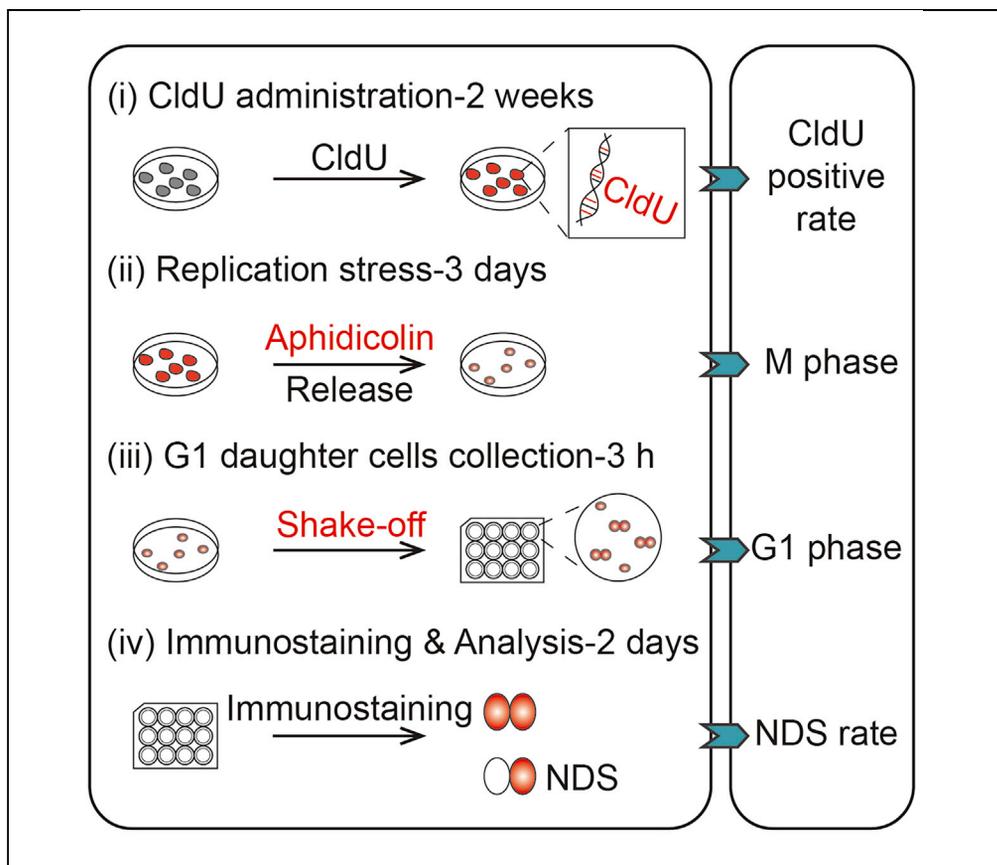


## Protocol

# Nonrandom DNA Segregation Detection under Replication Stress



Nonrandom DNA segregation (NDS) is a mitotic event in which sister chromatids carrying the old (parent) DNA strands are distributed exclusively to one of the two daughter cells. Although this phenomenon occurs in multiple organisms, the low frequency poses an obstacle to observation. Here, we present an improved protocol to induce NDS under replication stress. This protocol can be modified to accommodate various cell lines.

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**HIGHLIGHTS**  
Nonrandom DNA  
segregation is of  
significant relevance  
but difficult to  
observe

Efficient induction of  
nonrandom DNA  
segregation by  
replication stress

A step-by-step  
protocol for  
nonrandom DNA  
segregation  
detection by  
immunofluorescence

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## Protocol

## Nonrandom DNA Segregation Detection under Replication Stress

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## SUMMARY

**Nonrandom DNA segregation (NDS) is a mitotic event in which sister chromatids carrying the old (parent) DNA strands are distributed exclusively to one of the two daughter cells. Although this phenomenon occurs in multiple organisms, the low frequency poses an obstacle to observation. Here, we present an improved protocol to induce NDS under replication stress. This protocol can be modified to accommodate various cell lines.**

**For complete details on the use and execution of this protocol, please refer to Xing et al. (2020).**

## BEFORE YOU BEGIN

⌚ Timing: 1–2 days

1. Grow cells at 30% confluence in a 10 cm culture dish and make sure they are in good condition.
2. Prepare the required reagents and make certain that they are sufficient to carry out the whole experiment.
  - a. Prepare stock solutions of 1 mM CldU in DMEM, 200 mM thymidine in DMEM, 4 mM aphidicolin in DMSO and 10 mM RO3306 in DMSO. These solutions can be stored at  $-20^{\circ}\text{C}$  for up to several months.
  - b. Prepare 1  $\mu\text{g}/\text{mL}$  DAPI by diluting the stock solution in PBS (1:1000). Prepare 10% (vol/vol) Triton X-100 in distilled water. These solutions can be stored for up to several months at  $4^{\circ}\text{C}$ , protected from light.

## KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Anti-BrdU BU1/75	Abcam	Cat#ab6326
Alexa Fluor 594 goat anti-Rat	Abcam	Cat#ab150160

(Continued on next page)



*Continued*

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Chemicals, Peptides, and Recombinant Proteins		
RO3306	Selleck	Cat#S7747
Aphidicolin	Sigma	Cat#38966-21-1
Thymidine	Sigma	Cat#T1895
Triton X-100	Sangon Biotech	Cat#9002-93-1
Bovine Serum Albumin (BSA)	Sangon Biotech	Cat#9048-46-8
DAPI	Thermo Scientific	Cat#62248
CldU	Sigma	Cat#C6891
Penicillin-Streptomycin	Gibco	Cat#15140122
DMEM/HIGH GLUCOSE	HyClone	Cat#SH30243.01
HCl	Sinopharm Chemical Reagent Co., Ltd	Cat#7647-01-0
DMSO	Sigma	Cat#472301
CELLSAVING	New Cell & Molecular Biotech Co., Ltd	Cat#C40100
PBS	Solarbio	Cat#P1020-500
Experimental Models: Cell Lines		
U2OS	ATCC	ATCC ® HTB-96™
Software and Algorithms		
ImageJ	ImageJ	<a href="https://imagej.nih.gov/ij/download.html">https://imagej.nih.gov/ij/download.html</a>
Other		
Poly-lysine-coated glass coverslip	WHB SCIENTIFIC	Cat#WHB-12-CS
Fluoromount-G Mounting Medium	SouthernBiotech	Cat#0100-01
10 cm cell culture dish	Hangzhou Xinyou Biotechnology Co., Ltd	Cat#010100
Syringe	Chengdu Xinjin Shifeng Medical Apparatus & Instrument Co., Ltd	Cat#0205
Tweezer	Sinopharm Chemical Reagent Co., Ltd	Cat#92361134
Nikon Eclipse Ti microscope	Nikon	N/A
OLYMPUS FV31S-SW	OLMYPUS	N/A

## MATERIALS AND EQUIPMENT

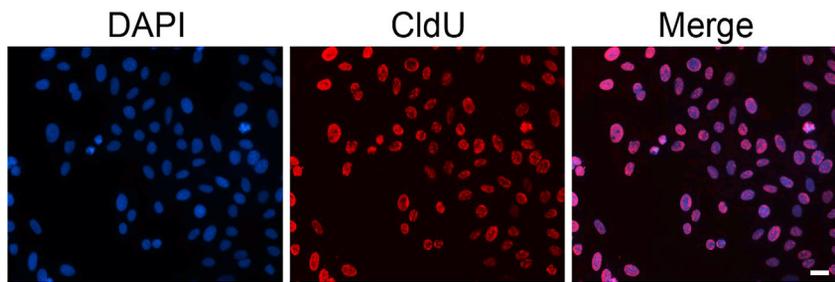
**Alternatives:** Aphidicolin can also be obtained from Abcam (ab142400); Triton X-100 can also be obtained from Sigma (T8787); BSA can also be obtained from Sigma (A1933); DAPI can also be obtained from Abcam (ab228549).

## STEP-BY-STEP METHOD DETAILS

### CldU Administration

⌚ Timing: 2 weeks

Cells are treated with CldU (1  $\mu$ M) for two consecutive weeks to ensure all the parent DNA strands are labeled (all steps in the below protocol are performed with U2OS cells. Adjustments can be made for other cell lines).



**Figure 1. Immunofluorescence Images Showing All Cells Are CldU Positive after Being Treated with CldU for 2 Weeks**

Scale bars, 20  $\mu\text{m}$ .

1. To label the DNA strands, add CldU to the culture media at a final concentration of 1  $\mu\text{M}$  for 2 weeks (Pine et al., 2010). During this period, cells are maintained in the exponential growth phase by passaging every 72 h. CldU-containing media is freshly prepared by diluting the stock at 1:1000 before passaging.
2. Two weeks later, seed  $1\text{--}2 \times 10^5$  cells on a 20 mm poly-lysine-coated glass coverslip in a 12 well plate for immunofluorescence, to verify that all cells are CldU positive (refer to steps 10–20, Figure 1).

**Note:** As the cell cycle length and CldU labeling efficiency vary among different cell lines, the labeling period should be modified accordingly. As for U2OS, the preliminary experiment showed that two-week CldU administration can achieve the highest labeling efficiency without causing significant cytotoxicity.

**△ CRITICAL:** It is important to make sure that all the chromosomes are labeled with CldU, so that the old parental DNA could be detected as CldU-positive DNA strands.

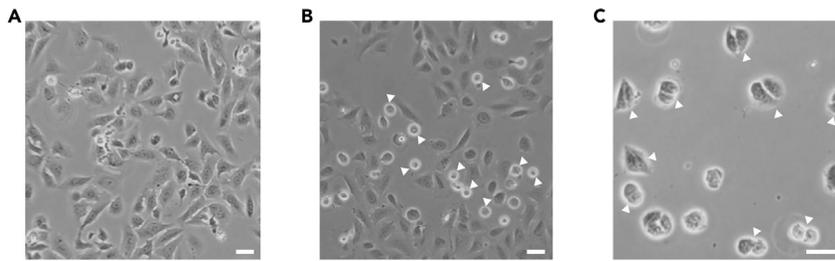
**▣ Pause Point:** The CldU-labeled cells can be cryopreserved for a couple of months at  $-80^\circ\text{C}$  using a standard protocol for cell cryopreservation with CELLSAVING.

### Cell Synchronization, Replication Stress Induction, and G1 Daughter Cells Collection

⌚ Timing: 3 days

After being cultured in the absence of CldU for one cell cycle (around 24 h), cells are treated with DNA polymerase inhibitor aphidicolin (0.3  $\mu\text{M}$ ) and CDK1 inhibitor RO3306 (9  $\mu\text{M}$ ) to induce replication stress (Okamoto et al., 2018, Minocherhomji et al., 2015) and G2/M arrest, respectively. Replication stress induction is the key step to increase the frequency of NDS (Xing et al., 2020).

3. Reseed the cells at 30%–50% confluence in CldU free media in a 10 cm dish for one cell cycle (about 24 h for U2OS cells), after which the nascent DNA strands are CldU negative but the old DNA strands are still CldU positive as the result of semi-conservative DNA replication.
4. In order to enrich mitotic cells in the next cell cycle, two enrichment steps are performed as followed. First, add thymidine directly to the culture media at a final concentration of 2 mM for 18 h (Chen and Deng, 2018) to synchronize cells in the S phase.
5. Second, wash the cells with PBS for two times and reseed into a new 10 cm dish at 50% confluence in culture media supplemented with 9  $\mu\text{M}$  RO3306 and 0.3  $\mu\text{M}$  aphidicolin. 16 h later, most of cells are at late S phase to G2/M boundary (Figure 2A).



**Figure 2. Collection of G1 Daughter Cells**

(A) The cellular morphology after being treated with APH and RO3306 for 16 h. Scale bars, 20  $\mu\text{m}$ .

(B) The cellular morphology after releasing for 30 min. The white arrows indicate mitotic cells. Scale bars, 20  $\mu\text{m}$ .

(C) The G1 daughter cells after reseeding for further 140 min. The white arrows indicate daughter cells. Scale bars, 20  $\mu\text{m}$ .

- Wash the cells with prewarmed PBS for three times within 5 min and add 6 mL of prewarmed culture media to the plate. Put the culture plate back into the 37°C incubator for another 30 min to release the cells into mitosis.

**△ CRITICAL:** To enrich mitotic cells, make sure PBS and culture media are preheated at 37°C and the wash procedure is finished within 5 min.

- 30 min after releasing the cells, many orbicular and bright mitotic cells can be seen under the microscope (Figure 2B). Remove 3 mL culture media from the culture dish and shake off the loosely attached mitotic cells by gently tapping the side of the plate (Macheret and Halazonetis, 2019). Rotate the plate between each tap to allow even removal of mitotic cells. Rinse the plate with the remaining 3 mL media and collect the mitotic cells in a 15 mL tube at 25°C–37°C.
- To ensure complete removal of mitotic cells, repeat the shake-off process for multiple rounds (1–3 times recommended), aspirating and adding 3 mL of fresh media between each round.

**Note:** When collecting the mitotic cells, gently aspirate and collect any liquid that is adhering to the lid or the edge of the plate to achieve better recovery.

**Note:** Verify under the microscope that few mitotic cells have remained on the culture plate after the collecting procedure.

- Centrifugate at 300  $\times$  g for 5 min and resuspend the collected mitotic cells in 3 mL of fresh media. Seed the cells into a 12 well culture plate with an appropriate number of coverslips and incubate them at the 37°C incubator for at least 140 min to allow the cells to progress into G1 phase (Figure 2C).

**Note:** If only a single shake-off is performed in step 8, the mitotic cells can be directly seeded into the 12 well plate without centrifugation.

**Note:** The number of coverslips can be decided according to the number of collected mitotic cells. Based on the preliminary experiment, 2–4  $\times$  10<sup>4</sup> mitotic cells can be recovered from a 10 cm dish and are suitable for 2 to 4 wells with coverslips.

**Note:** As the cell cycle varies among different cells, the release time should be optimized for the cell line used. We recommend a combination of microscopic examination of the cellular morphology and cell cycle analysis by flow cytometry to determine the optimal time for harvesting the daughter cells. Under bright field microscopy, the morphology of the reseeded mitotic cells can be examined at different time points. The time when most of the cells divide

into two neighboring cells separated by a clear cleavage furrow will be appropriate for collecting G1 daughter cells. With flow cytometry-based cycle analysis, the appropriate release time can be determined as when the DNA content of most cells decrease from 4 N to 2 N.

10. Wash cells with PBS once and fix them with ice-cold 70% ethanol at  $-20^{\circ}\text{C}$  for at least 2 h.

▮▮ **Pause Point:** Fixed cells can be stored at  $-20^{\circ}\text{C}$  for no more than 2 weeks.

### Immunostaining and Fluorescence Microscopy

⌚ **Timing:** 1–2 days

Immunostaining and nonrandom DNA segregation detection.

11. To denature the DNA, cells are incubated with 2 N HCl containing 0.5% Triton X-100 (500  $\mu\text{L}$  each well) at  $4^{\circ}\text{C}$  for 1 h (Pine et al., 2010).

⚠ **CRITICAL:** 2 N HCl treatment is indispensable because CldU can only be stained when the DNA is fully denatured.

12. After two washes with 0.5% Triton X-100/0.5% BSA in PBS and one wash in 0.5% Triton X-100/0.1% BSA in PBS, cells are blocked using 5% BSA/0.5% Triton X-100 in PBS (500  $\mu\text{L}$  each well) at  $4^{\circ}\text{C}$  for 1 h.
13. Prepare 3% BSA as the antibody dilution buffer: To prepare 10 mL, add 300 mg BSA to 10 mL PBS and mix well.
14. Incubate cells with anti-BrdU BU1/75 (1:1000 dilution in 3% BSA, 400  $\mu\text{L}$  each well) at  $4^{\circ}\text{C}$  for at least 8 h (Sarek et al., 2019).

*Optional:* Primary antibody (BU1/75) incubation time can be shortened to 1–2 h at  $25^{\circ}\text{C}$ .

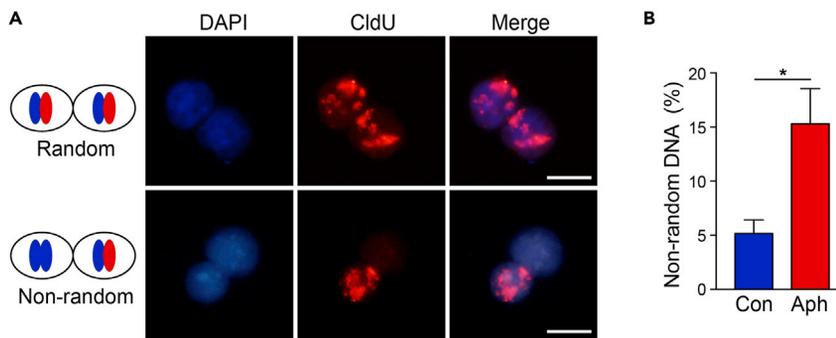
15. Wash the cells with PBS for three times and incubate with anti-rat Alexa Fluor 594 (1:2000 dilution in 3% BSA, 400  $\mu\text{L}$  each well) for 1 h at  $25^{\circ}\text{C}$ , protected from light.

⚠ **CRITICAL:** Wash cells for at least three times to reduce nonspecific binding.

16. Wash cells with PBS for three times and incubate with 1  $\mu\text{g}/\text{mL}$  DAPI (400  $\mu\text{L}$  each well) at  $4^{\circ}\text{C}$  for 10 min, protected from light.
17. Wash cells with PBS once.
18. Pipette 20–30  $\mu\text{L}$  of Fluoromount-G mounting media on each clean glass slide.
19. Take out the coverslips from the 12 well culture plate carefully using a syringe with curved needle and a tweezer. Mount the coverslips with the cells facing down on the glass slides. Seal the coverslips with nail polish.
20. Images are captured using the Nikon Eclipse Ti microscope. First, use DAPI channel (Ex 359 nm) to locate suspicious daughter cells whose nuclei are close to each other. Then use DIC channel to validate they are bona fide daughter cells separated by a cleavage furrow, rather than merely two adjacent cells. Finally, images are captured with DAPI channel (Ex 359 nm) and TRITC channel (Ex 550 nm) using OLYMPUS FV31S SW.

**Note:** Keep the exposure time same for each channel.

21. The chromatin segregation pattern is determined by ImageJ.
  - a. Open a stored image of TRITC channel and convert the image to 8-bit of gray.



**Figure 3. Schematic Representation of Different DNA Segregation Patterns and Quantification Result**

(A) Representative images of daughter cells that divide their CldU-labeled template DNA (red) randomly to both daughter cells (upper) and exclusively to one daughter cell (lower). Scale bars, 10  $\mu$ m.

(B) Quantification of the frequency of NDS without (n = 456) or with (n = 397) APH treatment. Data are the mean  $\pm$  SEM, \*p < 0.05 calculated by t test.

- Adjust the threshold using the slider. Choose the parameters of the Integrated density (IntDen) and shape descriptors in the “set measurement.”
- Select one of the daughter cells by the area selection tools and obtain its IntDen value by clicking “measure.”
- Calculate the relative fluorescence as  $\log_2$  (IntDen of one daughter cell / IntDen of another daughter cell). Daughter cells with a relative fluorescence above 8 are defined as NDS.

**Note:** According to the preliminary results, there were two distinct groups of data well separated at the cutoff of 8. So, we defined those cell pairs with a CldU ratio above 8 as NDS.

**Optional:** BF (Bright field) and PH (Phase contrast) are alternatives to DIC.

**Optional:** Other fluorescent microscopes are optional.

## EXPECTED OUTCOMES

Images for the expected outcomes of the protocol are shown in [Figure 3](#).

Under normal culture condition, NDS is detected in a small proportion of daughter cell pairs in U2OS (5%–8%). However, aphidicolin-induced replication stress elevates the frequency up to 15%–20% ([Figure 3B](#)). Besides, this protocol can be combined with other downstream applications. One application is immunofluorescence of DNA damage response proteins. Our previous study illustrated that those daughter cells exhibiting DNA damage response markers, such as the single-stranded DNA-binding protein replication protein A (RPA) and the FANCD2 protein, were generally CldU negative during NDS ([Xing et al., 2020](#)). Second, genomic sequencing following cell sorting based on FACS enables mutation analyses such as copy number variants (CNV) and loss of heterozygosity (LOH) to help to uncover the biological outcome and significance of NDS. And other applications such as fluorescence *in situ* hybridization (FISH) can be sequentially conducted to see the specific genomic elements that may play a role in NDS.

## LIMITATIONS

Highlighted below are some potential limitations to consider during experimental design and data analysis.

Considering that the old DNA strand and the new one need to be distinguished, it is essential to make sure all the old DNA strands are labeled with CldU, so the concentration and duration time

of CldU administration need to be optimized accordingly. Either too high or too low concentration may cause labeling failure. Too high concentration may give rise to poor cell state, especially for primary cells. Too low concentration or too short labeling duration may not be able to label the old DNA strands efficiently.

Mitotic cells can be easily collected by manual shake-off because they have much poorer adhesion than interphase cells. Therefore, this protocol is not available for suspension cells. Alternatively, there are some other ways to obtain mitotic cells, such as colcemid or nocodazole treatment (step 5). However, compared to the RO3306, these agents arrest cells in prometaphase to metaphase instead of G2/M boundary, which precludes the time window for intervening with the very early mitotic events upstream of NDS in a temporally controlled manner.

Finally, data collection and analysis are relatively time-consuming and labor intensive.

## TROUBLESHOOTING

### Problem 1

The proportion of CldU-positive cells is too low in step 2.

### Potential Solution

This may happen for several reasons: 1) poor cell status; 2) low CldU concentration; 3) CldU labeling time is too short. To avoid this situation, please make sure cells are in good condition and CldU is added at right concentration for adequate time.

### Problem 2

No CldU-positive cells at all.

### Potential Solution

2 N HCl is crucial for CldU immunostaining. The double-stranded DNA may not denature completely if the HCl concentration is too low, so make sure that HCl is stored well considering the evaporation and prepare fresh working solution for each use.

### Problem 3

The number of daughter cells is too few (step 9).

### Potential Solution

Most cells may have not progressed into the G1 phase if the releasing time is too short in step 9. To overcome this problem, we recommend optimizing the incubation time according to the length of mitosis. Taking U2OS for instance, its mitotic duration is around 40 min to 60 min.

Besides, either cold PBS or inadequate wash time in step 6 may also lead to the failure of entering mitosis.

## RESOURCE AVAILABILITY

### Lead Contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, Songmin Ying ([yings@zju.edu.cn](mailto:yings@zju.edu.cn)).

### Materials Availability

This study did not generate new unique reagents.

### Data and Code Availability

This study did not generate any unique datasets or code.

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## AUTHOR CONTRIBUTIONS

Methodology, M.X.; Validation, S.C., F.J., and G.C.; Writing – Original Draft, S.C. and F.J.; Writing – Review & Editing, S.C., F.J., J.L., X.G., C.Z., and M.Z.S.; Supervision, S.Y.

## DECLARATION OF INTERESTS

The authors declare no competing interests.

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