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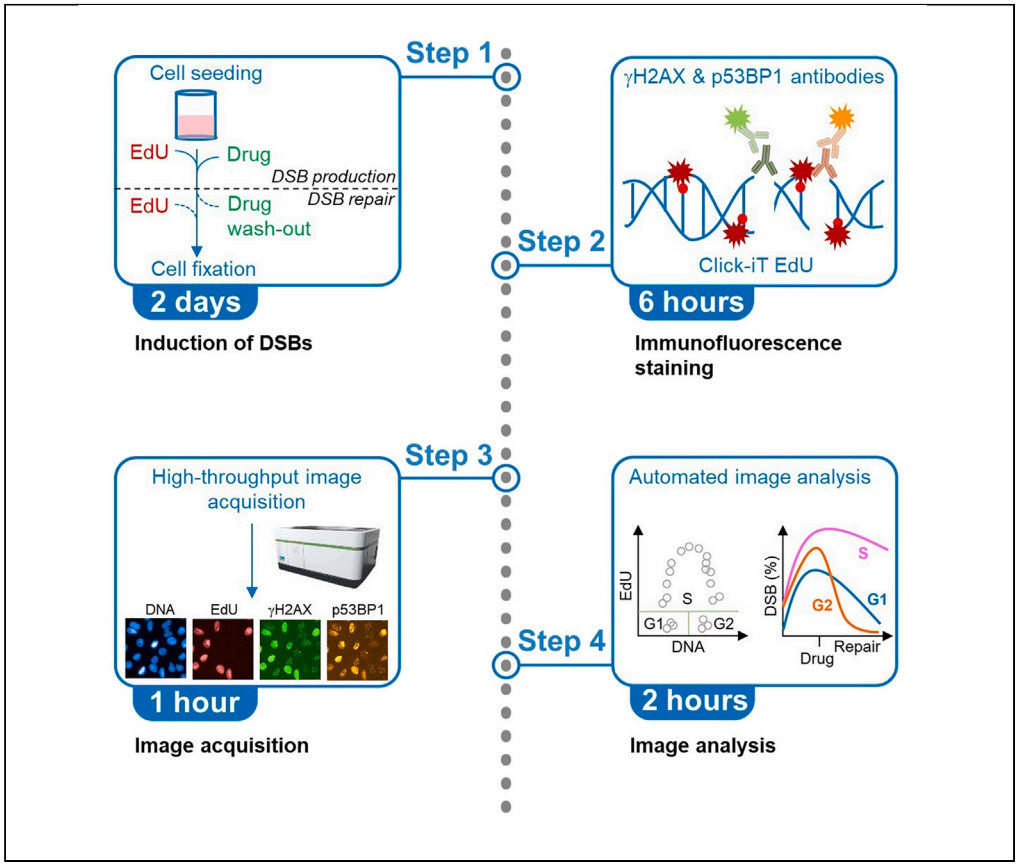
Protocol for single-cell analysis of DNA double-strand break production and repair in cell-cycle phases by automated high-content microscopy



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

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Highlights

Steps for quantification of DSB production and repair in cell-cycle phases

Instructions for automated high-content imaging

Guidance on cell seeding, staining, and data analysis

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Protocol for single-cell analysis of DNA double-strand break production and repair in cell-cycle phases by automated high-content microscopy

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SUMMARY

The mechanisms of DNA double-strand break (DSB) production and repair vary throughout the cell cycle. Here, we provide a protocol to quantify DSB production and repair in G1, S, and G2 phases of asynchronous adherent cells by coupling the staining of DSBs and cell-cycle markers with automated high-content fluorescence microscopy. We describe steps for cell seeding, treatment, staining, imaging, and analysis. This protocol is broadly applicable for monitoring DSB dynamics at single-cell level throughout the cell cycle.

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

BEFORE YOU BEGIN

The mechanisms of production and repair of DNA double-strand breaks (DSBs) can vary in different phases of the cell cycle.[2] Thus, in several contexts, it is essential to apply an assay that can simultaneously assess DSB level and distinguish the different phases of the cell cycle. This is for example useful when studying DSB production by transcription- or replication-dependent mechanisms,[3] and DSB repair, which can involve distinct pathways depending on the cell cycle.[2] Hence, such assay is particularly important for identifying mechanisms of DSB production and repair under physiological and pathological conditions, like those involving DNA damage and repair deficiencies. The most common method to quantify DSBs is based on the analysis of DNA damage response (DDR) proteins by fluorescence microscopy. DDR proteins rapidly accumulate and/or are modified at DSB sites, forming nuclear foci that can be visualized.[4] Phosphorylated histone H2AX on S139 (known as γH2AX) is the most commonly used marker of DSBs, as it is independent of the DSB repair pathway employed by the cell.[5] Another widely used DSB marker is 53BP1, which is recruited at DSB site where it serves as a recruitment platform for other DDR proteins and plays a role in DSB repair pathway choice.[4] The colocalization of γH2AX and 53BP1 foci is currently recognized as the most reliable marker of DSBs. Despite their extensive usage to dissect DSB production and repair, they have been mainly used to analyze DSBs independently of the cell cycle phases or, if the cell cycle was examined, only to study DSB production and not repair.[6–9] Here, we describe an automated protocol to analyze both DSB production and repair at single-cell level throughout the cell cycle. It couples the immunodetection by fluorescence microscopy of γH2AX and p53BP1 (53BP1 phosphorylated at S1778) together with the labeling of newly synthesized DNA with 5-ethynyl-2′-deoxyuridine (EdU) to mark S-phase cells and the staining of DNA content (Hoechst 33342) to further

discriminate G1 and G2 cells in asynchronous adherent growing cells. For DSB repair kinetic analysis in cell cycle phases, we further culture cells after drug removal in medium containing EdU. We also describe how to acquire images by using a high-throughput imaging system and perform analysis of both γH2AX and p53BP1 foci and fluorescence intensity per nucleus in an automated manner, to obtain DSB distribution dynamics in thousands of single cells under up to 96 different experimental conditions. This protocol describes the specific steps for using U2OS cancer cells in response to the DSB inducer camptothecin (CPT), with the Operetta CLS High-Content Imaging System for imaging and the Columbus software for analysis.[1] We further extend its area of application to other DSB inducers, such as to the restriction enzyme AsiSI[10,11] and etoposide,[12,13] and to other cell types, such as normal fibroblast cells. Furthermore, imaging and analysis steps can be adapted to employ other acquisition and analysis systems.[9] The application of this protocol for the quantification of DSB production and repair according to cell cycle phases has notably allowed to uncover that the accumulation of DSBs associated with the neurodegenerative syndrome SCAN1 caused by a mutation in the repair enzyme TDP1, occurs specifically in the G1 cell population due to increased DSB formation and lack of repair.[1] This key molecular mechanism associated with the G1 population and likely contributing to SCAN1 pathogenesis would have been masked by analysis of DSBs in the bulk population given the lower amount of DSBs produced in G1 compared to S and G2 cells and the different kinetics of repair between cell cycle phases.

Reagent and protocol preparation

1. Prepare buffers and reagents.
   a. Prepare stock and working solutions according to the recipes provided in the materials and equipment setup section.
   b. Store solutions and buffers that can be made ahead of time as indicated.

   Note: The full list of required materials and equipment is provided in the key resources table.

2. Prepare cells and maintain them in an exponentially growing phase (60–80% confluence).
3. Optimize primary antibodies used to label DSBs and cell cycle phases.
   a. Optimize antibody concentration starting from the instructions from the manufacturer.
   b. Test different antibody dilutions in the chosen cell line.

   Note: Antibodies suitable for immunofluorescence applications are compatible with this protocol.

4. Determine the kinetic range for DSB repair analysis, i.e., the time after drug removal and before fixation.

   ⚠ CRITICAL: The time required for DSB repair can vary depending on the cell line and the DNA damaging agent and should be first established by including both short and long time points.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
p53BP1 (phospho-Ser1778) rabbit polyclonal antibody (used for IF); 1:500	Cell Signaling Technology	Cat# 2675; RRID:AB_490917
CENPF (D6X4L) rabbit monoclonal antibody (used for IF); 1:500	Cell Signaling Technology	Cat# 58982; RRID:AB_2799552

(*Continued on next page*)

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Cyclin B1 (GNS1) mouse monoclonal antibody (used for IF); 1:100	Santa Cruz Biotechnology	Cat# sc-245; RRID:AB_627338
γH2AX (phospho-Ser139) mouse monoclonal antibody (clone JBW301) (used for IF); 1:500	Merck Millipore	Cat# 05-636; RRID:AB_309864
PCNA (10) mouse monoclonal antibody (used for IF); 1:50	Santa Cruz Biotechnology	Cat# sc-56; RRID:AB_628110
Donkey anti-mouse secondary antibody, Alexa Fluor 488 (used for IF); 1:500	Thermo Fisher Scientific	Cat# A-21202; RRID:AB_141607
Goat anti-rabbit secondary antibody, Alexa Fluor 594 (used for IF); 1:500	Thermo Fisher Scientific	Cat# A-11037; RRID:AB_2534095
Goat anti-mouse secondary antibody, Alexa Fluor 594 (used for IF); 1:500	Thermo Fisher Scientific	Cat# A-11032; RRID:AB_2534091
Goat anti-rabbit secondary antibody, Alexa Fluor 488 (used for IF); 1:500	Thermo Fisher Scientific	Cat# A-11070; RRID:AB_2534114
Chemicals, peptides, and recombinant proteins		
4-OHT [(Z) 4-Hydroxytamoxifen]	Sigma-Aldrich	Cat# H7904; CAS: 68047-06-3
Alexa Fluor 647 azide	Thermo Fisher Scientific	Cat# A10277
Auxin (indole-3-acetic acid sodium salt)	Sigma-Aldrich	Cat# I5148; CAS: 6505-45-9
Bovine serum albumin (BSA)	Euromedex	Cat# 04-100-812-E; CAS: 9048-46-8
CPT [(S)-(+)-camptothecin]	Sigma-Aldrich	Cat# C9911; CAS: 7689-03-4
$CuSO_4$	Sigma-Aldrich	Cat# 203165; CAS: 7758-99-8
Dimethyl sulfoxide (DMSO)	Sigma-Aldrich	Cat# D8418; CAS: 67-68-5
DMEM with 4.5 g/L glucose, L-glutamine, sodium pyruvate, and sodium bicarbonate	Sigma-Aldrich	Cat# D6429
EdU	Thermo Fisher Scientific	Cat# A10044; CAS: 61135-33-9
Ethanol	Sigma-Aldrich	Cat# 51976; CAS: 64-17-5
Ethanol 70%	VWR	Cat# 83801.360; CAS: 64-17-5
Etoposide (ETO)	Sigma-Aldrich	Cat# E1383; CAS: 33419-42-0
Fetal bovine serum (FBS)	Dutscher	Cat# S1900-500C
Formaldehyde, 36.5%–38% solution	Sigma-Aldrich	Cat# F8775; CAS: 50-00-0
Hoechst 33342	Thermo Fisher Scientific	Cat# H3570; CAS: 23491-52-3
Phosphate-buffered saline (PBS)	Sigma-Aldrich	Cat# D8537; CAS: 24867-26-3
Nuclease-free water	Thermo Fisher Scientific	Cat# 10977035; CAS: 7732-18-5
Penicillin-streptomycin solution	Sigma-Aldrich	Cat# P0781
Poly-L-lysine, 0.01% solution	Sigma-Aldrich	Cat# P4707; CAS: 25988-63-0
Triton X-100	Sigma-Aldrich	Cat# T8532; CAS: 9036-19-5
Tris-buffered saline (TBS) 10X	Euromedex	Cat# ET220-B
Trypsin-EDTA solution	Sigma-Aldrich	Cat# T3924
Critical commercial assays		
Click-iT™ EdU Alexa Fluor 647 imaging kit	Thermo Fisher Scientific	Cat# C10340
Experimental models: Cell lines		
Human: U2OS cells	ATCC	Cat# HTB-96; RRID:CVCL_0042
Human: U2OS AID-DIvA cells	Gaëlle Legube laboratory; Aymard et al.[10]	N/A
Human: HCC4006 subclone cells	Olivier Sordet laboratory; Figarol et al.[14]	N/A
Human: WI38-hTERT cells	Carl Mann laboratory; Jeanblanc et al.[15]	N/A
Software and algorithms		
Columbus software (version 2.8.2)	PerkinElmer	N/A
GraphPad Prism 10	GraphPad Prism	http://www.graphpad.com/; RRID:SCR_002798
Harmony software (version 4.9)	PerkinElmer	RRID:SCR_023543
Other		
Filter 0.2 μm	Dutscher	Cat# 146560
PhenoPlate 96-well	PerkinElmer	Cat# 6055300
Operetta CLS high-content imaging system	PerkinElmer	https://www.perkinelmer.com
Countess II FL automated cell counter	Thermo Fisher Scientific	https://www.thermofisher.com

MATERIALS AND EQUIPMENT

- Basic laboratory materials, such as a laminar culture hood, CO_2 incubator and plasticware have not been mentioned below but are required for the protocol.
- Operetta CLS High-Content Imaging System. Our system is equipped with 10X, 20X and 40X objectives and 4 excitation LEDs:
  - LED 365: excitation 355-385, emission 430-500.
  - LED 475: excitation 460-490, emission 500-550.
  - LED 550: excitation 530-560, emission 570-650.
  - LED 630: excitation 615-645, emission 655-760.

Note: Operetta can be equipped with up to 8 excitation LEDs: 365, 405, 440, 475, 510, 550, 630 and 660 nM.

Recipe of solutions to prepare

- DMEM complete medium for culture of U2OS cells: 500 mL DMEM medium (containing 4.5 g/L glucose, L-glutamine, sodium pyruvate, and sodium bicarbonate) supplemented with 55.5 mL (10%, v/v) Fetal Bovine Serum (FBS).

  Optional: Add 5.5 mL (1%, v/v) of penicillin/streptomycin solution.

  ⚠ CRITICAL: All cell culture solutions must be prepared and maintained in sterile conditions.

- 20 mM camptothecin (CPT) stock solution: dissolve 5 mg CPT (MW: 348.35 g/mol) in 717.5 μL dimethyl sulfoxide (DMSO). Store in aliquots protected from light at −20°C for up to several weeks. Aliquots should not be refrozen after thawing.

  ⚠ CRITICAL: CPT is toxic if swallowed and may cause genetic defects. Please read the MSDS before working with this chemical.

- 50 mM etoposide (ETO) stock solution: dissolve 5 mg ETO (MW: 588.56 g/mol) in 170 μL DMSO. Store in aliquots protected from light at −20°C for up to several weeks.

  ⚠ CRITICAL: ETO is harmful if swallowed and may cause cancer. Please read the MSDS before working with this chemical.

- 10 mM 4-hydroxytamoxifen (4-OHT) stock solution: dissolve 5 mg 4-OHT (MW: 387.51 g/mol) in 1.29 mL ethanol and store protected from light at −20°C for a few weeks.

  ⚠ CRITICAL: 4-OHT is harmful if swallowed and may cause cancer. Please read the MSDS before working with this chemical.

- 500 mM indole-3-acetic acid sodium salt (auxin) stock solution: dilute 10 mg auxin (MW: 197.17 g/mol) in 100 μL DMEM complete medium. The auxin solution requires extemporaneous preparation.
- 3.7% formaldehyde fixation solution: dilute 1 volume of 36.5–38% formaldehyde solution in 9 volumes of phosphate-buffered saline (PBS). The formaldehyde-PBS fixation solution requires extemporaneous preparation.

  ⚠ CRITICAL: Formaldehyde is toxic. Gloves and safety glasses should be worn and always handle it in a fume hood. Please read the MSDS before working with this chemical.

- 0.5% Triton X-100 permeabilization solution: dilute 1 volume of Triton X-100 in 4 volumes of PBS to obtain a 20% Triton X-100 stock solution, which can be stored at 18°C–25°C for a few weeks. To obtain a 0.5% Triton X-100, dilute 1 volume of the 20% Triton X-100 stock solution in 39 volumes of PBS. This solution should be prepared fresh before the experiment.

⚠ CRITICAL: Triton X-100 is harmful if swallowed and causes skin irritation and serious eye damage. Please read the MSDS before working with this chemical.

- 8% BSA blocking buffer: dissolve 0.8 g BSA (w/v) in 10 mL PBS. This solution can be prepared extemporaneously or filtered through a 0.2 μm filter, and stored for a few weeks at 4°C.
- 3% BSA wash buffer: dissolve 0.3 g BSA (w/v) in 10 mL PBS. This solution can be prepared extemporaneously or filtered through a 0.2 μm filter, and stored for a few weeks at 4°C.
- 1% BSA antibody dilution buffer: dissolve 1 g BSA (w/v) in 100 mL of PBS. This solution can be prepared extemporaneously or filtered through a 0.2 μm filter, and stored for a few weeks at 4°C.
- Click-iT EdU Alexa Fluor 647 Imaging Kit: prepare and store the stock solutions of 10 mM 5-ethynyl-2′-deoxyuridine (EdU), 1X Click-iT EdU reaction buffer, Alexa Fluor 647 azide, 10X Click-iT EdU buffer additive, and Hoechst 33342 according to the manufacturer's protocol, except that Hoechst 33342 was diluted 1:10,000 in PBS to obtain a 1X solution, instead of 1:2,000. $CuSO_4$ provided in the kit is ready to use.

Alternatives: EdU can be purchased separately (#A10044; Thermo Fisher Scientific). To prepare a 10 mM EdU stock solution, dissolve 5 mg EdU (MW: 252.23 g/mol) in 2 mL DMSO and store in aliquots at −20°C for up to 1 year. Alexa Fluor 647 azide can be purchased separately (#A10277; Thermo Fisher Scientific). To prepare a working stock solution, dissolve 0.5 mg Alexa Fluor 647 azide in 1.8 mL DMSO and store in aliquots at −20°C for up to 1 year. $CuSO_4$ can be purchased separately (#203165; Sigma-Aldrich). To prepare a 100 mM $CuSO_4$ working stock solution, dissolve 24.97 mg $CuSO_4$ (MW: 249.69 g/mol) in 1 mL nuclease-free water and store in aliquots at −20°C for up to 1 year. Hoechst 33342 (10 mg/mL) can be purchased separately (#H3570; Thermo Fisher Scientific). To prepare a 1X solution, dilute the 10 mg/mL Hoechst 33342 solution at 1:10,000 in PBS and store the solution at 4°C protected from light for a few weeks. 1X Click-iT EdU reaction buffer can be replaced by 1X Tris-buffered saline (TBS). To obtain a 1X TBS solution, dilute 1 volume of 10X TBS in 9 volumes of nuclease-free water.

- **Click-iT reaction cocktail** (using components of the Click-iT EdU Alexa Fluor 647 Imaging Kit).

	Amount					
	Number of wells in a 96-well plate					
Reagents	1	2	5	10	25	Concentration
1X Click-iT EdU reaction buffer	60 μL	120 μL	300 μL	600 μL	1.5 mL	N/A
$CuSO_4$	2.8 μL	5.6 μL	14 μL	28 μL	70 μL	N/A
Alexa Fluor 647 azide	0.2 μL	0.4 μL	0.9 μL	1.8 μL	4.4 μL	N/A
1X Click-iT EdU buffer additive	7 μL	14 μL	35 μL	70 μL	175 μL	N/A
Total Volume	70 μL	140 μL	350 μL	700 μL	1.75 mL	N/A

Prepare at 18°C–25°C protected from light and use within 15 min of preparation.

Note: Prepare extemporaneously the 1X Click-iT EdU buffer additive by diluting 1 volume of the 10X Click-iT EdU buffer additive in 9 volumes of nuclease-free water. This solution should be used on the same day.

⚠ CRITICAL: Add components in the order listed in the table.

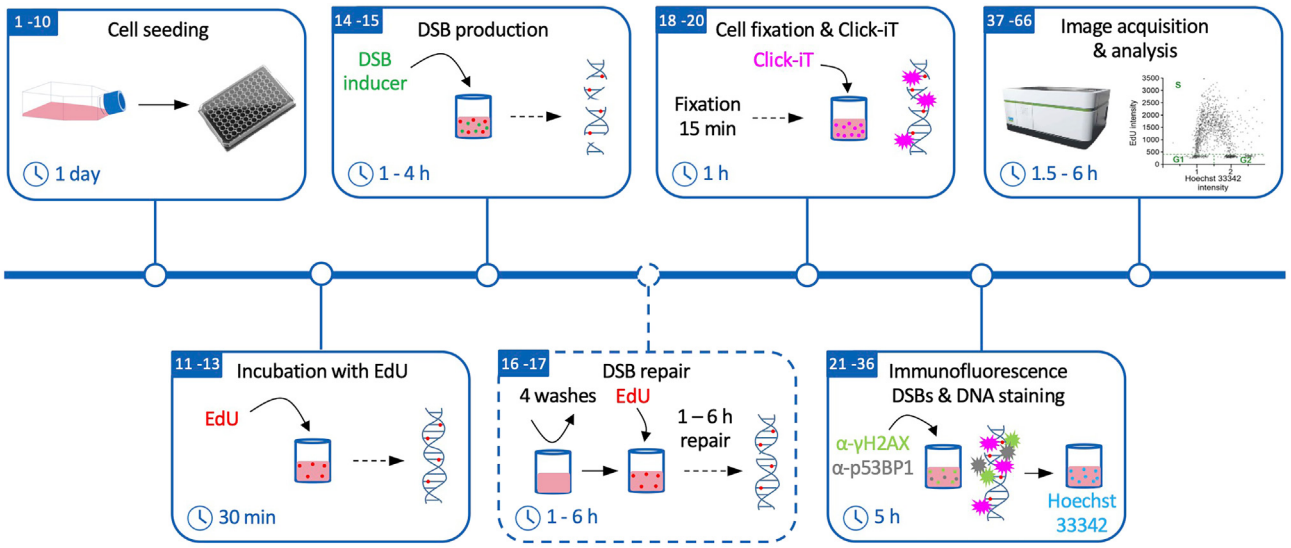

Figure 1. Diagram of the protocol workflow

The numbers in the top left-hand corner of each square correspond to the steps of the procedure. The dotted line in square border (steps 16-17) indicates that these steps are required only when DSB repair kinetics are analyzed.

STEP-BY-STEP METHOD DETAILS

For an overview of the quantitative single-cell analysis of DNA double-strand break workflow, see Figure 1.

Part 1: Cell seeding—Day 1

Timing: 24 h (for steps 1 to 10)

The following steps describe the procedure for seeding U2OS cells on 96-well plates suitable for fluorescence microscopy and coated with poly-L-lysine (optional). For culture and seeding of the other cell types shown in the "expected outcomes" section, refer to the following publications: U2OS AID-DIvA cells,[10] HCC4006,[14] WI38-hTERT.[1,3]

1. Add 60 μL of 0.01% poly-L-lysine solution in each well of a phenoplate 96-well and incubate at 18°C–25°C for 5 min, lid on.
2. Remove the poly-L-lysine solution and let dry for 15 min at 18°C–25°C, lid on.
3. Wash each well once with 200 μL DMEM complete medium at 18°C–25°C. Remove the medium.
4. Add again 200 μL of complete DMEM medium to each well, which will be removed just before seeding the cells.
5. Detach and dissociate exponentially growing U2OS cells from maintenance culture flasks (e.g., T75 flasks).
   a. Wash the cells once with PBS at 18°C–25°C.
   b. Cover the cells with trypsin-EDTA solution.
   c. Incubate for a few minutes in a 37°C incubator with 5% CO_2.
6. Inactivate trypsin by adding at least twice a volume of DMEM complete medium to the trypsin-EDTA solution.
7. Further dissociate and homogenize cells by pipetting up and down 3 to 5 times.
8. Determine cell concentration, e.g., with an Invitrogen Countess II FL cell counter.
9. Seed 10 x 10^3 cells in each well in 200 μL DMEM complete medium.
   a. Prepare a suspension of 50 x 10^3 cells/mL in DMEM complete medium.
   b. Add 200 μL of this cell suspension per well.

10. Incubate cells for 24 h in a 37°C incubator with 5% CO_2.

Note: Incubation time depends on the cell line and should be long enough for cells to adhere to the well and complete at least one cell cycle.

Note: The number of cells at seeding depends on the cell type and on the overall experiment duration and it should be optimized in order to have around 60–80% confluence the day of the treatment (see Troubleshooting 1).

Part 2: Cell treatment with DSB inducer and EdU and cell fixation—Day 2

Timing: 3–12 h (for steps 11 to 20; Time varies depending on the duration of DSB-inducing treatment and repair)

The following steps describe the treatment protocol to quantify DSB production and repair in cell cycle phases in 96-well plates. Cells are incubated with EdU, which incorporates into newly synthesized DNA, during treatment with the DSB inducer (analysis of production) as well as after drug removal (analysis of repair). This approach allows to identify cells that were in or that have passed through S-phase during the chosen time frame. G1 and G2 phases are further distinguished by their DNA content.

DSB production

Note: A concentration of 10 μM EdU was used for U2OS cells. Optimal EdU concentration should be adjusted depending on experimental conditions, as growth medium, cell density and cell type may influence EdU labeling.

11. Prepare a reaction mix of 200 μL per well of DMEM complete medium pre-warmed to 37°C with 10 μM EdU (i.e., a 1:1,000 dilution of the 10 mM EdU stock solution).
12. Aspirate cell medium and add 200 μL of the EdU-containing medium to each well.
13. Incubate for 30 min in a 37°C incubator with 5% CO_2.
14. Treat the cells with camptothecin (CPT) (step 14a), etoposide (ETO) (step 14b) or 4-hydroxytamoxifen (4-OHT) (step 14c). In parallel, treat cells with the drug vehicle (dissolution/dilution agent) only, which corresponds to "untreated cells".

Note: Prepare drugs and vehicles just before use.

a. Camptothecin (CPT).
   i. To treat cells with 25 μM CPT, dilute an aliquot of the 20 mM CPT stock solution at 1:16 (v/v) in sterile water.
   ii. Vortex vigorously and add 4 μL of the diluted CPT solution to each well containing the 200 μL of medium with EdU and resuspend homogeneously the drug in the well by pipetting up and down few times.
   iii. Incubate for 1 h in a 37°C incubator with 5% CO_2.

Note: A concentration of 25 μM CPT allows induction of discrete γH2AX foci in G1 cells. In contrast, this concentration of CPT induces a pan-nuclear staining of γH2AX in S cells rather than well-defined foci, as CPT is a very potent inducer of replication-dependent DSBs. To visualize individual γH2AX foci in S cells, a 0.1 μM CPT concentration should be used.[1] To obtain this concentration, follow the procedure in step 14a. but dilute the 20 mM CPT stock solution at 1:4,000 in sterile water instead of 1:16.

⚠ CRITICAL: CPT is unstable in aqueous solutions, so dilution of CPT in water should be prepared fresh just before cell treatment. CPT is also light-sensitive and should be protected from light at all times.

b. Etoposide (ETO).
   i. To treat cells with 10 μM ETO, dilute an aliquot of the 50 mM ETO stock solution at 1:125 (v/v) in sterile water (dilution #1). To treat cells with 1 μM ETO, dilute the dilution #1 at 1:10 (v/v) in sterile water (dilution #2).
   ii. Vortex and add 5 μL of the diluted ETO solution (dilution #1 for 10 μM final; dilution #2 for 1 μM final) to each well containing the 200 μL of medium with EdU.
   iii. Incubate for 1 h in a 37°C incubator with 5% CO_2.

c. 4-hydroxytamoxifen (4-OHT).
   i. To treat cells with 300 nM 4-OHT, dilute an aliquot of the 10 mM 4-OHT stock solution at 1:16.67 (v/v) in 70% ethanol (4-OHT dilution #1: concentration of 0.6 mM).
   ii. Vortex and make a second dilution at 1:40 in DMEM complete medium (4-OHT dilution #2: concentration of 15 μM).
   iii. Vortex and add 4 μL of the 4-OHT dilution #2 to each well containing the 200 μL of medium with EdU.
   iv. Incubate for 4 h in a 37°C incubator with 5% CO_2.

Note: The use of 4-OHT is restricted to the U2OS auxin-inducible degron (AID)-DIvA cells to induce the restriction enzyme AsiSI, which in turn will induce DSBs.[10] To analyze DSB production only, the U2OS DIvA cells[11] can also be used.

15. To analyze DSB production only, go on to step 18. To analyze both DSB production and repair, continue to next step.

DSB repair

16. Aspirate cell medium containing drugs and EdU and proceed to cell washes and incubation in the appropriate pre-warmed drug-free culture medium to allow DSB repair (see Troubleshooting 2).
   a. Cell washes and incubation protocol after CPT or ETO treatment.
      i. Wash the wells four times with 200 μL of 37°C pre-warmed DMEM complete medium in each well, with no incubation time between the washes.
      ii. After the last wash, remove the cell medium and add 200 μL of 37°C pre-warmed DMEM complete medium containing 10 μM EdU in each well, as in step 12.
   b. Cell washes and incubation protocol after 4-OHT treatment.
      i. Wash the wells three times with 200 μL of 37°C pre-warmed PBS in each well: first wash at 18°C–25°C with no incubation time, second wash in a 37°C incubator with 5% CO_2 for 2–3 min, and third wash at 18°C–25°C with no incubation time.
      ii. After the last wash, remove the PBS and add 200 μL of 37°C pre-warmed DMEM complete medium containing 500 μM auxin and 10 μM EdU in each well.

Note: Prepare the stock solution of 500 mM auxin fresh, and the culture medium containing 500 μM auxin (1:1,000 dilution) just before to apply on cells.

Note: The use of auxin is restricted to the U2OS AID-DIvA cells to degrade the restriction enzyme AsiSI.[10]

17. Incubate cells for the chosen time to analyze repair (e.g., 1, 2, 4, 6 h; see example in Table 1) in a 37°C incubator with 5% CO_2.

Table 1. Schedule of treatments to study CPT-induced DSB repair for up to 6 h

	Cell treatment				
Time (h)	Repair (6 h)	Repair (4 h)	Repair (2 h)	CPT (1 h)	Untreated (vehicle 1 h)
0.0	EdU (steps 11-13)				
0.5	CPT (step 14a)				
1.0					
1.5	Release + EdU (steps 16a and 17)				
2.0		EdU (steps 11-13)			
2.5		CPT (step 14a)			
3.0					
3.5		Release + EdU (steps 16a and 17)			
4.0			EdU (steps 11-13)		
4.5			CPT (step 14a)		
5.0					
5.5			Release + EdU (steps 16a and 17)		
6.0				EdU (steps 11-13)	EdU (steps 11-13)
6.5				CPT (step 14a)	DMSO (step 14a)
7.0					
7.5	Proceed to cell fixation (steps 18-20)				

Cell fixation

18. Remove culture medium and wash the cells with 200 µL of PBS in each well at 18°C–25°C, with no incubation time. Remove the PBS.
19. Incubate cells with 200 µL of freshly prepared 3.7% formaldehyde fixation solution in each well for 15 min at 18°C–25°C.
20. Remove the fixation solution and wash each well twice for 5 min with 200 µL of PBS at 18°C–25°C, with no incubation time.

Note: Carry out treatments in reverse kinetics to add the fixative solution simultaneously to all wells of a 96-well plate containing different time points of the DSB production and repair kinetics. Start by treating the wells with the longest DSB repair time (e.g., "repair (6 h)" in Table 1), and proceed sequentially to the wells with the shortest treatment time (e.g., "CPT (1 h)" in Table 1). See Table 1 for an example of a treatment schedule to study CPT-induced DSB repair for up to 6 h (see Troubleshooting 3).

Pause Point: Fixed cells can be kept in PBS at 4°C for few days.

Part 3: Cell staining—Day 3

Timing: 6 h (for steps 21 to 36)

This protocol describes the labeling of DNA content in blue (Hoechst 33342), γH2AX in green (Alexa Fluor 488), p53BP1 in red (Alexa Fluor 594) and newly synthesized DNA in far-red (Click-iT EdU Alexa Fluor 647). Other color combinations can be chosen depending on the excitation LEDs of the automated fluorescence microscope.

Click-iT reaction for EdU detection

21. Remove the PBS, then wash cells twice for 5 min with 200 µL of 3% BSA in PBS per well at 18°C–25°C. Remove the wash solution.

22. Permeabilize cells with 200 μL of 0.5% Triton X-100 solution per well for 25 min at 18°C–25°C. During the permeabilization, prepare the Click-iT reaction cocktail and protect it from light.

⚠ CRITICAL: Use the Click-iT reaction cocktail within 15 min of preparation.

23. Remove the permeabilization solution, then wash cells twice for 5 min with 200 μL of 3% BSA in PBS per well at 18°C–25°C. Remove the wash solution.

Note: From the next step, protect the 96-well plate from light.

24. Add 70 μL of Click-iT reaction cocktail to each well. Incubate for 30 min at 18°C–25°C.
25. Remove the Click-iT reaction cocktail and wash cells once for 5 min with 200 μL of 3% BSA in PBS in each well at 18°C–25°C.

γH2AX and p53BP1 staining

26. Remove the wash buffer and add 200 μL of 8% BSA in PBS solution (blocking buffer) to each well. Incubate for 1 h at 18°C–25°C.
27. Remove the blocking buffer and wash cells twice with 200 μL of PBS per well at 18°C–25°C, with no incubation time for the first wash and 5 min for the second wash.
28. During the washes, prepare a mix of 70 μL per well of primary antibody solution, containing a mouse anti-γH2AX antibody and a rabbit anti-p53BP1 antibody, both diluted at 1:500 in 1% BSA in PBS.
29. Remove the PBS and incubate each well with 70 μL of primary antibody solution for 2 h at 18°C–25°C.

Note: Primary antibodies against other DNA damage response proteins can be used in combination. If so, determine the optimal primary antibody concentration as well as incubation time and temperature (see Troubleshooting 4).

30. Remove the primary antibody solution and wash cells three times with 200 μL of PBS per well for 5 min at 18°C–25°C.
31. During washes, prepare a mix of 70 μL per well of secondary antibody solution, containing an anti-mouse Alexa Fluor 488 antibody (to detect γH2AX) and an anti-rabbit Alexa Fluor 594 antibody (to detect p53BP1), both diluted at 1:500 in 1% BSA in PBS.
32. Remove the PBS and incubate each well with 70 μL of secondary antibody solution for 1 h at 18°C–25°C.
33. Remove the secondary antibody solution and wash cells three times with 200 μL of PBS per well for 5 min at 18°C–25°C.
34. Remove the PBS and incubate each well with 200 μL of Hoechst 33342 working solution for 15 min at 18°C–25°C.
35. Remove the Hoechst 33342 solution and wash each well twice with 200 μL of PBS at 18°C–25°C, with no incubation time. Add 200 μL of PBS in each well.
36. Proceed immediately to image acquisition or store the plate at 4°C protected from light until image acquisition.

Note: For optimal image acquisition, we recommend to proceed with image acquisition within 2 days of step 36. However, plates can be stored in PBS at 4°C in the dark (to avoid photobleaching) for few weeks before image acquisition. Change the PBS once a week to prevent the wells drying out or becoming contaminated.

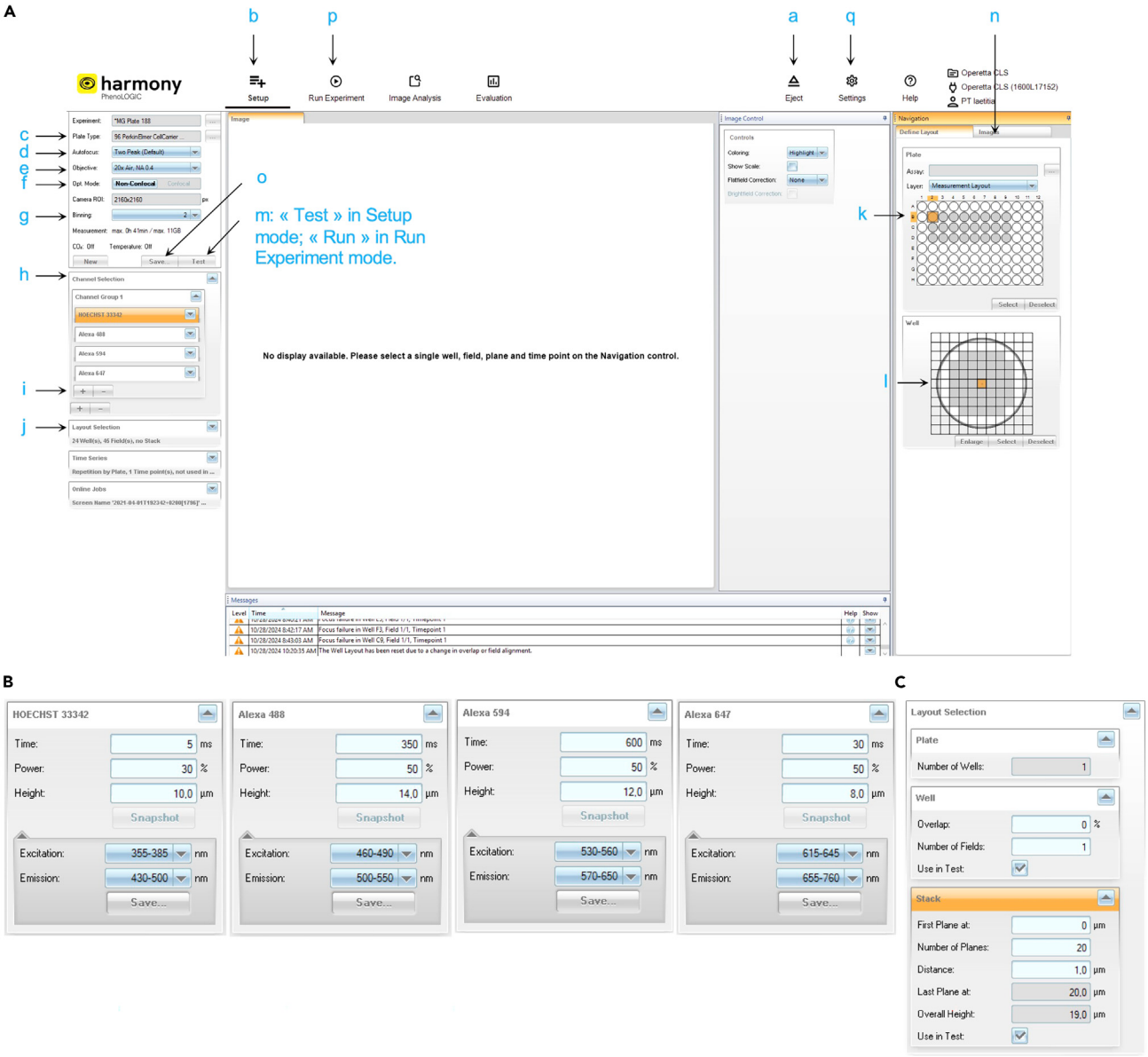

Figure 2. Screenshots of the Harmony software for image acquisition

(A) Full screenshot of the Harmony Setup mode. Lower case letters (referred as "annotation" in the text) indicate the elements for setting up the acquisition parameters described in the text.

(B) Screenshots from the drop-down menu from the "Channel Selection", annotation h in Figure 2A. Excitation and emission ranges as well as parameters for acquisition are shown for each channel.

(C) Screenshot of the drop-down menu from the "Layout Selection", annotation j in Figure 2A.

Part 4: Image acquisition—Day 3

Timing: 0.5–2 h (for steps 37 to 46; Time varies depending on the number of wells and fields per well to be scanned and the acquisition parameters chosen)

The steps below describe image acquisition for 96-well plates using an Operetta CLS High-Content Imaging System (PerkinElmer) with Harmony software (version 4.9). Acquisition can be done with other widefield high-content fluorescence microscopes.

Optional: If the plate has been stored at 4°C, allow it to warm up for about 30 min at 18°C–25°C before acquisition to avoid condensation phenomenon that could affect image quality.

37. Clean the bottom of the plate with a precision wipe soaked with 70% ethanol.
38. Load the plate inside the Operetta device.
    a. Click on "Eject" button (Figure 2A, annotation a) to release the tray from the Operetta device. The "Eject" button then switches to "Load" button.
    b. Place the plate on the tray.
    c. Click on "Load" button.
39. With the Harmony software interface, set up the acquisition parameters using the "Setup" panel (Figure 2A, annotation b).
40. Select the appropriated type of plate (Figure 2A, annotation c) to configure the acquisition, here the "Perkin Elmer Phenoplate 96 wells".

    Note: This plate was previously called "96 PerkinElmer CellCarrier Ultra".

41. Select parameters for the acquisition.
    a. Select "Two Peak (Default)" in the "Autofocus" item (Figure 2A, annotation d).
    b. Select "20X air" objective in the "Objective" item (Figure 2A, annotation e).
    c. Select "Non-Confocal" (epifluorescence) optical mode in the "Opt. Mode" item (Figure 2A, annotation f).
    d. Select "2" in the "Binning" item (Figure 2A, annotation g).
    e. Select channels for acquisition in the "Channel Selection" panel (Figure 2A, annotation h).
        i. Click on the "+" button (Figure 2A, annotation i) until 4 channels are displayed.
        ii. Select "Hoechst 33342" (for DNA content), "Alexa 488" (for γH2AX), "Alexa 594" (for p53BP1), and "Alexa 647" (for EdU).

    Note: The excitation and emission wavelengths of these four channels are shown in Figure 2B.

    Note: The use of the 20X objective (non-confocal mode) is sufficient to analyze γH2AX and p53BP1 within cell cycle phases. However, bigger magnifications and confocal mode acquisition can also be used. For confocal mode, use the 40X objective. 10X air objective has not been tested yet.

42. Adjust the focus height for each channel.
    a. Click on a single well (Figure 2A, annotation k) and a single field in this well (Figure 2A, annotation l) to select them.

    Note: Once you click, the chosen well and field become orange, indicating that they have been selected.

    b. In the "Layout Selection" panel (Figure 2A, annotation j), select the "Stack" acquisition (zoom in Figure 2C, in orange).
    c. Set the parameters: First Plane = 0 μm; Number of Planes = 20; Distance = 1.0 μm.
    d. Select "Use in Test" mode.
    e. Select "Test" (Figure 2A, annotation m) to start the stack acquisition.
    f. Click on the up-right icon ''Images'' (Figure 2A, annotation n) to visualize the images of each channel.

    Note: Images of each channel appear within 20 plans.

    g. Choose the height value corresponding visually to the best focus of the cells for each channel.

h. Note this value on the "Channel Selection" panel (Figure 2A, annotation h; Figure 2B).

Note: Perform initial focus calibration on the chosen well layout field of the well with expected highest signal in samples across experiment. Test the calibrated focus on several fields for a single well to confirm the selection of the most accurate focus, as it will be used for automated acquisition of the defined channel for all wells of the plate.

43. Define the plate layout by selecting the wells in the "Define Layout" panel (Figure 2A, annotation k), i.e., the wells in the plate to be acquired.
44. Define the well layout by selecting the fields in the "Define Layout" panel (Figure 2A, annotation l), i.e., the fields in the plate to be acquired.

Note: After selection, the wells and fields appear in gray.

Note: The plate and well layout depends on the experiment, mainly the number of conditions across experiment (plate layout) and the number of cells necessary for the experimental analysis (well layout). When selecting the well layout, it is recommended to exclude fields in the well border (see example in Figure 2A, annotation l).

45. Calibrate the time of exposure and the excitation power for each channel. To that end, use the "Snapshot" button (Figure 2B) to test and visualize the chosen exposure settings of each channel.

Note: No range indicator is available. However, a histogram for each channel is displayed and can be used to set the appropriate exposure time. Example settings are shown in Figure 2B.

Note: For γH2AX and p53BP1 corresponding channels, adjust the time of exposure on the condition with the highest expected signal, i.e., cells treated with the DSB inducer.

46. Check your chosen settings in another well and field with the "Snapshot" function.
    a. When settings are chosen, if satisfied, click "Save" (Figure 2A, annotation o) to save your parameters of acquisition.
    b. Click "Run Experiment" in the navigator bar (Figure 2A, annotation p).
    c. Click "Save" to save your experiment.
    d. Click "Run" (Figure 2A, annotation m) for the automatic acquisition of all the selected wells and fields of the sample plate.

Part 5: Image analysis and data extraction—Day 3

Timing: 1–4 h (for steps 47 to 66; Time varies depending on the number of wells and fields per well to be analyzed)

The steps below describe image analysis using Columbus software (version 2.8.2). Analysis can also be done using Harmony software (version 4.9), which is used for data acquisition (steps 37-46) and is very similar to Columbus.

47. Transfer the acquired data from Harmony to Columbus software by clicking on "Settings" (Figure 2A, annotation q), "Data Management", "Transfer to Columbus".
48. Click on "Image Analysis" icon (Figure 3, annotation a) on the Columbus software to create a sequence of analysis.
49. Select a measurement (i.e., the acquired images) in the data tree (Figure 3, annotation b).

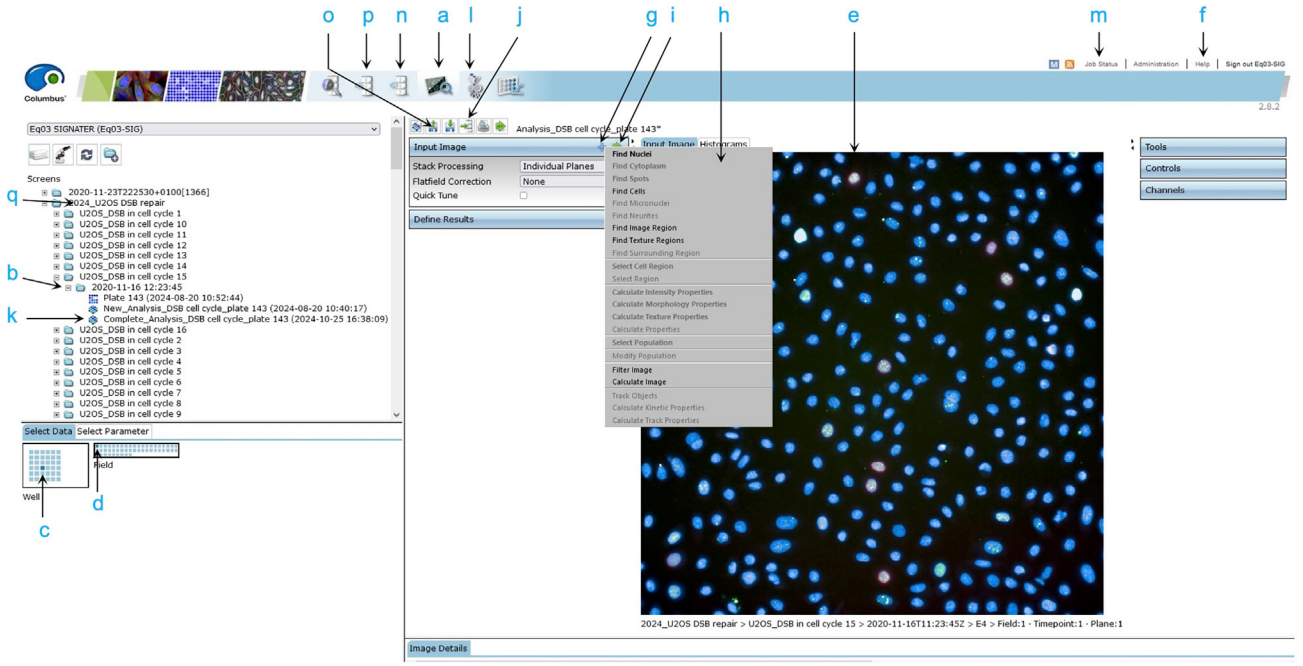

Figure 3. Overview of the Columbus software interface for image analysis

Lower case letters (referred as "annotation" in the text) indicate the elements to open acquired images (annotations a-e), select one well (annotation c) and one field (annotation d) and start building a sequence of analysis (annotations g-k), launch and export the analysis and images (annotations l-n), and re-import the analysis and images (annotations o-q), as described in detail in the text.

50. Select one single image, i.e., one well (Figure 3, annotation c) and one field (Figure 3, annotation d). The selected image is displayed in the "image view" section (Figure 3, annotation e). This is the beginning of the analysis.

Note: The following steps describe the protocol and example settings for the analysis of γH2AX and p53BP1 in U2OS cells in different phases of the cell cycle, based on DNA content (Hoechst 33342 staining) and EdU incorporation into newly synthesized DNA. Adjust all parameter settings for each new analysis and for the use of other cell lines. For further details, consult Columbus help by clicking on "Help" (Figure 3, annotation f) and selecting "Columbus help" from the pop-up list.

51. Detection of nuclei.
    a. Click on the "+" button on the "Input Image" building block (Figure 3, annotation g) to add another building block.
    b. Select the "Find Nuclei" building block from the pop-up list (Figure 3, annotation h) to create a "Find Nuclei" building block.
    c. Define the parameters: "Channel" = "Hoechst 33342"; "Method" = "B" with the associated parameters "Common Threshold", "Area", "Split Factor", "Individual Threshold" and "Contrast" to enable identification of the largest number of individual nuclei.

Note: The following thresholds are suitable for U2OS cells: Common Threshold: 0.3; Area: >100 μM^2, Split Factor: 2.0; Individual Threshold: 0.3 and Contrast: 0.00.

    d. Name the output population "Nuclei".
    e. Click on the green arrow icon at the top right of the "Find nuclei" building block (same icon as in Figure 3, annotation i) to validate the parameters.

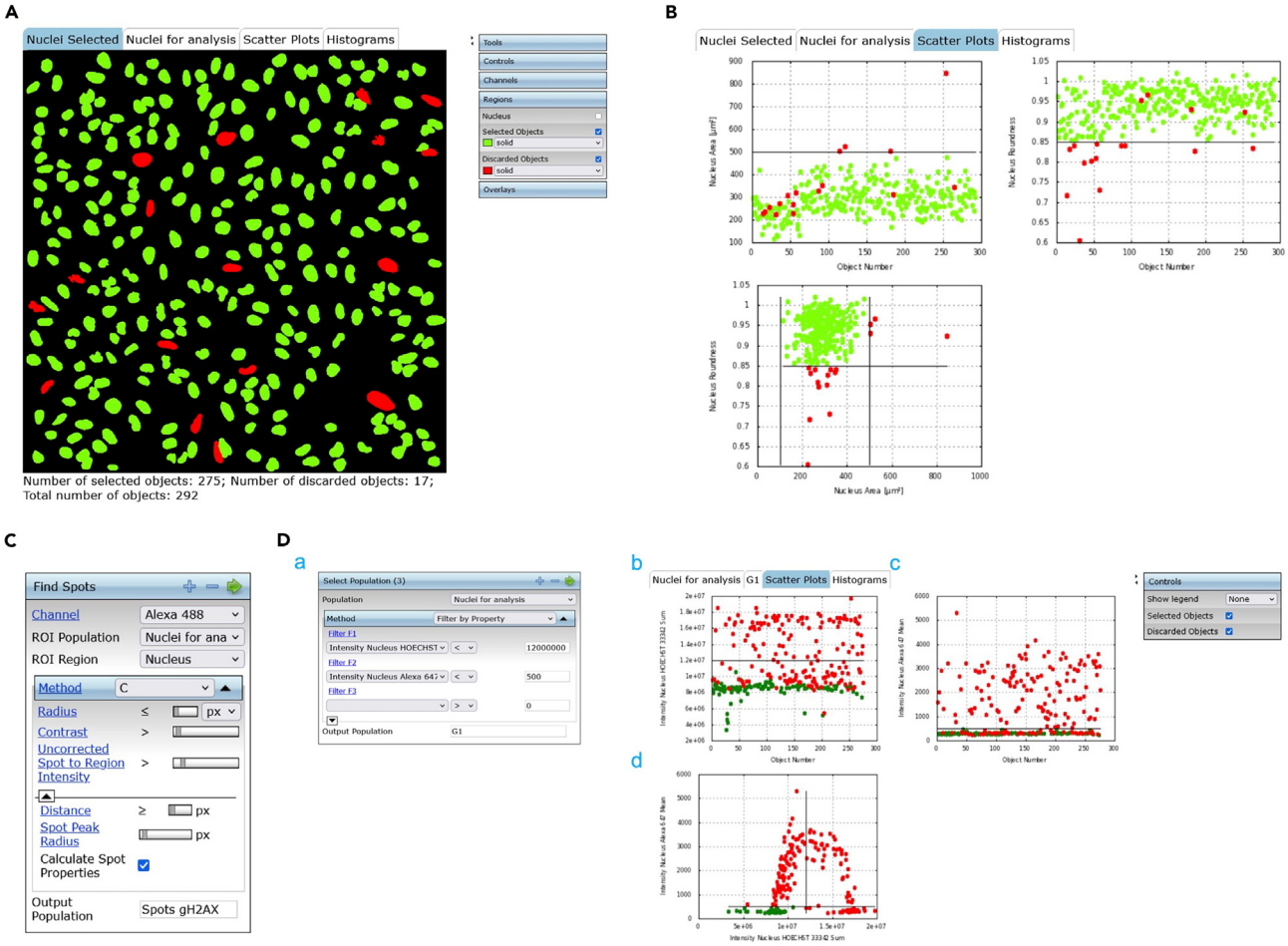

Figure 4. Screenshots of the Columbus software to illustrate the individual steps for nuclei and population selection

(A and B) Steps from the sequence of analysis of the Columbus software showing how to select nuclei based on morphology properties (steps 53 and 54). (A) Field overview with nuclei selected in function of their morphological properties ("Nuclei for analysis"), which are colored in green, and nuclei discarded, which are in red. (B) Examples of scatter plot windows displaying the area and roundness properties of each nucleus in one field of a well.
(C) Building block and example of setting parameters to select γH2AX spots within nuclei.
(D) Steps showing how to select a cell phase population, here exemplified for G1 cells. (a) Setting parameters for EdU and Hoechst 33342 intensity per nucleus; (b-d) Examples of scatter plot windows displaying the EdU and Hoechst 33342 intensity properties of each nucleus in one field of a well. Cells selected as G1 are depicted in green, and cells that are not selected as G1 are in red.

Note: In the "image view" section (same position as in Figure 3, annotation e), the nuclei corresponding to the output population "Nuclei" are circled.

52. Remove cropped nuclei from the edges of an image.
    a. Click on the "+" button on the "Find Nuclei" building block to add another building block.
    b. Select the "Select Population" building block from the pop-up list to create a "Select Population" building block.
    c. Define the parameters: "Population" = "Nuclei"; "Method" = "Common Filters" with the associated selection of "Remove Border Objects".
    d. Name the output population "Nuclei Selected".
    e. Click on the green arrow icon at the top right of the "Select Population" building block to validate the parameters.

Note: In the "image view" section: (a) "Nuclei" tab: nuclei corresponding to the output population "Nuclei Selected" are colored in green whereas nuclei discarded at this step are

colored in red (same representation as in Figure 4A); (b) "Nuclei Selected" tab: nuclei corresponding to the output population "Nuclei Selected" only are highlighted by a colored border.

53. Calculate nucleus morphology properties.
    a. Click on the "+" button on the "Select Population" building block to add another building block.
    b. Select the "Calculate Morphology Properties" building block from the pop-up list to create a "Calculate Morphology Properties" building block.
    c. Define the parameters: "Population" = "Nuclei Selected"; "Region" = "Nucleus"; "Method" = "Standard" with the associated selection of "Area" and "Roundness"; "Output Properties" = "Nucleus".
    d. Click on the green arrow icon at the top right of the "Calculate Morphology Properties" building block to validate the parameters.
54. Discard nuclei with unusual morphology properties (see Troubleshooting 5).
    a. Click on the "+" button on the "Calculate Morphology Properties" building block to add another building block.
    b. Select the "Select Population" building block from the pop-up list to create a "Select Population (2)" building block.
    c. Define the parameters: "Population" = "Nuclei Selected"; "Method" = "Filter by Properties" with the associated filters "Nucleus Area [μm^2], >", "Nucleus Area [μm^2], <", and "Nucleus Roundness, >".
    d. Apply the appropriate thresholds to select individual nuclei with usual morphology properties.

    Note: The following thresholds are suitable for U2OS cells: 100 < "Nucleus Area [m^2]" < 500 and "Nucleus Roundness" > 0.85.

    e. Name the output population "Nuclei for analysis".
    f. Click on the green arrow icon at the top right of the "Select Population (2)" building block to validate the parameters.

    Note: In the "image view" section: (a) "Nuclei Selected" tab (Figure 4A, left panel): nuclei corresponding to the output population "Nuclei for analysis" are in green, and nuclei discarded at this step are in red. Colors of nuclei can be changed by clicking on the "Regions" item in the top right section, within "Selected Objects" and "Discarded Objects" items (Figure 4A, right panel). (b) "Nuclei for analysis" tab: nuclei corresponding to the output population. "Nuclei for analysis" are highlighted by a colored border. (c) "Scatter Plots" tab: it shows the area and roundness properties of each nucleus, helping to define the parameters in step 54c. Nuclei corresponding to the output population "Nuclei for analysis" are in green, and nuclei discarded at this step are in red (Figure 4B).

    Note: The setting of the maximum "Nucleus Area [μm^2]" allows to remove multiple nuclei identified by the software as a single object (one nucleus). The setting of the minimal "Nucleus Area [μm^2]" allows to remove cell debris. The "Nucleus Roundness" filter allows to exclude cells that are partially detached, cell aggregates and unappropriated masks of nuclei.

55. Calculate fluorescence parameters per nucleus.
    a. Click on the "+" button on the "Select Population (2)" building block to add another building block.
    b. Select the "Calculate Intensity Properties" building block from the pop-up list to create a "Calculate Intensity Properties" building block.

c. Define the parameters: "Channel" = "Hoechst 33342"; "Population" = "Nuclei for analysis"; "Method" = "Standard" with the associated selection of "Mean" and "Sum".
d. Name the output properties "Intensity Nucleus Hoechst 33342".
e. Click on the green arrow icon at the top right of the "Calculate Intensity Properties" building block to validate the parameters.
f. Repeat steps 55a to 55e for each channel ("Alexa 488", "Alexa 594", "Alexa 647") and name the output properties accordingly.

56. Find γH2AX and p53BP1 foci per nucleus (see Troubleshooting 6).
a. Click on the "+" button on the "Calculate Intensity Properties (4)" building block (which is the last building block created in step 55) to add another building block.
b. Select the "Find Spots" building block from the pop-up list to create a "Find Spots" building block.
c. Define the parameters: "Channel" = "Alexa 488" (for γH2AX); "ROI Population" = "Nuclei for analysis"; "ROI Region" = "Nucleus"; "Method" = "C" and the associated parameters "Radius", "Contrast", "Uncorrected Spot to Region Intensity", "Distance", and "Spot Peak Radius", and select "Calculate Spot Properties" (Figure 4C).
d. Name the Output Population "Spots γH2AX".
e. Click on the green arrow icon at the top right of the "Find Spots" building block to validate the parameters.
f. Repeat steps 56a to 56e for the "Channel" = "Alexa 594" (for p53BP1) and name the output population "Spots p53BP1". To do this, use the "Find Spots" building block to create the "Find Spots (2)" building block.

Note: The following thresholds are indicative for γH2AX spot detection in U2OS cells treated or not with CPT: Radius: ≤ 2.0 px; Contrast: > 0.04; Uncorrected Spot to Region Intensity: 1.2; Distance: ≥ 2.5 px; and Spot Peak Radius: 0.5 px. The following thresholds are indicative for p53BP1 spot detection in U2OS cells treated or not with CPT: Radius: ≤ 6.0 px; Contrast: > 0.04; Uncorrected Spot to Region Intensity: 1.1; Distance: ≥ 2.5 px; and Spot Peak Radius: 0.5 px. Thresholds must be adjusted for each experiment due to technical variations in staining from one experiment to another.

Note: It is recommended to optimize the parameter setting for foci identification on a few fields from different conditions, ideally on a negative condition (i.e., untreated), which should display the lower number of spots per nucleus, and a positive condition (i.e., treatment with a DSB inducer), which should display the higher number of spots per nucleus.

57. Identify G1 cells.
a. Click on the "+" button on the "Find Spots (2)" building block to add another building block.
b. Select the "Select Population" building block from the pop-up list to create a "Select Population (3)" building block.
c. Define the parameters: "Population" = "Nuclei for analysis"; "Method" = "Filter by Properties" with the associated filters "Intensity Nucleus Hoechst 33342 Sum, <" and "Intensity Nucleus Alexa 647 Mean, <" and apply the appropriate thresholds to select individual nuclei with low Hoechst 33342 and EdU-negative (Figure 4D, annotation a).
d. Name the output population "G1".
e. Click on the green arrow icon at the top right of the "Select Population (3)" building block to validate the parameters.

Note: The thresholds displayed in Figure 4D are indicative for cell cycle phase identification in U2OS cells. Thresholds must be adjusted for each experiment.

Note: G1 cells are identified as low Hoechst 33342 (below the "Intensity Nucleus Hoechst 33342 Sum" threshold) and EdU-negative (below the "Intensity Nucleus Alexa 647 Mean"

threshold). To select the best threshold, visualize the intensity of fluorescence in individual nuclei by clicking on the "Scatter Plots" tab in the "image view" section (Figure 4D, annotations b-d). Selected G1 nuclei appear in green and excluded nuclei in red. The scatter plot "Intensity Nucleus Alexa 647 Mean" *vs* "Intensity Nucleus Hoechst 33342 Sum" (Figure 4D, annotation d) is used to define the threshold for the Hoechst intensity, which should be set in the middle of the distributed population between the highest Hoechst intensity corresponding to 2 N DNA content and the lowest corresponding to 1 N DNA content, as shown in Figure 4D, annotations b and d. The scatter plot displaying "Intensity Nucleus Alexa 647 Mean" *vs* "Object Number" (nuclei) is used to define the intensity threshold for the Alexa 647. This threshold should be set on the upper limit of the population of nuclei with the lowest intensity values (values close to 0, Figure 4D, annotation c). In the "G1" tab of the "image view" section, check that the circled cells corresponding to the G1 population are EdU-negative (i.e., not labeled with Alexa 647).

58. Identify G2 cells.
    a. Click on the "+" button on the "Select Population (3)" building block to add another building block.
    b. Select the "Select Population" building block from the pop-up list to create a "Select Population (4)" building block.
    c. Define the parameters: "Population" = "Nuclei for analysis"; "Method" = "Filter by Properties" with the associated filters "Intensity Nucleus Hoechst 33342 Sum, >" and "Intensity Nucleus Alexa 647 Mean, <" and apply the same threshold as in step 57c to select individual nuclei with high Hoechst 33342 and EdU-negative.
    d. Name the output population "G2".
    e. Click on the green arrow icon at the top right of the "Select Population (4)" building block to validate the parameters.

    Note: G2 cells are identified as high Hoechst 33342 (above the "Intensity Nucleus Hoechst 33342 Sum" threshold) and EdU-negative (below the "Intensity Nucleus Alexa 647 Mean" threshold).

59. Identify S cells.
    a. Click on the "+" button on the "Select Population (4)" building block to add another building block.
    b. Select the "Select Population" building block from the pop-up list to create a "Select Population (5)" building block.
    c. Define the parameters: "Population" = "Nuclei for analysis"; "Method" = "Filter by Properties" with the associated filter "Intensity Nucleus Alexa 647 Mean, >" and apply a slightly higher threshold that in step 57c to select individual nuclei EdU-positive.
    d. Name the output population "S".
    e. Click on the green arrow icon at the top right of the "Select Population (5)" building block to validate the parameter.

    Note: S cells are identified as EdU-positive (above the "Intensity Nucleus Alexa 647 Mean" threshold).

    Note: Leave a gap between the chosen Alexa 647 intensity threshold between G1/G2 and S to ensure a more selective discrimination of cell cycle phases by excluding cells at the threshold edge. Pay attention that, as a consequence of this gap, the sum of the number of G1, S and G2 nuclei will be lower than the number of "Nuclei for analysis" initially selected.

    Note: It is recommended to optimize the parameter setting for cell cycle phase discrimination on a few fields from different wells.

60. Define results.
    a. Click on "Define Results" building block.

    Note: This is always the last building block in the sequence of analysis.

    b. Fill in the "Methods" tab.
        i. Define the parameters "Method" = "List of Outputs".
        ii. Select the measurement values to be extracted and saved from the analysis by selecting the parameter "Apply to all" = "Individual Selection" in each population (i.e., "Nuclei for analysis", "G1", "S", G2").
        iii. For the tab "Population: Nuclei for analysis", select "Number of Objects" and set the parameters "Intensity Nucleus Hoechst 33342 Sum", "Intensity Nucleus Alexa 488 Sum", "Intensity Nucleus Alexa 594 Sum", "Intensity Nucleus Alexa 647 Mean", "Number of Spots" (referred to γH2AX Spots) and "Number of Spots (2)" (referred to p53BP1 Spots) = "Mean".

    Note: The "Number of Object" measurement corresponds to the number of nuclei in the "Nuclei for analysis" population. The other parameters provide the nuclear intensity and the number of foci per nucleus in the "Nuclei for analysis" population (i.e., independently of the cell cycle).

    Note: Although additional measurements can be obtained and saved, DSB production and repair analysis requires the extraction of these measurements.

        iv. Repeat the previous step for the tabs "Population: G1", "Population: G2", and "Population: S" to have this analysis done in each phase of the cell cycle.
    c. Fill in the "Object Results" tab. Set the parameters "Population: Nuclei for analysis", "Population: G1", "Population: G2" and "Population: S" = "ALL".
    d. Click on the green arrow icon at the top right of the "Define Results" building block to validate the parameters.
61. Click the icon "Save Analysis to Database" (Figure 3, annotation j) to save the analysis sequence in the database.
62. Rename the analysis sequence (e.g., "DSB foci in the cell cycle phases").
    a. Right-click on the analysis file (Figure 3, annotation k).
    b. Select "Rename" in the pop-up list.

    Note: The saved analysis sequence can be re-used and modified to analyze other data sets by clicking on it at the end of step 49, instead of creating a new sequence of analysis (steps 51-62).

63. Click "Batch Analysis" in the Navigator bar (Figure 3, annotation l) to start the automated analysis of the plate. A new window appears with the following tabs: "Select Data", "Select Analysis Options", "Start Analysis".
    a. Select the measurement on the data tree (Figure 3, annotation b).
    b. Select analysis on the data tree (Figure 3, annotation k).

    Note: The selected measurement and analysis are automatically loaded in the "Select Data" and "Select Analysis" tabs, respectively.

    c. Click on the green arrow icon at the top right of the "Start Analysis" tab to start the batch analysis.

Note: The status of the ongoing analysis can be viewed by clicking on the "Job Status" icon at the top right (Figure 3, annotation m).

64. Click on "Export" in the Navigator bar (Figure 3, annotation n) to export the data when the batch analysis is completed. A pop-up window "Columbus Helper Required" appears. Click on "connection file". A "Columbus Helper" window appears confirming the connection with the Columbus server.
65. Define the data and analysis to export. When a new window with the "Select Data", "Select Export Options" and "Start Export" tabs appears:
    a. Select the measurement on the data tree (Figure 3, annotation b), which will be automatically loaded in the "Select Data" tab.
    b. In the "Select Export Options" tab.
        i. Sequentially click on "Methods" and select "Export to Disk".
        ii. Click on the "…" icon in the "Export Folder" section.
        iii. Select the local disk to store your data and analysis.
        iv. Click on "Image Data" = "SinglePlane TIF" (export of raw images); "Results" = "Excel (txt)" (export of the analysis measurements) and "Analysis" = "Script" (export of the analysis sequence).
    c. Click on the green arrow icon at the top right of the "Start Export" tab to start the export.

Note: The exported "Results" consist of: i) a ".txt" summary file including all the mean measurements per well (i.e., mean for all the nuclei of an analyzed population) defined in the sequence of analysis (see step 60), e.g., mean number of γH2AX foci per nucleus in the G1, G2 or S population per well etc…; ii) a ".txt" individual well file for each analyzed population including single-cell values for each measurement, e.g., number of γH2AX foci per nucleus in the G1 population in a well, etc…); iii) a ".analysis.aas" file, which is the sequence of analysis. This sequence can be reused by clicking on "load Analysis from Disk" (Figure 3, annotation o) at the end of step 49.

66. Import images on Columbus. When images have been exported (step 65), they can be re-imported for further analysis if needed.
    a. Click on "Import" in the Navigator bar (Figure 3, annotation p). A pop-up window "Columbus Helper Required" appears. Click on "connection file". A "Columbus Helper" pop-up window appears confirming the connection with Columbus server.
    b. A new window with the "Select Data", "Select Import Options" and "Start import" tabs appears.
    c. In the "Select Data" tab, click on "Methods".
        i. Click on "Import type" and select "Columbus IDX/TIF".
        ii. Click on the "…" icon and select the folder with the images to be imported.
    d. Select the folder on Columbus where the images will be re-imported.
        i. Right-click on the name of the chosen folder in the data tree (Figure 3, annotation q).
        ii. Click on "Select". The name of the folder then appears in the "Select Import Option" tab in the window "screen name".
    e. Click on the green arrow icon at the top right of the "Start Import" tab to start the import.

EXPECTED OUTCOMES

DSB-inducing agents can lead to DSB formation either directly (e.g., IR or radiomimetics) or through transcription- and/or replication-dependent mechanisms (e.g., CPT),[3,16–19] resulting in differences in γH2AX staining, foci number and repair kinetics in G1, S and G2 phases of the cell cycle. Our quantitative image-based cytometry (QIBC) pipeline protocol for analysis of DSB production and repair enables simultaneous single-cell quantification of γH2AX foci number and γH2AX intensity distinctly in G1, S and G2 phases. This protocol has been applied to analyze DSB production and repair

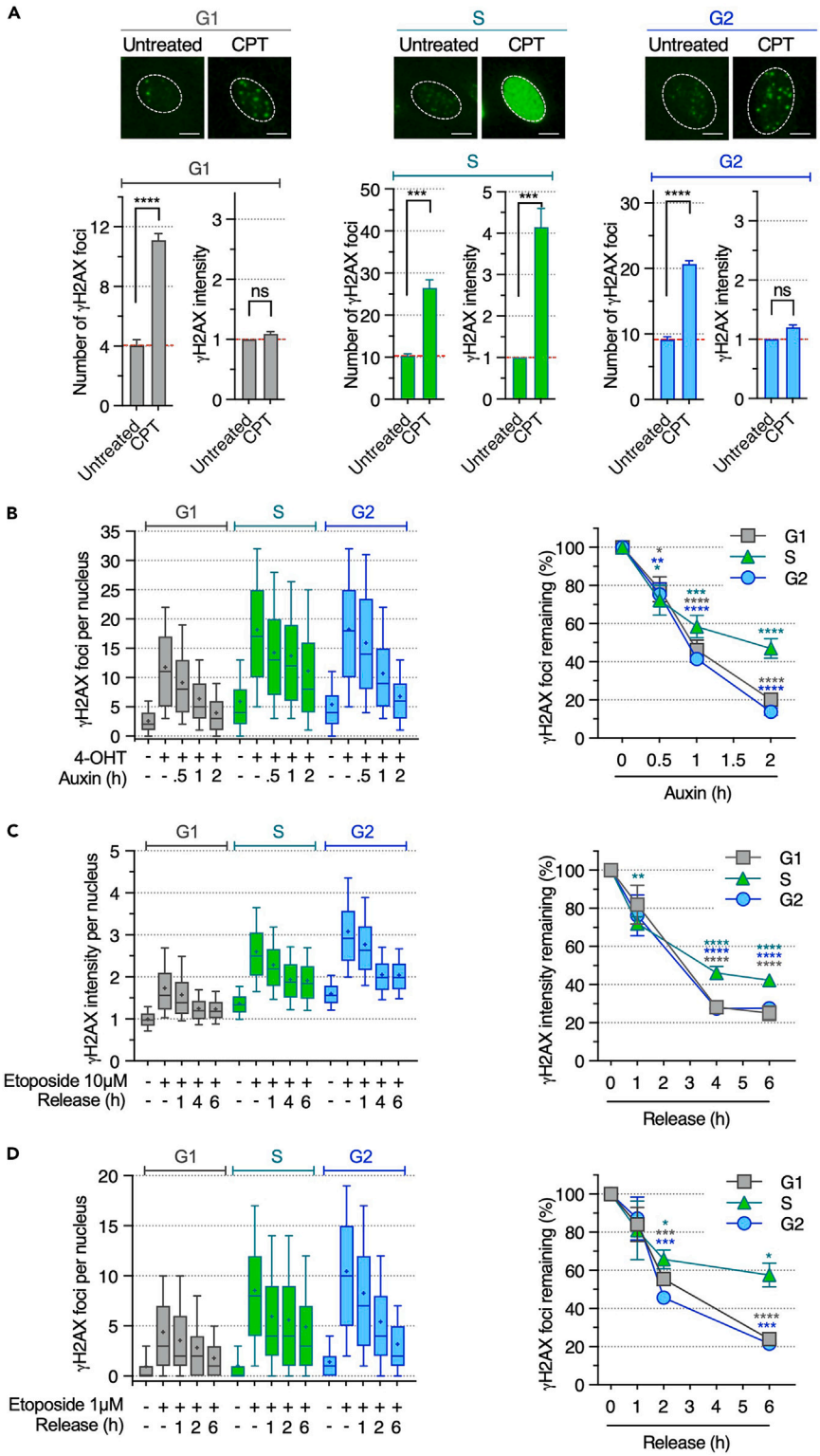

Figure 5. Analysis of DSB production and repair in response to CPT, AsiSI, and etoposide

(A) U2OS cells were incubated with EdU (10 μM) for 30 min before the addition of CPT (25 μM) for 1 h. Cells were then stained for EdU, γH2AX and Hoechst 33342 (DNA). Top panels: Representative images of γH2AX labeling in G1, S and

Figure 5. *Continued*

G2 cells. Scale bars: 10 μm. Bottom panels: comparison between the number of γH2AX foci and γH2AX intensity in G1, S and G2 cells (means ± SEM; $n = 4$). ns, not significant; ***$p < 0.001$, ****$p < 0.0001$ (two-tailed unpaired t-test).

(B) U2OS cells were incubated with EdU (10 μM) for 30 min, then 4-OHT (300 nM) was added for 4 h while maintaining EdU in the media, washed, and cultured in 4-OHT-free medium containing auxin (500 μM) and EdU (10 μM) for up to 2 h. Cells were then stained for EdU, γH2AX, and Hoechst 33342 (DNA). Left panel: number of γH2AX foci per G1, S and G2 nucleus. Right panel: percentages of γH2AX foci remaining in G1 (gray line), S (green line) and G2 (blue line) nuclei following auxin addition normalized to 4-OHT-treated cells (means ± SEM; $n = 3$). *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$ (two-tailed unpaired t-test).

(C and D) U2OS cells were incubated with EdU (10 μM) for 30 min, then with 10 μM (C) or 1 μM (D) of etoposide (ETO) for 1 h while maintaining EdU in the media, washed, and cultured in ETO-free medium (Release) containing EdU (10 μM) for up to 6 h. Cells were then stained for EdU, γH2AX, and Hoechst 33342 (DNA). (C) Left panel: γH2AX signal intensity per G1, S and G2 nucleus. Right panel: percentages of γH2AX intensity remaining in G1 (gray line), S (green line) and G2 (blue line) nuclei following ETO removal normalized to ETO-treated cells (means ± SEM; $n = 3$). (D) Left panel: number of γH2AX foci per G1, S and G2 nucleus. Right panel: percentages of γH2AX foci remaining in G1 (gray line), S (green line) and G2 (blue line) nuclei following ETO removal normalized to ETO-treated cells (means ± SEM; $n = 2$). *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$ (two-tailed unpaired t-test).

kinetics in the context of an asynchronous U2OS cell population treated with CPT.[1] An important question is when to choose γH2AX foci counting *vs* γH2AX fluorescence intensity. We demonstrate here that when the number of foci induced by CPT is low, as in G1 and G2 phases, γH2AX fluorescence intensity does not reliably reflect γH2AX induction. Therefore, γH2AX foci counting is the preferred approach (Figure 5A). Conversely, when the number of foci induced by CPT is high, such as in S phase (pan-staining), all γH2AX foci cannot be accurately discriminated and counted, and γH2AX fluorescence intensity is the preferred approach (Figure 5A). Nevertheless, we have reported that low doses of CPT, which allow individual foci to be counted, display similar DSB repair kinetics to high doses of CPT when measured by γH2AX fluorescence intensity,[1] indicating that both parameters can be used to analyze DSB production and repair.

To test the workability of our protocol, we extended the analysis of DSB production and repair by CPT[1] to other DSB inducers, including restriction enzymes and etoposide (Figures 5B–5D). To analyze DSB induced by restriction enzymes, we used AID-DIvA (auxin-inducible degron-DSB inducible via AsiSI) cells,[10] which enables the induction of multiple DSBs by the AsiSI enzyme throughout the cell cycle by addition of 4-OHT. The analysis of repair kinetics can be done through degradation of the AsiSI by addition of auxin.[10] We show that AsiSI induced γH2AX foci in all phases of the cell cycle (Figure 5B, left panel). These foci disappeared within 2 h upon auxin addition (Figure 5B). It further shows that AsiSI induced more γH2AX foci in S and G2 cells (Figure 5B, left panel) and that the repair kinetics appeared to be faster in G1 and G2 compared to S phase (Figure 5B, right panel), likely reflecting different repair mechanisms. We also tested etoposide (ETO), which is a topoisomerase II (TOP2) inhibitor and allows to assess our protocol in the analysis of DSBs characterized by a TOP2-bound 5′-end.[12,13] QIBC analysis illustrates that ETO induced γH2AX foci in all the cell cycle phases and that, similarly to what is described for CPT,[1] the number of γH2AX foci decreased rapidly after ETO removal (Figures 5C and 5D). As in the case of CPT,[1] analysis of the γH2AX foci number and fluorescence intensity at low and high ETO doses, respectively, showed comparable results (Figures 5C and 5D). Finally, we demonstrate that our protocol to analyze γH2AX in different cell cycle phases can be applied to various cell types beyond U2OS, including normal cells like WI38-hTERT cells (Figure 6A).

In our protocol, to distinguish cell cycle phases, we used the intensity of Hoechst 33342, which is proportional to DNA content, and the intensity of EdU incorporated into newly replicated DNA. As a result, the G1 cell population is defined as EdU-negative with low Hoechst 33342 intensity, the S population as EdU-positive, and the G2 population as EdU-negative with high Hoechst 33342 intensity. To assess the robustness of our QIBC protocol, we additionally stained cells with two cell cycle-specific markers, CENPF (a late S and G2/M marker)[20] and cyclin B1 (a late G2 and M marker). Scatter plots showed that, in different cell lines, CENPF effectively labeled G2 cells and a percentage of the S-phase cells identified using the Hoechst/EdU approach (HCC4006 and WI38-hTERT cells in

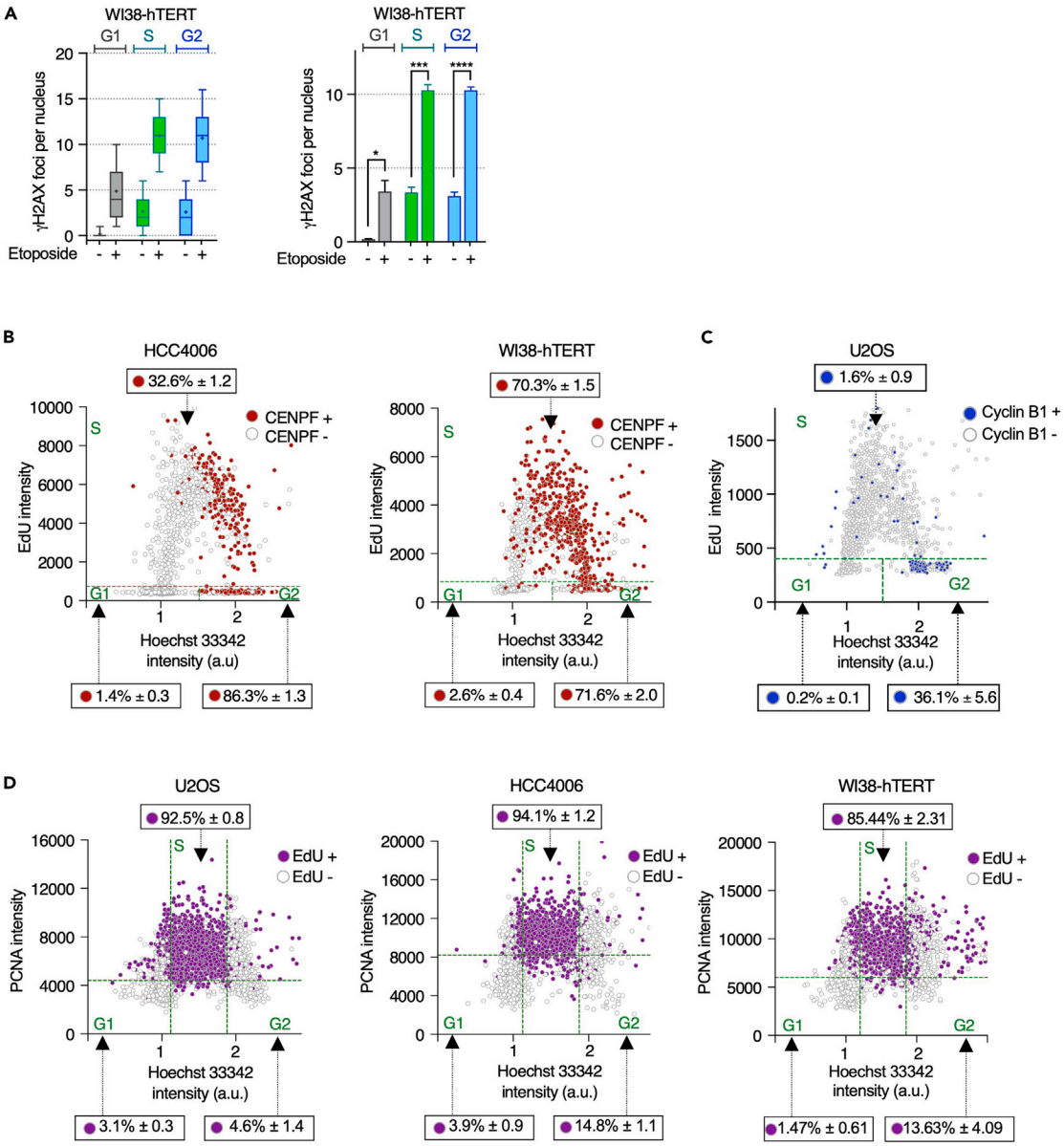

Figure 6. Analysis of DSB production in etoposide-treated WI38-hTERT cells, and QIBC analysis of the cell cycle

(A) WI38-hTERT cells were incubated with EdU (10 μM) for 30 min before the addition of etoposide (10 μM) for 1 h. Cells were then stained for EdU, γH2AX and Hoechst 33342 (DNA). Number of γH2AX foci per G1, S and G2 nucleus in a representative experiment (left panel) and in 3 independent experiments (mean ± SEM; $n = 3$; right panel). *$p < 0.05$, ***$p < 0.001$, ****$p < 0.0001$ (two-tailed unpaired t-test).

(B) Cells were incubated with EdU (10 μM) for 30 min before staining for EdU, CENPF and Hoechst 33342 (DNA). Representative scatter plot of HCC4006 (left) and WI38-hTERT (right) cells from QIBC analysis showing cell cycle distribution. Cell cycle phases were identified by Hoechst 33342 intensity in DNA to distinguish G1 from G2 cells, and EdU incorporation in newly replicated DNA to identify S phase. Cells positive for CENPF, a late S and G2/M marker, are colored in red. The percentages of CENPF-positive cells in G1 cells (EdU-negative and low Hoechst 33342), S (EdU-positive) and G2 cells (EdU-negative and high Hoechst 33342) are indicated (mean ± SEM; $n = 3$).

(C) Same experiment as in (B) in U2OS cells, except that cells were labeled with cyclin B1 (colored in blue), a late G2 marker, instead of CENPF (mean ± SEM; $n = 3$).

(D) Cells were incubated with EdU (10 μM) for 30 min before staining for EdU, PCNA (S marker) and Hoechst 33342 (DNA). Representative scatter plot of U2OS (left), HCC4006 (middle) and WI38-hTERT (right) cells from QIBC analysis showing cell cycle distribution. Cell cycle phases were identified by Hoechst 33342 intensity in DNA to distinguish G1 from G2 cells, and PCNA to identify S phase. EdU labeling (colored in purple) was used for comparison. The percentages of EdU-positive cells in G1 cells (low PCNA and low Hoechst 33342), S (high PCNA) and G2 cells (low PCNA and high Hoechst 33342) are indicated (mean ± SEM; $n = 3$).

Figure 6B; U2OS cells in Geraud et al.[1]). Similarly, cyclin B1 effectively labeled a percentage of the G2 cells (Figure 6C), indicating the reliability of our protocol. In addition, the use of these markers could be useful to specifically analyze a subpopulation of a cell cycle phase (e.g., late S cells with CENPF; late G2 cells with cyclin B1). They could also substitute the Hoechst or the EdU staining to identify the cell cycle phases when their use is not technically possible. Indeed, we have reported that cell cycle distribution is similar when assessing cell cycle phases by Hoechst/EdU or Hoechst /CENPF in U2OS cells.[1] Furthermore, we demonstrated that, when using stringent parameters (which can lead to exclusion of some G1 and G2 cells), PCNA could efficiently replace EdU for the identification of cell cycle phases in all tested cell lines (Figure 6D).

QUANTIFICATION AND STATISTICAL ANALYSIS

The figure legends specify the number of independent biological replicates (n), statistical methods applied, and how center (mean, median) and variability (SD, SEM) are represented. For immunofluorescence microscopy, each experiment analyzed an average of at least 1,000 nuclei per condition. Statistical significance of differences between experimental groups was determined using GraphPad Prism 10 software. Non-significant differences are marked as "ns". Values of $p < 0.05$ were considered significant: *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$.

LIMITATIONS

The primary limitation of this protocol for analyzing DSB dynamics during DNA repair lies in the increasing difficulty of accurately distinguishing cell cycle phases when the repair kinetics are prolonged in time. Indeed, when performing prolonged EdU incorporation in a drug-free medium for repair analysis, G1 phase cells can progress to S phase, and S phase cells to G2, leading to EdU-positive cells that may have undergone repair in either G1 or G2, respectively. In particular, extended EdU pulsing can artificially reduce the apparent percentage of G2 phase cells. Therefore, while this protocol is valuable for assessing DSB production and repair in an asynchronous growing cell population, cell synchronization may be required for longer DSB repair kinetics (e.g., longer that 8 h). Synchronization can be achieved through various strategies, such as CDK4/6 inhibition[21] or single/double thymidine block and release.[22] However, it is important to note that synchronization protocols may induce undesired side effects, such as transcriptomic and proteomic changes, or DNA damage.

This protocol focuses on analyzing DSB dynamics in the G1, S, and G2 phases of the cell cycle and does not include DSB detection in mitosis. Although we have not yet applied this analysis to mitotic cells, we have demonstrated that our QIBC protocol is compatible with various cell cycle-specific markers (Figures 6B–6D). Hence, this approach could be adapted to include the staining of mitotic markers, such as histone H3 phosphorylated on Ser10.[23]

In this protocol, we used Columbus software, which is associated with the Operetta high-content microscope and is not free, to generate the sequence of analysis for cell cycle and γH2AX/p53BP1 foci discrimination. However, this analysis sequence can be adapted to other softwares associated with different imaging devices or an open-source Fiji script has recently been developed to perform this type of analysis.[9]

TROUBLESHOOTING

Problem 1

Cells are not homogeneously seeded and/or form clusters on the edges of wells in 96-well plates (steps 9-10).

To obtain high-quality images, cells must be evenly distributed over the entire well surface, with an average confluence of 60–80%.

Potential solution

Depending on the cell line used, it is recommended to gently resuspend the cells using a P1000 micropipette before plating to break up any clumps that may have formed in the suspension, and to maintain the seeded plate at 18°C–25°C for 30 min before placing it in the incubator at 37°C.

Problem 2

Few cells remaining after drug washout for DSB repair kinetic analysis (step 16).

Multiple washes with drug-free medium are necessary to analyze DSB repair kinetics prior to cell fixation, and may result in cell detachment and loss.

Potential solution

- Coating the plate with poly-L-lysine (steps 1-4) and seeding cells at a slightly higher density could eliminate or reduce this problem.
- Additionally, it is recommended to check cell density with a phase-contrast microscope before and after drug washout, fixation and permeabilization.

Problem 3

DSBs are repaired too quickly or too slowly (steps 16-17).

The time frame to analyze DSB repair may not be appropriate for the DSB inducer studied. Additionally, the inability of cells to repair DSBs could be due to insufficient drug washout resulting in continued DSB production.

Potential solution

- Optimizing the repair kinetics for each DSB inducer and cell line can solve this issue, e.g., when repair is fast, it is recommended to use short kinetics and closer time points.
- Increasing the number of washes with drug-free medium can improve drug removal and favor repair. Alternatively, if the drug induces a very high amount of DSBs, drug concentration could be decreased, as shown for CPT[1] and ETO (Figures 5C and 5D).

Problem 4

Antibody signal is weak (steps 28 and 29).

This may indicate suboptimal conditions related to a low concentration of primary antibody or insufficient incubation time, resulting in a low signal-to-noise ratio. Alternatively, it could be due to inadequate permeabilization, which reduces antibodies from accessing the nucleus, or incorrect exposure settings during acquisition.

Potential solution

- To improve the signal, consider increasing the primary antibody concentration and/or extending the incubation time, e.g., 12–15 h at 4°C. It is advisable to optimize the antibody concentration by testing various dilutions based on the manufacturer's guidelines.
- Permeabilization time with Triton X-100 could be extended up to 30 min.
- Change setting for image acquisition, e.g., by increasing the exposure time and/or laser power (Figure 2B).

Problem 5

The number of nuclei to be analyzed varies significantly depending on the conditions, and does not reflect the number of cells in the wells (step 54).

Some drugs can induce changes in nucleus morphology, such as size and shape, leading to misidentification of nuclei in treated conditions.

Potential solution

Testing morphological parameters on few test images of each condition within the same experiment (plate) until the selected morphological criteria are suitable for all conditions will help to obtain a consistent number of nuclei across conditions (step 54). If it is not possible to find satisfactory morphological criteria for all conditions, different morphological parameters may be set for untreated and treated conditions to minimize cell loss and reduce potential bias in the analysis.

Problem 6

The number of γH2AX/p53BP1 foci increases during DSB repair (step 56).

When the number of DSBs is too high, individual foci cannot be distinguished and counted accurately, resulting in an underestimation of their number (e.g., Figure 5A, middle panels). However, as DSBs are repaired, the number of γH2AX/p53BP1 foci decreases, making them discrete and countable. This may lead to an apparent but artificial increase in their number during the repair process.

Potential solution

- Using γH2AX fluorescence intensity instead of γH2AX foci helps prevent artifacts when the number of γH2AX foci is excessively high or when staining patterns differ significantly between DSB induction and repair conditions (Figure 5A, middle panels; Figure 5C).
- In addition, comparing fluorescence intensity measurement with number of γH2AX/p53BP1 foci could help reduce bias and ensure a more accurate analysis.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Olivier Sordet (olivier.sordet@inserm.fr).

Technical contact

Technical questions on executing this protocol should be directed to and will be answered by the technical contacts, Agnese Cristini (agnese.cristini@inserm.fr) and Olivier Sordet (olivier.sordet@inserm.fr).

Materials availability

This study did not generate new unique reagents.

Data and code availability

- Original images and source data included in Figures 5 and 6 of the present article are available upon request to the lead contact, Olivier Sordet (olivier.sordet@inserm.fr).
- Example images of untreated and CPT-treated U2OS cells, which can be imported to the Columbus software for the users as a starting point for setting up the pipeline with the settings provided in this protocol, and which corresponds to Figure 4, will be available without restrictions upon request: lead contact, Olivier Sordet (olivier.sordet@inserm.fr).
- This study did not generate new code.

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AUTHOR CONTRIBUTIONS

M.G., A.C., and O.S. developed and optimized the method with input from L.F.M. and A.C.A. L.F.M. and A.C.A. performed the experiments and analyzed the data. M.G., A.C., and O.S. wrote the manuscript with contribution from all authors. A.C. and O.S. supervised the study and acquired funding.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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