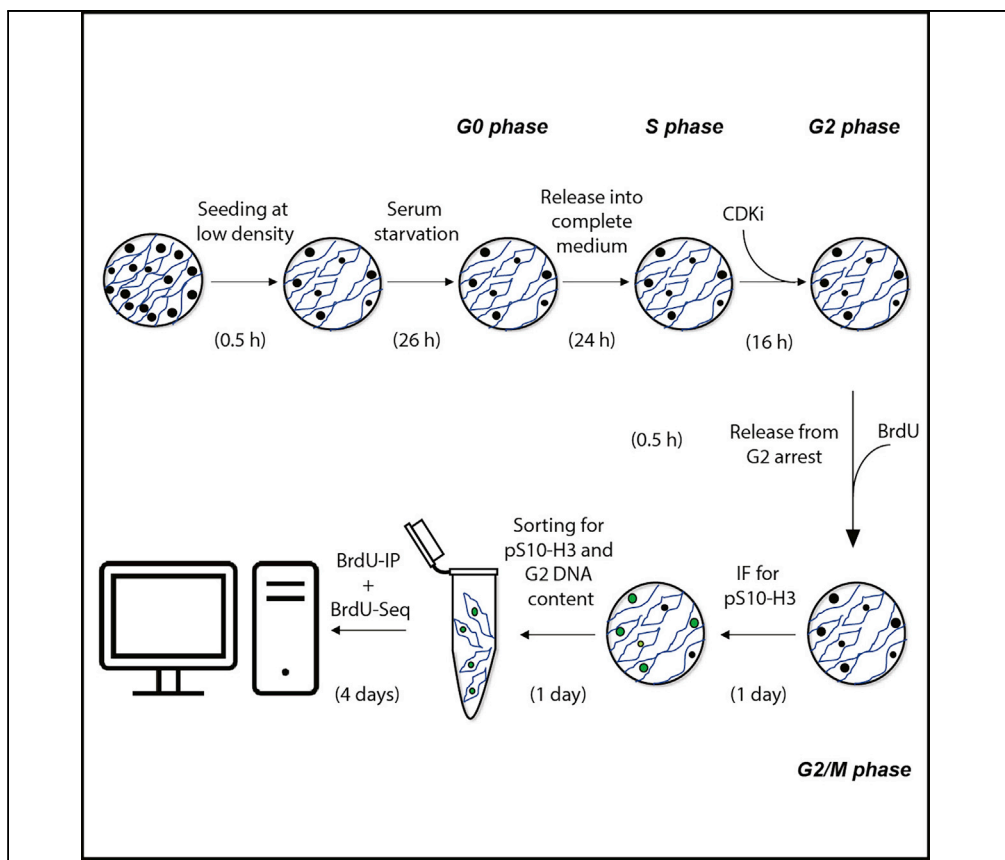


Protocol

Protocol for analysis of G2/M DNA synthesis in human cells



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Highlights

How to arrest cells in G0/G1 and to get a synchronous release into S-phase

FACS strategy to identify cells in G2/M performing DNA synthesis

Pull-down of DNA synthesized in G2/M

Next-generation sequencing of sites of G2/M DNA synthesis

G2/M DNA synthesis (G-MiDS) can be observed in one in five G2/M cells in unperturbed conditions by immunofluorescence microscopy. However, little is known of the genomic sites undergoing G-MiDS. Here, we describe a protocol which allows enriching for G2/M cells and investigating the sites of G-MiDS using BrdU-seq. This method can also be used to study the role of DNA replication or transcription-associated factors in affecting G-MiDS levels in different cell lines.

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Protocol

Protocol for analysis of G2/M DNA synthesis in human cells

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SUMMARY

G2/M DNA synthesis (G-MiDS) can be observed in one in five G2/M cells in unperturbed conditions by immunofluorescence microscopy. However, little is known of the genomic sites undergoing G-MiDS. Here, we describe a protocol which allows enriching for G2/M cells and investigating the sites of G-MiDS using BrdU-seq. This method can also be used to study the role of DNA replication or transcription-associated factors in affecting G-MiDS levels in different cell lines. For complete details on the use and execution of this protocol, please refer to Wang et al. (2021).

BEFORE YOU BEGIN

- Before starting the experiment, it is necessary to maintain cells in consistent growth conditions. Moreover, it is important to ensure that DMEM medium and FBS are from the same batch for reproducible cell growth.
- Unless specified, cells were cultured in DMEM (Sigma-Aldrich) supplemented with 10% FBS, 2 mM L-glutamine and antibiotics (50 U/mL Penicillin-Streptomycin), in a humidified incubator with 5% CO₂ at 37°C.
- The protocol below describes the specific steps with human immortalized fibroblasts (BJ-hTERT).
- We combine cells grown in parallel in two 150 mm dishes for G2/M cell sorting.

Thaw cell stocks

⌚ Timing: 0.5–1 h

1. Thaw the cell stock quickly by gently swirling the cryovial in a 37°C water bath and dilute them with 15 mL warm culture medium in a T75 flask.
2. Keep cells growing in the incubator and replace with fresh medium next day.

Note: Passage the cells at least twice to allow them to recover from thawing and resume consistent cell cycle progression before using them.

Maintenance of cell culture

⌚ Timing: 5 days



Day 1

3. When cells reach 100% confluency remove the media from T75 flask and rinse the cells once with 10 mL of warm PBS.
4. Add 2 mL trypsin-EDTA to the cells and place the T75 flask into the incubator for 2 min, gently flap the flask to detach the cells.

Note: Double check the detachment of cells after trypsinization under a microscope. If some cells are not detached, incubate them in the incubator with trypsin for longer time.

5. Add 5 mL complete media to the flask and detach cells by slowly pipetting up and down. Split the cells at around 14% confluency by transferring and mixing 1 mL detached cells into a new T75 flask with 15 mL of complete DMEM by pipetting.

Note: This starting confluency allows BJ-hTERT to reach full confluency in 5 days, so it may need to be adjusted according to the cell growth of the specific cell line used. The rationale behind this approach is to get a partial pre-synchronization of the cell culture before the actual serum starvation stage.

6. Incubate cells in the incubator for five days.

Day 5

7. After five days, split the cells at around 33% confluency into another T75 flask.
 - a. Trypsinize cells by repeating step 4.
 - b. Add 7 mL culture medium and detach cells by slowly pipetting up and down, and split cells 1:3 by transferring and mixing 3 mL detached cells into a new T75 flask with 12 mL complete medium by pipetting.
 - c. Incubate in the incubator until when cells reach 100% confluency.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Rabbit monoclonal anti-phospho-histone H3 (Ser10)	Cell Signaling Technology	Cat#3377S; RRID: AB_1549592
Mouse monoclonal anti-BrdU	Sigma-Aldrich	Cat#B8434; RRID: AB_476811
Alexa Fluor 488-conjugated Goat Anti-Rabbit IgG (H+L)	Thermo Fisher Scientific	Cat#A-11070; RRID: AB_2534114
Chemicals, peptides, and recombinant proteins		
DMEM	Sigma-Aldrich	Cat#D6546
L-Glutamine (200 mM)	Thermo Fisher Scientific	Cat#25030024
Penicillin-Streptomycin (5,000 U/mL)	Thermo Fisher Scientific	Cat#15070063
FBS	Sigma-Aldrich	Cat#F7524
Trypsin-EDTA (0.25%)	Thermo Fisher Scientific	Cat#25200056
RNase A	AppliChem	Cat#A2760,0100
BrdU	Sigma-Aldrich	Cat#B5002
EdU	Sigma-Aldrich	Cat#900584
Ro 3306	Adooq Bioscience	Cat#A14437
Violet Stain	Thermo Fisher Scientific	Cat#V35003
Proteinase K	Promega	Cat#MC5008
BSA	VWR	Cat# 9048-46-8

(Continued on next page)

Continued		
REAGENT or RESOURCE	SOURCE	IDENTIFIER
Agencourt AMPure XP	Beckman Coulter	Cat#A63881
Dynabeads Protein A	Thermo Fisher Scientific	Cat#10002D
Critical Commercial Assays		
DNA Clean & Concentrator-5	Zymo Research	Cat#D4013
Random Primed DNA Labeling Kit	Roche	Cat#11004760001
Qubit dsDNA HS Assay Kit	Thermo Fisher Scientific	Cat#Q32851
Click-iT RNA Alexa Fluor 594 Imaging Kit	Thermo Fisher Scientific	Cat#C10330
QIAseq Ultralow Input Library Kit	QIAGEN	Cat#180492
Deposited data		
G2M BrdU-Seq	This paper	GEO: GSE136293
Experimental models: cell lines		
Human immortalized fibroblasts (BJ-hTERT)	ATCC	CRL-4001
Other		
1.5-mL Low DNA Binding Tube	Sarstedt	Cat#72.706.700
5-mL Polypropylene round bottom tube	Corning	Cat#352063
15-mL Centrifuge tube	Sarstedt	Cat#62.554.502
DynaMag™-2 Magnet	Thermo Fisher Scientific	Cat#12321D
Bench-top centrifuge	VWR	Cat#521-1752P
BD Accuri C6 software	BD Biosciences	n/a
Qubit Fluorometer	Thermo Fisher Scientific	Cat#15387293
BD FACSAria cell sorter	BD Biosciences	n/a
Ultrasonic processors VCX130	Sonics & Materials, Inc	n/a

MATERIALS AND EQUIPMENT

Note: Due to the instability of formaldehyde in aqueous solution, we recommend preparing fresh PTEMF buffer (Bhowmick et al., 2016) each time before use keeping the buffer at 15°C–30°C.

Cell synchronization	Final concentration	Amount
Ro-3306 (9 mM)	9 μ M	20 μ L
DMEM complete medium	N/A	20 mL
Total	N/A	20 mL

Prepare on the day of experiment.

DNA synthesis labeling	Final Concentration	Amount
BrdU (10 mM)	50 μ M	75 μ L
EdU (10 mM)	25 μ M	37.5 μ L
DMEM complete medium	N/A	15 mL
Total	N/A	15 mL

Prepare on the day of experiment.

PTEMF buffer	Final concentration	Amount
PIPES pH 6.8 (0.5 M)	20 mM	0.4 mL
EGTA (0.5 M)	10 mM	0.2 mL
Triton X-100 (10%)	0.2%	0.2 mL
MgCl ₂ (1 M)	1 mM	0.01 mL
Formaldehyde (37%)	4%	1.08 mL
ddH ₂ O	N/A	8.11 mL
Total	N/A	10 mL

Prepare on the day of experiment.

FACS samples preparation	Final concentration	Amount
RNase A (20 mg/mL)	0.1 mg/mL	2.5 μ L
Violet DNA (5 mM)	5 μ M	0.5 μ L
1 \times PBS	N/A	497 μ L
Total	N/A	500 μL

Prepare on the day of experiment.

Click-iT [®] reaction buffer (Thermo Fisher Scientific)	Final concentration	Amount
Click-iT [®] RNA reaction buffer (labeled Component C in the kit)	1 \times	214 μ L
CuSO ₄ (labeled Component D in the kit)	4 mM	10 μ L
Alexa Fluor [®] 594 azide	N/A	0.9 μ L
Click-iT [®] reaction buffer additive	1 \times	25 μ L
Total	N/A	250 μL

Prepare on the day of experiment.

TE buffer	Final concentration	Amount
Tris-Cl (1 M) (pH 8.0)	10 mM	0.5 mL
EDTA (0.5 M) (pH 8.0)	1 mM	0.1 mL
ddH ₂ O	N/A	49.4 mL
Total	N/A	50 mL

Can be stored at 15°C–30°C for several years.

Lysis buffer 1 (BrdU-pull down)	Final concentration	Amount
Na Deoxycholate (10%)	0.1%	0.5 mL
EDTA (0.5 M) (pH 8.0)	1 mM	0.1 mL
HEPES-KOH (1M) (pH 7.5)	50 mM	2.5 mL
NaCl (5 M)	140 mM	1.4 mL
Triton X-100 (10%)	1%	5 mL
ddH ₂ O	N/A	40.5 mL
Total	N/A	50 mL

Can be stored at 15°C–30°C for several months.

Lysis buffer 2 (BrdU-pull down)	Final concentration	Amount
Na Deoxycholate (10%)	0.1%	0.5 mL
EDTA (0.5 M) (pH 8.0)	1 mM	0.1 mL
HEPES-KOH (1M) (pH 7.5)	50 mM	2.5 mL
NaCl (5 M)	500 mM	5 mL
Triton X-100 (10%)	1%	5 mL
ddH ₂ O	N/A	36.9 mL
Total	N/A	50 mL

Can be stored at 15°C–30°C for several months.

Wash buffer (BrdU-pull down)	Final concentration	Amount
Na Deoxycholate (10%)	0.5%	2.5 mL
EDTA (0.5 M) (pH 8.0)	1 mM	0.1 mL
LiCl (10 M)	250 mM	1.25 mL
Igepal CA-630	0.5%	0.25 mL
Tris-Cl (1 M) (pH 8.0)	10 mM	0.5 mL
ddH ₂ O	N/A	45.4 mL
Total	N/A	50 mL

Can be stored at 15°C–30°C for several months.

Elution buffer (BrdU-pull down)	Final concentration	Amount
EDTA (0.5 M) (pH 8.0)	10 mM	1 mL
Tris-Cl (1 M) (pH 8.0)	50 mM	2.5 mL
SDS (20%)	1%	2.5 mL
ddH ₂ O	N/A	44 mL
Total	N/A	50 mL

Can be stored at 15°C–30°C for several years.

Note: For the Click-iT reaction, we used Click-iT RNA Alexa Fluor 594 Imaging Kit (Thermo Fisher Scientific), as we had that kit already in the laboratory and it contains all the required Click-reaction ingredients except EdU, which can be purchased separately. An alternative kit is the Click-iT® EdU Imaging Kit (Thermo Fisher Scientific, catalogue number C10086).

△ **CRITICAL:** Formaldehyde should always be added into PTEMF buffer in a fume hood to avoid inhalation of formaldehyde vapor; add components of the Click-iT reaction cocktail in the order listed in the table above according to manufacturer recommendation (<https://www.thermofisher.com/order/catalog/product/C10330#/C10330>).

STEP-BY-STEP METHOD DETAILS

Cell synchronization by serum starvation

⌚ Timing: 3 days

This step is used to synchronize cells into G0/G1.

Day 1

1. Cells fully confluent were seeded at 20% confluence into 150 mm dish and grown in the incubator for around 20 h.

△ **CRITICAL:** When starting the protocol cells are fully confluent but not over confluent. We recommend seeding cells at 20% confluence to avoid contact inhibition for later stage of cell cycle progression.

Day 2–3

2. Remove the media from 150 mm dish, and wash the cells once with 10 mL prewarmed PBS.
3. Add 20 mL of serum starvation media (DMEM supplemented with 0.2% FBS, 2 mM L-glutamine and 50 U/mL penicillin/streptomycin) to 150 mm dish and keep cells in the incubator for 26 h.

△ **CRITICAL:** To ensure the majority of cells are arrested in G0/G1 after the serum starvation, but meanwhile maintain the ability of cell cycle progression with only a small fraction of cells remaining into G0/G1 after release, we recommend performing optimizations for cell serum starvation; these include optimizing FBS concentration in starvation medium, length of starvation time. Lower FBS concentration and longer serum starvation time increase the number of cells arrested in G0/G1 but also increase the number of cells that will not re-enter the cell cycle remaining in G0. Therefore, it is important to identify for the specific cell line the optimal conditions for serum starvation to achieve a high level of G0/G1 synchronization (>75%–80% cells in G0/G1) and a high percentage of cells going into the S phase after release (>60%–65%). The synchronization-release procedure needs to be validated by fluorescence-activated cell sorting (FACS) (Figure 1).

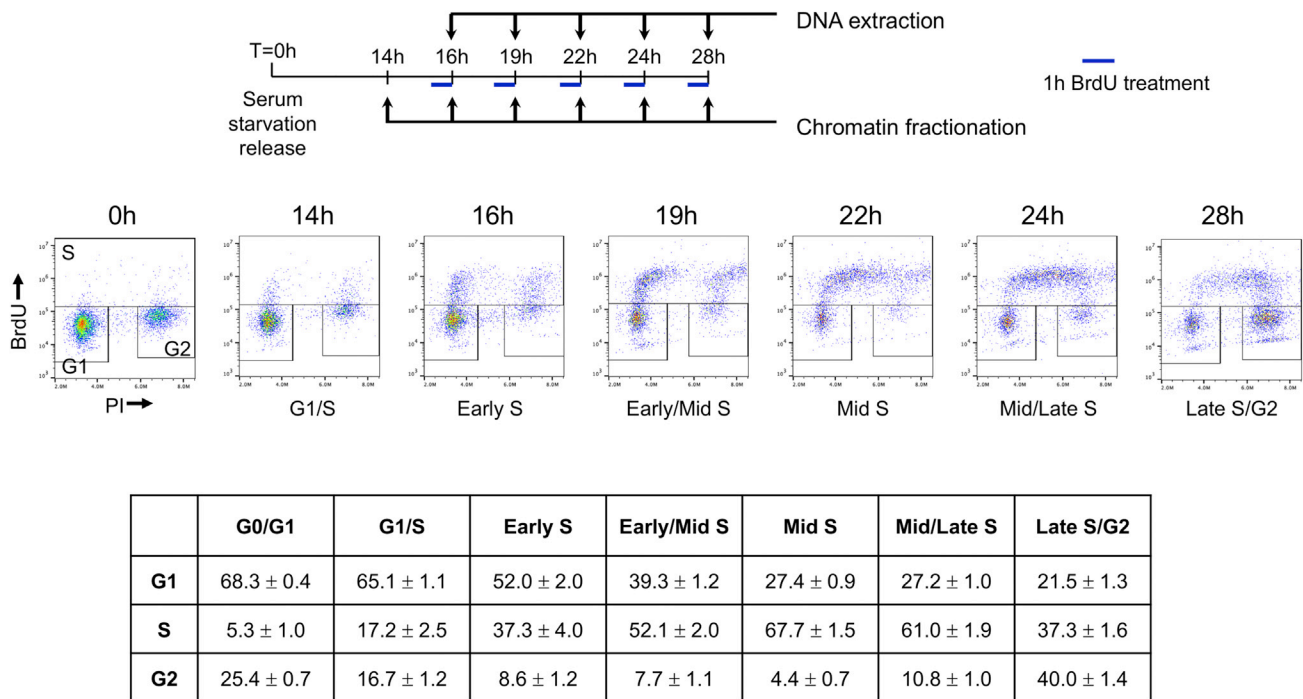


Figure 1. FACS analysis of S-phase progression following cells synchronization by serum starvation

The top panel showing the FACS results using propidium iodide (PI) and BrdU after serum starvation (0 h) (bottom left, G0/G1 phase; top, S phase; bottom right, G2 phase); the bottom panel showing the quantification of cells in each stage; n = 3; average mean ± standard error of the mean. (Adapted from Wang et al. 2021).

Cell serum starvation release and G2 arrest

⌚ Timing: 2 days

This step is used to release cells into S phase followed by a block of cycling cells into G2 phase using CDK1 Inhibitor Ro-3306.

4. Remove starvation media and add 20 mL of prewarmed culture media to 150 mm dish.

⚠ **CRITICAL:** The culture media should be warmed to 37°C before replacing the starvation medium to allow cells progression into S phase without stress (Figure 1).

5. 24 h after the release (corresponding to the time needed for the majority of the cells to reach mid-late S phase) add Ro-3306 at a final concentration of 9 μM into the 150 mm dish, and keep cells in the incubator for 16 h to arrest cells in G2.

EdU/BrdU incorporation and formaldehyde fixation of G2/M cells

This step is used to release cells into G2/M, label G2/M DNA synthesis sites with BrdU, and fix cells with formaldehyde.

⌚ Timing: 1.5 h

6. Remove the media containing Ro-3306 from 150 mm dish and wash cells 4 times with prewarmed PBS for 5 min.

7. Pulse cells with both 25 μM EdU (Sigma-Aldrich) and 50 μM BrdU (Sigma-Aldrich) in fresh pre-warmed media for 30 min in the incubator.
8. Quickly wash cells once with ice-cold PBS, and incubate the cells with 4 mL trypsin for 10 min in 5% CO_2 at 37°C followed by mixing with 10 mL ice-cold regular DMEM to inactivate trypsin.

△ CRITICAL: During trypsinization, ensure all cells are detached from 150 mm dish checked under microscope to increase cells recovery. Otherwise incubate cells for longer time with trypsin.

9. Transfer cells into a 15 mL centrifuge tube (Sarstedt) and pellet the cells by centrifugation at 900 $\times g$, 4°C for 3 min.
10. Wash cells once with 10 mL ice-cold PBS followed by centrifugation at 900 $\times g$ at 4°C for 3 min. After removing the PBS, resuspend the pellet in 1 mL ice-cold PBS and transfer into one 1.5 mL Low DNA Binding tube.
11. Pellet cells again by centrifugation at 900 $\times g$ at 4°C for 3 min followed by formaldehyde fixation and permeabilization for 20 min at 15°C–30°C by gently resuspending cell pellet in 1 mL freshly made PTEMF buffer.
12. Pellet fixed cells by centrifugation as in step 11 and discard the supernatant.
13. Wash the fixed cells twice by gently resuspending with 1 mL of ice-cold PBS and keep at 15°C–30°C for 5 min and pellet cells each time by centrifugation at 1000 $\times g$, 4°C for 3 min.
14. Store the cells in 1 mL ice-cold PBS at 4°C until use.

⏸ Pause point: Fixed cells in PBS can be kept at 4°C for several days before use.

Note: We recommend using a centrifuge with swinging-bucket rotors to pellet cells when necessary in this and the following sections to reduce cell loss.

△ CRITICAL: EdU is not used for the pull-down of DNA synthesis sites, but it is important later during FACS to identify the appropriate population of cells in G2/M performing DNA synthesis and to assess the frequency of G2/M cells performing DNA synthesis. Another positive control for FACS sorting is a population of asynchronous cells pulsed with 25 μM EdU for 30 min, fixed and processed as above, to determine the signal intensity of a large population of cells positive for EdU incorporation (S-phase cells). As a negative control for FACS sorting, an asynchronous population of untreated cells is fixed as above.

Cell sorting by flow cytometry

⌚ Timing: 1 day

This step is used to prepare G2/M phase cells for FACS.

15. Remove supernatant from fixed cells in step 13 after centrifugation at 1000 $\times g$, 4°C for 3 min, and resuspend the pellet with 600 μL 10% FBS/PBS by gently pipetting.
16. Incubate cells in 10% FBS/PBS for 1 h at 15°C–30°C (to reduce non-specific binding of antibodies at later stages of the protocol).
17. Remove the 10% FBS/PBS from cells by centrifugation at 1000 $\times g$ at 15–30°C for 3 min and resuspend cells with 600 μL 1:2000 diluted rabbit monoclonal anti-Phospho-Histone H3 (pS10-H3) (Cell Signaling Technology) in 1% FBS/PBS, incubating them for 1 h at 15°C–30°C.
18. Wash cells three times with 800 μL 1% FBS/PBS for 5 min and pellet cells each time by centrifugation at 1000 $\times g$ at 15°C–30°C for 3 min.
19. After the third wash, incubate the cells with 600 μL 1:1000 diluted Alexa Fluor488-conjugated goat anti-rabbit antibody (Thermo Fisher Scientific) in 1% FBS/PBS in the dark, for 1 h at 15°C–30°C.

20. Wash cells three times with 800 μ L 1% FBS/PBS for 5 min and pellet cells each time by centrifugation at 1000 \times g at 15°C–30°C for 3 min.
21. After the third wash, gently resuspend the cells in 800 μ L 1% FBS/PBS.
 - a. Aliquot 100 μ L cells to a new DNA Low Binding tube for EdU Click-iT reaction.
 - i. Prepare the Click-iT reaction buffer in the order listed in the table below.

Click-iT® reaction buffer (Thermo Fisher Scientific)	Final concentration	Amount
Click-iT® RNA reaction buffer (labeled Component C in the kit)	1 \times	214 μ L
CuSO ₄ (labeled Component D in the kit)	4 mM	10 μ L
Alexa Fluor® 594 azide	n/a	0.9 μ L
Click-iT® reaction buffer additive	1 \times	25 μ L
Total	n/a	250 μL

- ii. Remove the remaining 100 μ L 1% FBS/PBS from cells by centrifugation at 1000 \times g at 15°C–30°C for 3 min. Resuspend the pellet with 250 μ L reaction buffer by gently pipetting and incubate at 15°C–30°C in the dark for 30 min.
- iii. Quickly wash the cells with 1 mL Rinse buffer from the Click-iT Kit by centrifugation at 1000 \times g at 15°C–30°C for 3 min.
- iv. Remove most of Rinse buffer and gently resuspend the pellet in 500 μ L PBS containing 0.1 mg/mL RNase A and 0.5 μ L Vybrant DyeCycle Violet Stain (Thermo Fisher Scientific) and transfer the cells to one 5 mL FACS tube for cell cycle analysis (Sample E).
- b. Remove the remaining 700 μ L 1% FBS/PBS from cells by centrifugation at 1000 \times g at 15°C–30°C for 3 min. Gently resuspend the pellet in 700 μ L PBS containing 0.1 mg/mL RNase A and 0.7 μ L Vybrant DyeCycle Violet Stain (Thermo Fisher Scientific) and transfer the cells to one 5 mL FACS tube for sorting according to DNA content and positive pS10-H3 signal (Sample F) (Figure 2).

▮▮ Pause point: Immunostained cells in PBS should be stored in the dark at 4°C, for up to several days, before cell sorting.

▴ CRITICAL: For colors compensation a series of controls are required, with cells positive for one dye alone at a time, to determine the range of signal observed in positive and negative cells.

22. Cells were sorted using a BD FACSAria cell sorter machine (Beckton Dickinson). Samples A-D are used to determine the signal intensities of cells positive and negative for pS10-H3 and in G2/M by DNA content. Once these parameters are identified, a gating is set to specifically select cells positive for both pS10-H3 and G2/M DNA content, so that Sample F can be sorted. Sorting was performed at 15°C–30°C, and cells were collected in a 5 mL FACS tube containing 2 mL of 10% FBS/PBS, to help the collection and centrifugation of the cells later in the protocol (step 26).
23. Four samples prepared as control samples are acquired sequentially to determine the signal intensities for each signal and allow for color compensation:
 - a. Sample A: half of fixed asynchronous cells grown in one 150 mm dish with around 70% confluency treated with 0.1 mg/mL RNase A and used as a no dye control.
 - b. Sample B: half of fixed asynchronous cells grown in one 150 mm dish treated with 0.1 mg/mL RNase A and stained only with 5 μ M Vybrant DyeCycle Violet Stain (Thermo Fisher Scientific) to measure signal intensity of violet signal according to the DNA content.
 - c. Sample C: around half of fixed asynchronous cells grown in one 150 mm dish were clicked with Alexa Fluor 594 azide following the same steps in 21a except treating the cells only with 0.1 mg/mL RNase A in 500 μ L PBS in the last step. This sample is used to measure 594 intensity signals in cells that have incorporated EdU and perform DNA synthesis.

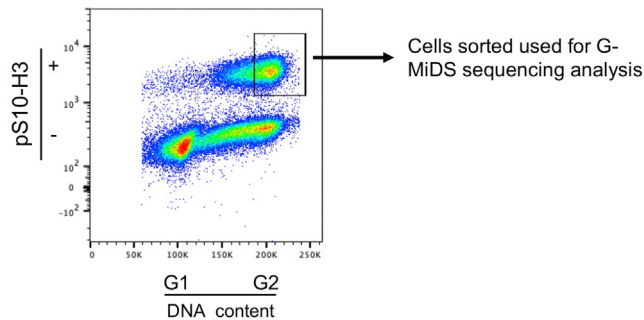


Figure 2. Gate set for G2/M cells sorting

Representative image of the mitotic cell sorting strategy by FACS. Cells that are both in G2 by DNA content and pS10-H3 positive (highlighted in the box) are sorted followed by BrdU sequencing. (Adapted from Wang et al. 2021).

- d. Sample D: a quarter of fixed synchronous cells grown in one 150 mm dish treated with 0.1 mg/mL RNase A and immunostained with pS10-H3 to determine pS10-H3 intensity signal in G2/M cells.
24. Following color compensation with the single control samples, Sample E is acquired. This will determine the frequency of cells in G2/M performing DNA synthesis, but will also ensure that the gating strategy to select pS10-H3 positive cells is including also cells actively performing DNA synthesis in G2/M (G-MiDS).
25. Finally, Sample F with cells labeled for pS10-H3 and DNA content is acquired; cells sorted are gated as from the acquisitions of Samples A-D, according to their positivity to pS10-H3 and G2 DNA content.

G2/M BrdU-seq

⌚ Timing: 4 days

This step is used to purify genomic DNA from G2/M cells collected after sorting (around 30,000 cells) and to prepare immunoprecipitated BrdU labeled G2/M DNA for sequencing.

26. DNA purification.
 - a. Pellet cells in 5 mL FACS tube by centrifugation at $2800 \times g$ at 15°C – 30°C for 10 min. Carefully remove most of the supernatant with around 1 mL remaining in the tube to avoid disrupting the pellets.
 - b. Gently resuspend the cell pellet and transfer to 1.5 mL Low DNA Binding tube by using 1 mL tip and pellet the cells by centrifugation at maximum speed at 15°C – 30°C for 3 min.
 - c. Carefully remove most of the supernatant with around 30 μL supernatant left in the tube to avoid disrupting the pellets.
 - d. Add 200 μL TE to the 1.5 mL Low DNA Binding tube containing the cells.
 - e. Sonicate the cells using VCX130 (Sonics & Materials, Inc) with the following settings: 30% Amp, 5 cycles of 15 s on and 10 s off, for a total pulsing on time of 1 min 15 s.
 - f. Spin cells for 10 s at the maximum speed of the centrifuge at 15°C – 30°C in the 1.5 mL Low DNA Binding tube and put at 65°C for 16–20 h to reverse-crosslink.
 - g. Spin cells for 10 s at the maximum speed of the centrifuge at 15°C – 30°C , add 0.7 mg/mL proteinase K and incubate at 55°C for 3 h.
 - h. Purify the DNA by using DNA Clean & Concentrator-5 and elute twice by adding 10 μL EB (10 mM Tris-HCl pH 8.5) each time.
 - i. Quantify the purified dsDNA DNA using 2 μL DNA in Qubit 3.0 Fluorometer (Thermo Fisher Scientific).

Alternatives: We used the Qubit 3.0 Fluorometer for the DNA quantification but in alternative any other Qubit Fluorometer (Qubit 1.0, Qubit 2.0, and Qubit 4 fluorometers), Bioanalyzer (Agilent Technologies) and TapeStation (Agilent Technologies) instrument can be used.

Pause point: Purified DNA can be kept at -20°C until further use.

Note: Due to the low number of sorted and collected cells, it is hard to visualize them by eye after centrifugation. We recommend leaving some liquid in the tube when discarding the supernatant to reduce loss of cells.

27. BrdU-pull down.

- a. Aliquot 10 μL Dynabeads Protein A (Thermo Fisher Scientific) into a 1.5 mL Low DNA Binding tube.
- b. Wash beads twice with 1 mL of 0.5% BSA/PBS/0.1% Tween 20, remove the washed solution by spinning the tube at $400 \times g$ for 1 min at 15°C – 30°C followed by placing it on the Magnet (Thermo Fisher Scientific) until the solution is clear (~ 1 min).
- c. Mix beads with 200 μL 1:150 diluted anti-BrdU antibody (Sigma-Aldrich) in 0.5% BSA/PBS/0.1% Tween 20 and incubate at 4°C for 16–20 h with rotation.

Note: Prepare beads on the same day as DNA sonication to save time.

- d. Wash beads twice as in step b. After washes, add 10 μL 0.5% BSA/PBS/0.1% Tween 20 to the beads and keep at 4°C until use.
- e. Dilute the purified DNA to 200 μL final volume with TE and denature at 100°C for 10 min. Immediately put the DNA on ice for 5 min. After a quick spin, aliquot 10 μL into a new 1.5 mL Low DNA Binding tube as Input for DNA library construction and store at -20°C until use.
- f. Mix the beads with the remaining 190 μL denatured DNA and 200 μL 2 \times blocking solution (2% BSA, 2 \times PBS, 0.2% Tween 20) in DNA low binding tubes, and incubate at 4°C for 16–20 h with rotation.
- g. After immunoprecipitation, wash BrdU-labeled DNA bound to beads twice with 1 mL of lysis buffer 1 (50 mM HEPES-KOH pH 7.5, 140 mM NaCl, 1 mM EDTA, 1% Triton X-100, 0.1% Sodium Deoxycholate), twice with 1 mL of lysis buffer 2 (50 mM HEPES-KOH pH 7.5, 500 mM NaCl, 1 mM EDTA, 1% Triton X-100, 0.1% Sodium Deoxycholate), twice with 1 mL of wash buffer (10 mM Tris-HCl pH 8.0, 250 mM LiCl, 1 mM EDTA, 0.5% Sodium Deoxycholate, 0.5% Igepal CA-630).
- h. Resuspend the beads in 1 mL of TE and centrifuge for 3 min at $900 \times g$ at 4°C . Remove the supernatant completely before resuspending the beads in 50 μL elution buffer and incubating at 65°C for 10 min.

Note: To remove completely the last wash, use a 1 mL tip to first remove most of the TE without disturbing the beads, then quick spin the beads and remove the remaining liquid using a 10 μL tip.

- i. Centrifuge at $2400 \times g$ at 15°C – 30°C for 1 min and leave the tubes on Magnet for 1 min. Then transfer the eluted supernatant into a new DNA low binding tube.
- j. Mix the eluted DNA (IP DNA) with 50 μL TE and 2.5 μL Proteinase K (20 mg/mL, Promega) and incubate at 37°C for 1 h. Meanwhile, thaw the Input DNA (10 μL) that had been stored at -20°C the previous day and mix with 40 μL TE.
- k. Purify both the single-stranded IP and Input DNA by DNA Clean & Concentrator-5 (Zymo Research) and elute twice with 7 μL nuclease-free water each time.

Pause point: Purified DNA can be kept at -80°C until further use.

- l. Following purification, convert both the single-stranded BrdU-labeled DNA and the single-stranded Input DNA to double-stranded DNA by Random Primed DNA Labeling Kit (Roche).

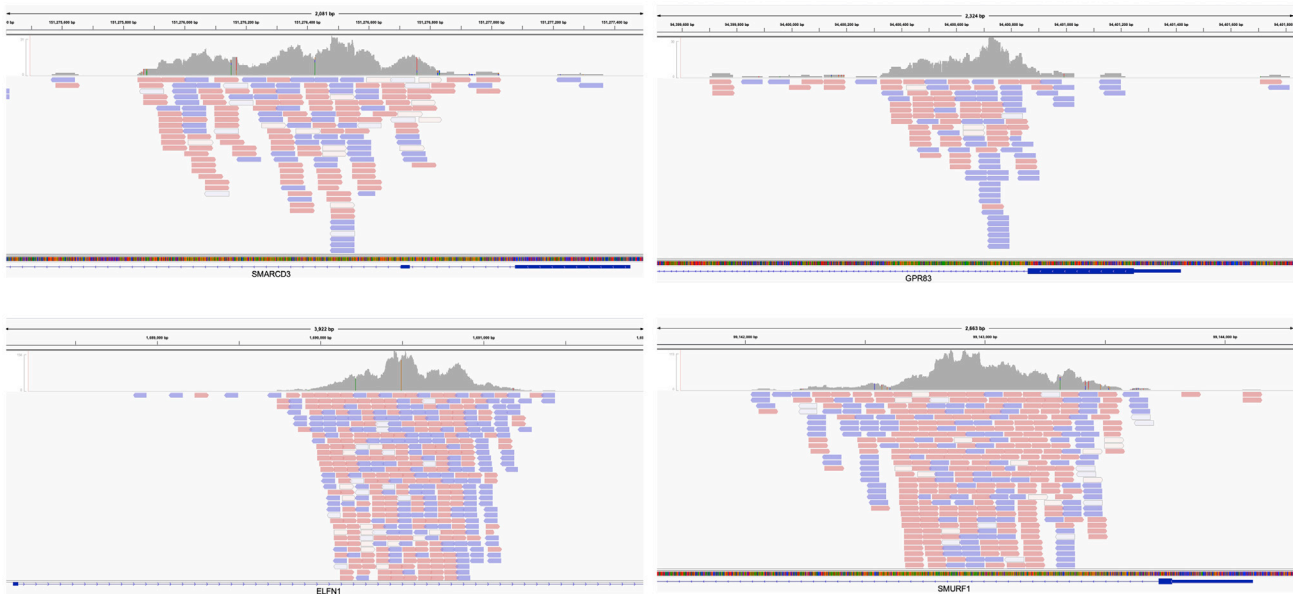


Figure 3. Snapshots with IGV of the G-MiDS sequencing files over the indicated G-MiDS hotspot sites

m. Purify the double-stranded DNA by DNA Clean & Concentrator-5 and elute once with 7 μ l EB (10 mM Tris-HCl pH 8.5).

▣▣ **Pause point:** Purified DNA can be kept at -20°C until further use.

- n. Quantify DNA with Qubit dsDNA HS Assay Kit (Thermo Fisher Scientific) using Qubit 3.0 Fluorometer (Thermo Fisher Scientific) (or alternatives as indicated in step 16.).
- o. Take 250 pg (or as much as available) of random-primed BrdU-DNA to prepare Illumina sequencing libraries according to the QIAseq Ultralow Input Library Kit protocol (QIAGEN).
- p. After being cleaned up, indexed libraries were quantified, normalized and pooled, sequenced on the Illumina NextSeq 500 system with a High Output Kit v2.5 (75 cycles) in a single lane on a single-end run (Figure 3).

EXPECTED OUTCOMES

The quantified yield of purified DNA from sorted and sonicated G2/M cells is around 500 ng. After BrdU pull down, and conversion to double-stranded BrdU-labeled DNA, the quantified yield of purified DNA is approximately 0.4 ng.

QUANTIFICATION AND STATISTICAL ANALYSIS

We recommend performing at least 2 biological replicates of this experiment in each specific condition, therefore at least 2 separate cell cultures grown and synchronized as above before sorting. We performed this experiment in control cells as well as cells knockdown for the promoter-proximal pausing regulating factor NELFA, and these samples were all sorted and processed together. Single-end BrdU-Seq data from G2/M DNA synthesis samples were aligned to the hg38 genome using Bowtie 2 v.2.3.4.2 on the online platform Galaxy (<https://usegalaxy.org>; Afgan et al., 2018). G2/M DNA synthesis (G-MiDS) peaks were called against the Input DNA using MACS2 v.2.1.1 with human genome size and the following parameters: (-m 8 30, -p 0.00001) on the online platform Galaxy (<https://usegalaxy.org>; Afgan et al., 2018). The bedtools intersect intervals function on the online platform Galaxy was used to identify the G-MiDS specific peaks against the BrdU peaks called in all the S-phase time points combined together.

LIMITATIONS

We used serum starvation to synchronize BJ-hTERT in G0/G1 phase. However, this synchronization strategy cannot be applied to all human cell lines, as not all cell lines are equally sensitive to serum starvation and/or will not resume cell cycle progression in a synchronous manner once released in complete medium.

TROUBLESHOOTING

Problem 1 (step 1)

Using a different cell line than BJ-hTERT.

Potential solution

We have performed sorting of G2/M cells following the above indicated conditions in BJ-hTERT and U2OS cell lines, followed by BrdU-Seq. With the BJ-hTERT we performed the sorting of G2/M cells both with and without serum starvation and synchronization in G2 with Ro-3306; for the U2OS we sorted G2/M cells out of an asynchronous population. In all cases, the sites of G2/M DNA synthesis were very consistent and conserved between the two cell lines. We expect therefore that the sorting and sequencing of BrdU labeled DNA as described above should be generally applicable to different cell lines. When applying the whole synchronization procedure from serum starvation to G2 arrest, several considerations are important, as doubling time varies between cell lines.

- For faster growing cells we would recommend seeding at lower confluency (less than 20%), and prolong the starvation time up to 72 h. This will allow a high level of G0/G1 synchronization but will also provide cells with space to grow once undergoing into S phase, as a dense population will contact inhibit re-entering the cell cycle. Efficiency of G0/G1 synchronization and S-phase progression need to be monitored by FACS.
- In addition, different cell lines may differ in the time needed to reach G1/S transition following serum starvation release or to reach the mid-late S phase timepoint, which is when we add Ro-3306 to the cell culture. Also in this case, it is important to monitor by FACS cell cycle progression of the released cells to determine when the majority of the population is reaching the end of S phase, in order to add Ro-3306 at that time point without risking that CDK1 inhibition might affect DNA replication.

Problem 2 (step 3)

Following serum starvation, low percentage of cells is in G0/G1 (less than 65%).

Potential solution

Increase the starvation time and/or decrease the amount of FBS in the starvation medium. Alternatively, completely remove the FBS from the starvation medium.

Problem 3 (step 5)

High percentage of cells (around 90%) synchronized in G0/G1 at the end of serum starvation, but there is a large number of cells (more than 30%) that remain in G0/G1 and will not resume cell cycle progression even when most of cells reach late S phase once released in complete medium.

Potential solution

Cell density might be too high in the dish. Therefore, some cells can be contact inhibited and not progress into S phase. A solution would be seeding a lower number of cells in the dish at the beginning. Another possibility is that the starvation time is too long and more cells will remain in G0. A solution is to reduce the starvation time.

Problem 4 (step 25)

Low number of cells is obtained after cell preparation before sorting.

Potential solution

Many cells get lost during the procedure. It helps to reduce cell losses using a centrifuge with swing-bucket rotors, low binding tubes, 1% BSA/PBS precoated 15 mL centrifuge tubes (Sarstedt), 1% BSA/PBS, gently pipetting cell pellets around 4 times when necessary during cell washing procedures, as well as determining the most appropriate Triton X-100 concentration in the PTEMF buffer according to your specific cell line (0.02%–0.2%).

Problem 5 (step 27)

The yields or concentration of the purified random-primed BrdU-DNA are too low to construct DNA sequencing library.

Potential solution

- Scale up the initial amounts of cells for serum starvation.
- Elute the purified random-primed BrdU-DNA in lower volume of EB (10 mM Tris-HCl pH 8.5) using DNA Clean & Concentrator-5.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Marco Saponaro (m.saponaro@bham.ac.uk).

Materials availability

This study did not generate new unique materials.

Data and code availability

The accession number for the G2/M BrdU-seq data files reported in this paper is GEO: GSE136294.

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AUTHOR CONTRIBUTIONS

J.W. and M.S. designed the study and wrote the manuscript.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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