1X PBS.
j. Add 2 mL of drug-free DMEM medium supplemented with 10% heat-inactivated FBS.
k. Incubate in a humidified incubator for 24 hours.

Note: The recovery period in drug-free medium may vary depending on the cell cycle duration and the type of analysis being performed. For micronucleus detection, when working with unsynchronized cells, the recovery time should allow the cells to undergo at least one mitosis. For HeLa cells, which are used as the reference in this protocol, a 24 hour recovery period is sufficient to complete one mitosis.

Optional: Add cytochalasin B at a final concentration of 8 μg/mL during a 24 hour recovery period to later perform the micronucleus analysis in binucleated cells. Adjust the cytochalasin B concentration based on the recovery time.

Fixation and permeabilization

Timing: 1 h

Live cells are fixed with 3.7% formaldehyde (FA) and permeabilized with Triton X-100 to enable EdU and BrdU DNA staining, as well as immunostaining of intracellular protein targets.

3. Fixation.
   a. In a fume hood remove media from 6-well plate.
   b. Wash cells with 2 mL of 1X PBS.
   c. Add 1 mL of 3.7% FA-PBS solution.
   d. Incubate for 15 minutes at room temperature. Do not move the plate until fixation is over.
   e. Wash cells two times with 2 mL of 1X PBS, incubating for 5 minutes each time on a rotating plate at 100 rpm (gentle agitation).

   Note: If you are handling multiple plates, complete all the washes first and then remove the liquid at the end to minimize the time the cells remain without liquid.

   Pause Point: The coverslips can be stored at 4°C in 1X PBS for up to two weeks, provided the 6-well plate is sealed with Parafilm and aluminum foil.

4. Permeabilization.
   a. Add 1 mL of 0.5% Triton-PBS solution per well.
   b. Incubate for 15 minutes at room temperature on a rotating plate at 100 rpm.
   c. Wash cells with 2 mL of 1X PBS for 5 minutes on a rotating plate at 100 rpm.

Denaturation step

Timing: 30 min

An acid treatment is necessary to separate the DNA into single strands, allowing the primary antibody to bind to the incorporated BrdU. It is generally recommended to perform this treatment before applying antibodies or other compounds to preserve their integrity and effectiveness; however, since acid can interfere with the binding of certain antibodies, their compatibility should be tested beforehand. Moreover, milder alternatives to the harsh HCl treatment, such as enzymatic DNase I digestion or pH-based denaturation, can be employed.[8] These approaches minimize damage to subcellular structures and better preserve antigenicity, making them particularly suitable for downstream applications, such as protein localization studies using immunofluorescence.

5. Denaturation.
   a. Add 1 mL of 4 N HCl solution.
   b. Incubate for 8 minutes at 25°C.

   ⚠ CRITICAL: Suboptimal denaturation, whether insufficient or excessive, can result in poor BrdU or DAPI staining, respectively, and may therefore compromise the experiment.

   Note: The incubation time varies depending on cell confluence and the type of cell line used. The time indicated in this protocol is optimal for HeLa cells seeded and fixed according to the instructions described above. Additionally, the HCl denaturation step is temperature-dependent; therefore, it is recommended to use an incubator to maintain a controlled temperature of 25°C.

c. Immediately remove the HCl solution by performing a brief wash of the cells with 2 mL of 1X PBS.
d. Wash the cells again with 2 mL of 1X PBS for 5 minutes on a rotating plate at 100 rpm.
e. Add 1 mL of sodium phosphate-citric acid pH 7.4 solution.
f. Incubate for 5 minutes at 25°C.
g. Wash cells with 2 mL of 1X PBS for 5 minutes on a rotating plate at 100 rpm.
h. Wash cells with 2 mL of 3% BSA-PBS solution for 5 minutes on a rotating plate at 100 rpm.

EdU and BrdU staining

Timing: 20 h

Fixed cells are stained with EdU following the protocol provided in the Click-IT EdU Imaging Kit (other commercial kits or home-made solutions[9] can also be used), without modifications, and with BrdU using a specific primary antibody, followed by a secondary antibody to amplify the signal. This enables the classification of cells based on their cell cycle phase at the time of EdU/BrdU incorporation.

6. EdU staining.
   a. Prepare the EdU staining solution by combining the reagents provided in the Click-IT EdU Imaging Kit as described in "materials and equipment" section, using 100 μL per sample.
   b. Place a piece of parafilm in a petri dish and add 100 μL droplet of the EdU reaction cocktail. Then gently place the coverslip with the cell side down onto each droplet.
   c. Incubate for 30 minutes at room temperature, protected from light.
   d. Transfer the coverslips to a 6-well plate with the cell layer facing up and wash the cells twice with 2 mL of 3% BSA-PBS solution, incubating for 5 minutes each time on a rotating plate at 100 rpm.
7. Blocking.
   a. Add 2 mL of 3% BSA-PBS solution.
   b. Incubate for 1 hour at room temperature on a rotating plate at 100 rpm.
8. BrdU staining-primary antibody incubation.
   a. Dilute the BrdU primary antibody 1:200 in a 3% BSA-0.1% Triton-PBS solution.
   b. Place a piece of parafilm in a petri dish and add 100 μL droplet of primary antibody solution. Then gently place the coverslip with the cell side down onto each droplet.
   c. Incubate overnight (O/N) at 4°C, protected from light.

   Note: It is recommended to cover the petri dish with aluminum foil, not only to protect the samples from light but also to prevent the primary antibody solution from evaporating. This helps keep the samples from drying out, which could compromise primary antibody staining. For additional protection against drying, a slightly damp piece of paper towel can be placed over the dish, under the aluminum foil, to maintain humidity.

   Note: Depending on the experiment, samples can also be incubated with a second primary antibody. Be sure to check its species compatibility with the anti-BrdU primary antibody. If the second primary antibody does not require O/N incubation (e.g., γH2AX primary antibody, Millipore #05-636), follow the instructions in its datasheet and perform secondary antibody staining before the overnight incubation with the anti-BrdU antibody. For complete details on γH2AX detection protocol in EdU-BrdU labeled HeLa cells, please refer to Duardo et al.[2]

9. BrdU staining-secondary antibody incubation.
   a. After the primary antibody incubation is complete, transfer the coverslips to a 6-well plate with the cell layer facing up and wash the cells three times with 2 mL of 3% BSA-PBS solution, incubating for 5 minutes each time on a rotating plate at 100 rpm.

b. Dilute the secondary antibody 1:500 in a 3% BSA-PBS solution.
c. Place a piece of Parafilm in a Petri dish and add 100 μL droplet of secondary antibody solution. Then gently place the coverslip with the cell side down onto each droplet.
d. Incubate for 1 hour at room temperature, protected from light.
e. After completing the secondary antibody incubation, transfer the coverslips to a 6-well plate with the cell layer facing up and wash the cells three times with 2 mL of 3% BSA-PBS solution, incubating for 5 minutes each time on a rotating plate at 100 rpm.
f. Perform a final wash with 1X PBS solution for 5 minutes on a rotating plate at 100 rpm.

Note: The BrdU primary antibody used in this protocol is already conjugated with the Alexa Fluor 488 fluorophore, so secondary antibody incubation may not be necessary for fluorescence microscope visualization. However, we have observed that incubating with a secondary antibody amplifies the signal and provides better visualization of the staining.

10. Negative controls.

To ensure specificity and evaluate background fluorescence, include the following negative controls.

a. No-nucleoside analog control: cells not exposed to EdU or BrdU, processed through the full staining procedure to assess autofluorescence and non-specific background.
b. EdU-only with BrdU antibody: cells labeled only with EdU, followed by BrdU antibody staining, to test for potential cross-reactivity of the BrdU antibody with EdU.
c. EdU-only with DNA denaturation vs. no denaturation: cells labeled only with EdU, subjected to the DNA denaturation step prior to the Click-iT reaction, compared with non-denatured EdU-labeled samples, to determine whether denaturation affects EdU signal detection.
d. Single-nucleoside analog control: cells labeled with EdU only or BrdU only, followed by their respective detection protocols, to verify that fluorescence is restricted to the appropriate channel and to exclude antibody cross-reactivity or fluorophore bleed-through.

DNA staining

Timing: 1.5 h

Fixed cell nuclei are stained with DAPI, a critical step for accurate cell and micronuclei analysis and counting.

11. DAPI staining.

a. Dilute the 0.1 mg/mL DAPI stock solution in deionized water (ddH_2O) to achieve a final concentration of 3 μg/mL.
b. Place a piece of Parafilm in a Petri dish and add 100 μL droplet of DAPI solution. Then gently place the coverslip with the cell side down onto each droplet.
c. Incubate for 30 minutes at room temperature, protected from light.
d. Once DAPI staining is complete, transfer the coverslips with the cell layer facing up to a 6-well plate containing ddH_2O.
e. Remove as much liquid as possible from the coverslip and gently place it onto the drop of mounting medium (Mowiol) with the cell side facing down on the microscope slide. Allow the slides to dry for 1 hour at room temperature, protected from light.
f. The mounted slides should be stored at 4°C and kept protected from light until analysis.

Note: Typically, the slides can be stored at 4°C for up to 1 month. However, it is strongly recommended to perform the image acquisition as soon as possible, as the staining quality may decline over time.

Image acquisition

Timing: 30–40 min per sample

After completing the immunostaining, slide images are captured using a conventional fluorescence microscope (Figure 1B). In this protocol, we used a Nikon Eclipse 90i fluorescence microscope equipped with a 100 W super high-pressure mercury lamp as the fluorescence source. Images were acquired with a Nikon Digital Sight DS-U1 camera and using NIS-Elements software (version 3.1). The pixel size of the acquired images was 2560 × 1920. A 40X objective (planApo 40X/0.95) is sufficient for image acquisition; however, a 60X (planApo 60X/1.40) oil-immersion objective can be used for higher resolution, although this requires capturing a higher number of fields to be statistically significant.

12. Settings of microscope parameters.
    a. EdU signal is acquired using the TRITC/Texas red filter set (excitation 580 nm/emission 700 nm) for Alexa Fluor 594, or the CY5 filter set (excitation 628 nm/emission 692 nm) for Alexa Fluor 647. Exposure time is fixed throughout each experiment, ranging from 0.5 to 1.5 seconds. The required exposure time is influenced by staining quality, lamp power and efficiency, while the gain is consistently kept at the minimum setting.
    b. BrdU signal is acquired using the FITC filter set (excitation 470 nm/emission 570 nm) for Alexa Fluor 488. Exposure time is fixed throughout each experiment, ranging from 1 to 3 seconds. The required exposure time is influenced by staining quality, lamp power and efficiency, while the gain is consistently kept at the minimum setting.
    c. DAPI signal is acquired using the DAPI filter set (excitation 377 nm/emission 450 nm). Exposure time is fixed throughout each experiment, ranging from 0.5 to 2 seconds. The required exposure time is influenced by staining quality, lamp power and efficiency, while the gain is consistently kept at the minimum setting.
13. General guidelines for image acquisition to maintain consistency and minimize artifacts.
    a. Minimize exposure time and illumination intensity to reduce photobleaching while preserving a clear signal-to-background ratio.
    b. Capture images from equivalent positions in each well across all experimental replicates to ensure consistency.

Note: To ensure a representative analysis of cell populations classified by EdU and BrdU staining, a total of 500 to 1000 cells per sample should be analyzed in each of three independent culture experiments. Adjust the number of fields acquired per sample according to the cell density in each field.

Image analysis

Timing: 1 day per 30 fields

The acquired images are analyzed using ImageJ software (Figure 1C). Nuclei and micronuclei are counted manually, without automated segmentation or filtering, to ensure accurate classification into the four defined cell cycle phases. Cells are categorized as follows: early/mid S phase if both thymidine analogs (EdU and BrdU) were incorporated, late S phase if only EdU was incorporated, late G1 phase if only BrdU was incorporated, and early G1/G2M if neither EdU nor BrdU was incorporated, indicating that the cells were not undergoing DNA replication during the labeling pulses. Similarly, if micronuclei analysis is of interest, micronuclei can be manually classified as $EdU^{+/-}$ and/or $BrdU^{+/-}$ based on their respective staining patterns (Figures 2A–2C, 3A, 3B, 4A, and 4B). If γH2AX or another marker is under investigation, proceed with the appropriate analysis, such as γH2AX foci quantification or fluorescence intensity measurement, and report the results for each of the four defined cell cycle categories (Figure 5).

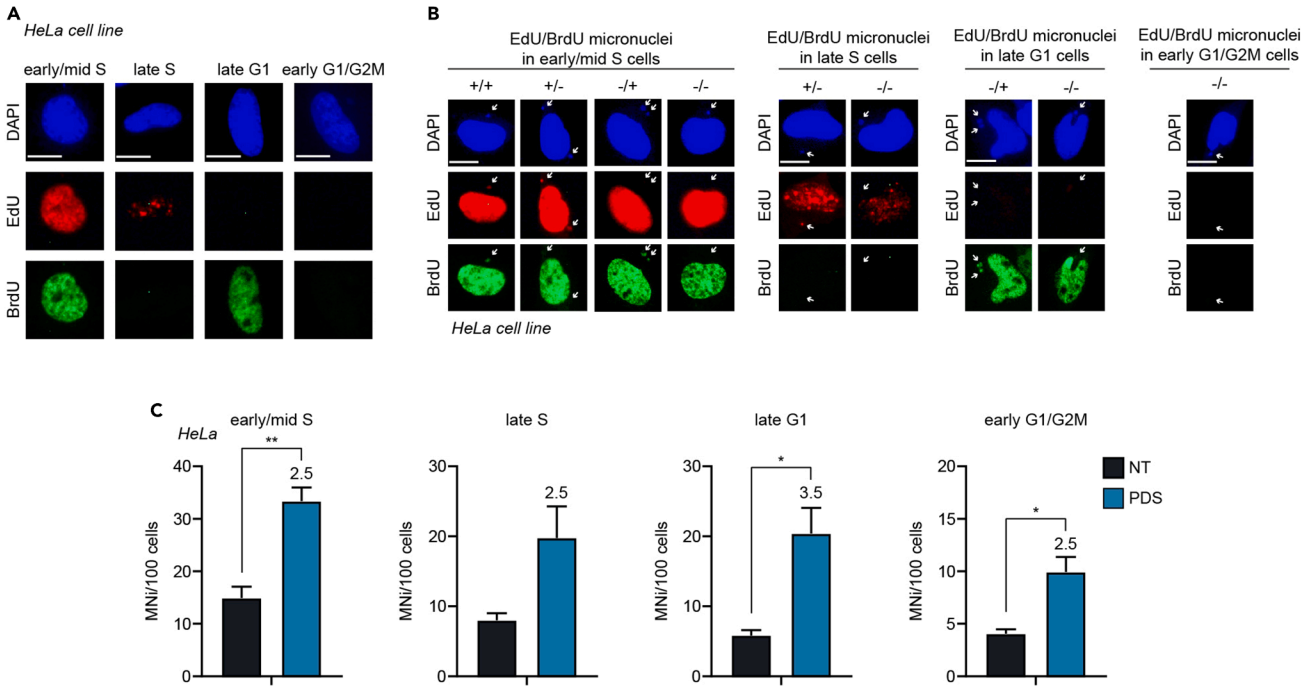

Figure 2. Cell cycle phase-specific micronuclei formation upon PDS treatment in HeLa cells

(A) Classification of HeLa cells in early/mid S, late S, late G1 and early G1/G2M phases based on EdU-BrdU staining.

(B) Classification of EdU-BrdU micronuclei (white arrows) in dual-labeled HeLa cells. Scale bar 10 μm.

(C) Micronuclei quantitation in HeLa cells, reported as MNi/100 cells, at early/mid S, late S, late G1, and early G1/G2M phases during 1 hour PDS treatment. Graphs show mean ± SEM, n=3. Average analyzed cells: 490 (early/mid S), 160 (late S), 116 (late G1), and 800 (early G1/G2M). *$p < 0.05$, **$p < 0.01$ (t test).

14. Guidance for image analyses.
    a. Open the acquired images in ImageJ.
    b. Count the nuclei and classify them according to EdU and BrdU incorporation patterns. If micronuclei analysis is included, manually count and categorize them as $EdU^{+/-}$ and/or $BrdU^{+/-}$ based on their staining profiles.

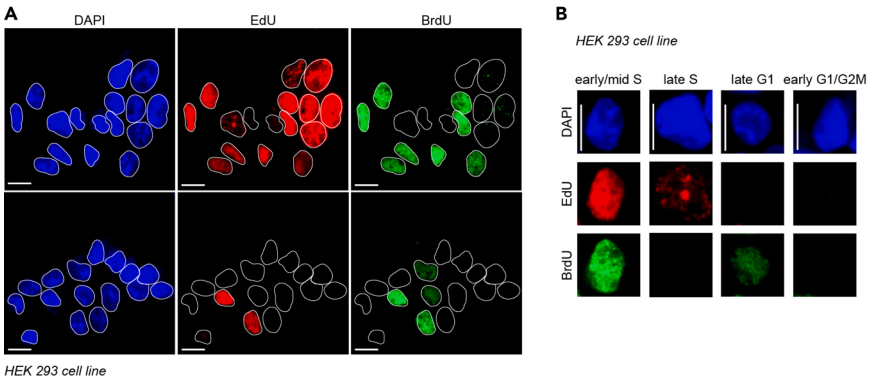

Figure 3. Immunofluorescence detection of EdU-BrdU labeled HEK 293 cells

(A) Representative immunofluorescence (IF) image showing HEK 293 cells stained with DAPI, EdU and BrdU. EdU incorporation was detected using the Click-IT EdU Imaging Kit-Alexa Fluor 594, while BrdU was visualized using specific primary and secondary antibodies. Images were acquired using a 40X objective (planApo 40X/0.95).

(B) Classification of HEK 293 cells in early/mid S, late S, late G1 and early G1/G2M phases based on EdU-BrdU staining. Scale bar 10 μm.

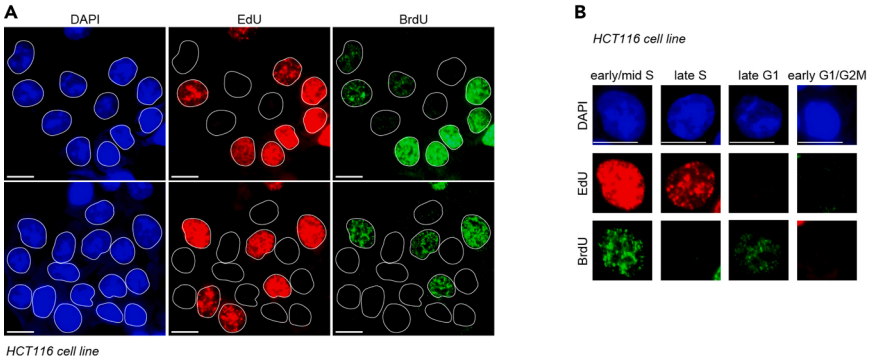

Figure 4. Immunofluorescence detection of EdU-BrdU labeled HCT116 cells

(A) Representative immunofluorescence (IF) image showing HCT116 cells stained with DAPI, EdU and BrdU. EdU incorporation was detected using the Click-IT EdU Imaging Kit-Alexa Fluor 594, while BrdU was visualized using specific primary and secondary antibodies. Images were acquired using a 40X objective (planApo 40X/0.95).
(B) Classification of HCT116 cells in early/mid S, late S, late G1 and early G1/G2M phases based on EdU-BrdU staining. Scale bar 10 μm.

c. For γH2AX analysis, nuclei are identified on the DAPI channel using the Wand Tool in ImageJ, which enables automated delineation of the nuclear area. The Tolerance value for the Wand Tool is set to 30 for HeLa cells but may be adjusted depending on the cell type used. Alternatively, nuclei can be selected using the Freehand Selections tool. The corresponding regions of interest (ROIs) are then transferred to the γH2AX channel (press SHIFT + E after selecting the relevant image). Within each nucleus, γH2AX staining is quantified as the mean fluorescence intensity across the nuclear area. To measure fluorescence intensity of selected nuclei, press CTRL + M (a new window with the measurements will open automatically). Background intensity is measured for each image, by drawing multiple ROI of comparable size in an area outside the nuclei, and these values are subtracted to obtain corrected nuclear intensities. Corrected intensities are subsequently normalized to the mean of the untreated control, allowing fold-change comparisons across experimental conditions. Quantification of γH2AX (fluorescence intensity measurement) is performed for each cell cycle category; however, the classification of nuclei into the four EdU/BrdU-defined cell cycle phases is carried out manually, as mentioned above.

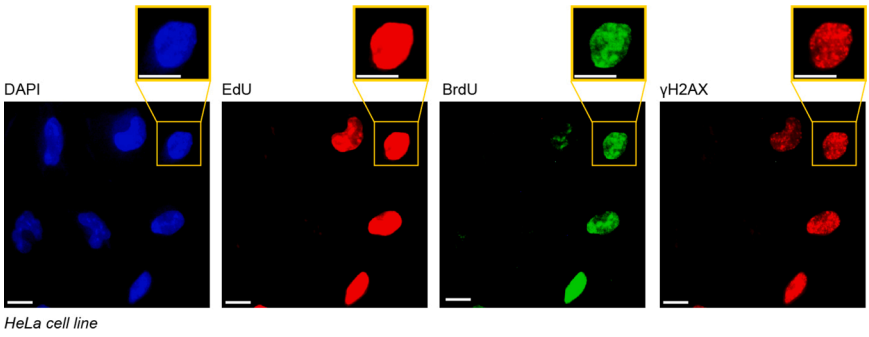

Figure 5. Immunofluorescence detection of γH2AX in EdU-BrdU labeled HeLa cells

Representative immunofluorescence (IF) image showing HeLa cells stained with DAPI, EdU, BrdU and γH2AX. DAPI, EdU, BrdU and γH2AX staining were performed at the end of BrdU incubation, without the 24 hour recovery period used for micronuclei detection. EdU incorporation was detected using the Click-IT EdU Imaging Kit-Alexa Fluor 647, BrdU was visualized using only primary antibody conjugated with Alexa Fluor 488. γH2AX detection (see note in step c of EdU and BrdU staining) was performed by incubating the samples with an anti-γH2AX primary antibody (1:1000, 2 hours at RT), followed by incubation with an anti-mouse Alexa Fluor 594 secondary antibody (1 hour at RT, 1:1000), prior to BrdU staining. The magnified area corresponds to the region highlighted in each IF image. Images were acquired using a 40X objective (planApo 40X/0.95). Scale bar 10 μm.

d. Record the counts in an Excel file or equivalent data table, ensuring clear differentiation between the four cell categories, documenting micronuclei and/or any additional markers of interest.
e. Plot the raw data using GraphPad Prism or equivalent software.

Note: Micronuclei data are usually reported as the number per 100 cells for each cell category, normalized or not with respect to the untreated control.

f. Perform the statistical analysis using GraphPad Prism or equivalent software. For micronucleus analysis in this protocol, we used a t-test.

EXPECTED OUTCOMES

This protocol allows for precise classification of cells into distinct cell cycle phases (early/mid S, late S, late G1, and G1/G2M) through the sequential incorporation of EdU and BrdU. It provides a powerful tool for investigating how various treatments affect cells during different phases of the cell cycle, enabling the identification of phase-specific responses. With its strong compatibility with immunofluorescence techniques, this protocol facilitates the simultaneous detection of damage-associated proteins, micronuclei, and chromosomal aberrations as markers of genomic instability. By combining dual-pulse labeling with immunofluorescence, we can examine micronuclei formation induced by G4s and R-loops stabilization across different cell cycle phases. This approach enhances the ability to track cell proliferation and DNA damage throughout the cell cycle, offering valuable insights into the mechanisms of chromosomal instability and the underlying causes of diseases such as cancer.

LIMITATIONS

A major drawback of this technique is that accurate result interpretation requires careful experimental design, as the labeling scheme must be optimized to accurately capture specific cell cycle phases. Notably, this approach only labels cells actively undergoing DNA replication, limiting its ability to provide information on G1 or G2 phase cells, unless combined with additional markers. Furthermore, the detection of EdU and BrdU requires specific methods, with BrdU detection often requiring DNA denaturation, which can compromise the integrity of certain epitopes making them more challenging to detect in immunofluorescence assays if the protocol is not properly optimized.

TROUBLESHOOTING

Problem 1: General

If the cells are not at the optimal confluence (either too low or too high), their replication rate may differ from usual, potentially compromising the effectiveness of treatments with EdU, BrdU, or with drugs (related to step 1), and may also affect the denaturation time (related to step 5) which is necessary for proper BrdU staining.

Potential solution

Re-seed the cells and ensure they reach the appropriate confluence based on the previous experiment.

Problem 2: Cell seeding

The cells adhere weakly and poorly to the glass coverslip (related to step 1).

Potential solution

For cells that exhibit poor or weak adhesion to glass coverslip (e.g., HEK293 cells), it is recommended to pre-treat the slides with poly-D-lysine before use. To do so, immerse the slides in a 0.1 mg/mL aqueous poly-D-lysine solution (prepared in ddH_2O) for 15-20 minutes, then rinse them with water and allow them to dry in a biosafety cabinet. Store at RT for up to 1-2 days. For longer storage, keep at 4°C.

Problem 3: Cell immunofluorescence staining

Significant amount of cell loss after each round of staining, especially for those that adhere poorly to the slide (related to step 3-9).

Potential solution

Be more precise during the washing steps by adding the liquid to the edges of the well rather than directly over the cells, and for example, use a 1000 μL pipette tip instead of a serological pipette.

Problem 4: DNA staining

DNA staining is suboptimal and too faint (related to step 10).

Potential solution

This could be due to excessive DNA denaturation, which prevents optimal nuclear staining with DAPI. Try incubating with a more concentrated DAPI solution for a longer period of time.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Jessica Marinello (jessica.marinello@unibo.it).

Technical contact

Technical questions on executing this protocol should be directed to and will be answered by the technical contact, Jessica Marinello (jessica.marinello@unibo.it).

Materials availability

This study did not generate new unique reagents.

Data and code availability

This study did not generate or analyze any sequencing data, and no original code is reported in this paper.

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AUTHOR CONTRIBUTIONS

G.C. and J.M. conceived the study, supervised the research, and secured fundings. S.P. conducted wet lab experiments and wrote the manuscript. R.C.D. contributed to the conduction of wet lab experiments. R.C.D., J.M., and G.C. reviewed and edited the manuscript.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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