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## Supplementary information

# Genome-wide and sister chromatid-resolved profiling of protein occupancy in replicated chromatin with ChOR-seq and SCAR-seq

In the format provided by the authors and unedited

# Supplementary Methods – ChOR-seq on HeLa S3 cells (suspension culture)

# MATERIALS

## Biological Material

- Human HeLa S3 cells (ATCC Cat. No. CCL-2-2; RRID: CVCL_0058) were adapted to grow in suspension.
- *D. melanogaster* S2-DRSC (Drosophila Genomics Resource, Center; Stock No. 181, RRID: CVCL_Z992)

**! CAUTION** The cell lines used in your research should be checked regularly to ensure they are authentic and mycoplasma-free.

## Reagents

### *Cell culture reagents for HeLa S3*

- DMEM (Thermo Fisher Scientific, Cat No. 11965092)
- FBS (Invitrogen, Cat No. 26400-036)
- Penicillin-Streptomycin (10,000 U/mL) (Gibco, Cat.No. 15140-122) **! CAUTION**. Irritant if contact with skin. Always wear gloves and a lab coat.

### *Cell culture reagents for D. melanogaster S2-DRSC cells*

- Bactopeptone (Thermo Fisher Scientific, Cat. No. 211677)
- FBS (Invitrogen, Cat No. 26400-036)
- Potassium bicarbonate ($KHCO_3$) (Sigma-Aldrich, Cat. No. 60339)
- Penicillin-Streptomycin (10,000 U/mL) (Gibco, Cat. No. 15140-122) **! CAUTION**. Irritant if in contact with skin. Always wear gloves and a lab coat.
- Shields and Sang M3 insect medium (Sigma-Aldrich, Cat. No. S8398) **! CAUTION.** Causes serious eye irritation. Wear appropriate gloves and a lab coat when handling.
- Yeast extract (Sigma-Aldrich, Cat. No. Y-1000)

***Common reagents***

- 10x PBS (pH 7.4; Thermo Fisher Scientific Cat. No. 70011044)
- 16% formaldehyde (wt/vol), methanol-free (Thermo Fisher Scientific, Cat. No. 28908) **!CAUTION** Formaldehyde is toxic if inhaled, ingested, or absorbed through the skin.; always wear a lab coat and gloves, and use it in a fume hood.
- 2´- Deoxycytidine (Sigma-Aldrich, Cat. No. D0776-1G)
- 5-Ethynyl-deoxy-uridine (5-EdU) 25 mg (Jena Bioscience, Cat. No. CLK-N001-25)
- Agencourt AMPure XP (Beckman Coulter, Cat. No. A63881)
- Aminoguanidine hydrochloride (Sigma-Aldrich, Cat. No. 396494) *(optional)*

  **! CAUTION** Skin and eye irritant. Wear gloves when handling.
- Aprotinin (Sigma-Aldrich, Cat. No. 10981532001) **! CAUTION** May cause an allergic skin reaction or breathing difficulties if inhaled. Wear gloves and a lab coat and work in a fume hood.
- Biotin-TEG-azide (Berry & Associates, Cat. BT1085)
- Bovine serum albumin (Sigma-Aldrich, Cat. No. A4503)
- Click-iT™ EdU Alexa Fluor™ 647 Flow Cytometry Assay Kit (Thermo Fisher Scientific, Cat. No. C10420)
- $CuSO_4$ (Jena Bioscience, Cat. No. CLK-MI004-50)
- Dynabeads MyOne Streptavidin T1 (Thermo Fisher Scientific Cat. No. 65602)
- EDTA Ultrapure (0.5 M, pH 8.0; Life Technologies, Cat. No. 15575-038) **! CAUTION** Toxic if swallowed. Wear gloves when handling.
- EGTA (0.5 M, sterile solution, pH 8.0; bioWorld, Cat. No. 40520008-2) **! CAUTION**. Toxic if swallowed or inhaled. Wear gloves when handling.
- Ethanol (Sigma-Aldrich, Cat. No. 51976) **! CAUTION** Ethanol is flammable. Keep away from open flames.
- Glycerol (for molecular biology, >99% (vol/vol); Sigma-Aldrich, Cat. No. 56-81-5)
- Glycine molecular biology grade (Applichem, Cat. No. A1067)

- High Sensitivity DNA kit for Bioanalyzer (Agilent, Cat. No. 5067-4626)
- IGEPAL® CA-630 (Sigma-Aldrich, Cat. No. 56741) **! CAUTION** IGEPAL® CA-630 is corrosive to the skin. Wear gloves when handling and thoroughly wash any skin exposed to this chemical.
- KAPA Hyperprep Kit (Kapa Biosystems, Roche Cat. No. KK8504)
- Leupeptin (Sigma-Aldrich, Cat. No. 11034626001) **! CAUTION** Toxic if swallowed, inhaled, or contact with skin. Wear a lab coat and gloves.
- Liquid nitrogen **! CAUTION**. Avoid contact with skin. Always wear appropriate gloves and eyeshields and handle the liquid nitrogen in a ventilated room.
- Lithium chloride (LiCl) (Sigma-Aldrich, Cat. No. L9650) **! CAUTION** Harmful if swallowed. Causes skin and eye irritation. Wear protective gloves and a lab coat.
- MinElute Reaction Cleanup Kit (Qiagen, Cat. No. 28204)
- Na ascorbate, (Jena Bioscience, Cat. No. CLK-MI005-1G)
- NGS indexed PentAdapters (PentaBase, Cat. No. SKU 310)
- Pepstatin (Sigma-Aldrich, Cat. No. 11524488001)
- Phenylmethylsulfonyl fluoride (PMSF) (Sigma-Aldrich, Cat. No. 93482-250ML)

  **! CAUTION** Toxic if swallowed. Serious burn if contact with skin and eye. Wear a lab coat, gloves, and eye shield when handling.
- Propidium iodide (PI) (Thermo Fisher Scientific, Cat. No. BMS500PI) **! CAUTION** Harmful if swallowed. Irritating to eyes, respiratory system, and skin. Wear gloves and protective clothes when handling.
- Protein A agarose beads (Thermo Fisher Scientific, Cat. No. 20333)
- Proteinase K (Sigma-Aldrich, Cat. No. P2308-100MG). Store at -20ºC for several months
- Qiagen Qiaquick PCR Purification kit (Qiagen, Cat. No. 28104)
- Qubit dsDNA BR Assay Kit (2-1000ng) (Cat. No. Q32853)
- Qubit dsDNA HS Assay Kit (0.2-100ng) (Cat. No. Q32854)
- RNase A (Sigma-Aldrich, Cat. No. R4875-100MG).

- Sodium chloride (NaCl) (Sigma-Aldrich, Cat. No. S7653)
- Sodium deoxycholate (Sigma-Aldrich, Cat. No. 30970) **! CAUTION** Harmful if swallowed or absorbed through the skin. May cause respiratory and eye irritation. Wear gloves and avoid inhaling.
- Sodium dodecyl sulfate (SDS) solution, 20% (wt/vol) (Sigma-Aldrich, Cat. No. 05030)

  **! CAUTION** SDS is corrosive to the skin and a respiratory irritant. Wear gloves when handling the solution. Thoroughly wash any skin exposed to this chemical.
- Thymidine (Sigma-Aldrich, Cat. No. T1895)
- Tris-HCl buffer (1 M, pH 7.5; Thermo Fisher Scientific, Cat. No. 15567027)
- Tris-HCl buffer (1 M, pH 8.0; Thermo Fisher Scientific, Cat. No. 15568025)
- Tris-hydroxypropyl triazolyl methylamine (THPTA) (Sigma-Aldrich-Aldrich, Cat. No. 762342) **! CAUTION** Skin and eye irritant. Wear gloves when handling.
- Triton X-100 (molecular-biology grade; Sigma-Aldrich, Cat. No. T8787-100ml)

  **! CAUTION** Skin and eye irritant. Wear gloves when handling.
- Trizma base (Sigma-Aldrich, Cat. No. T1503)
- Tween 20 (Sigma-Aldrich, Cat. No. P9416)
- Water, PCR Grade (Sigma-Aldrich, Cat. No. 3315959001)

**Equipment**

- 2100 Bioanalyzer Instrument (Agilent, Cat. No. G2939BA)
- BD FACSCalibur™ (BD Biosciences, discontinued)
- BD Microlance™ Stainless Steel Needles, 21G (BD, Cat. No. 304432)
- Bioruptor® Plus sonication device (Diagenode, Cat. No. B01020001)
- Brand™ Bürker Counting Chambers (Fisher Scientific, Cat. No. 718905)
- CELLSTAR Polypropylene tubes, conical-bottom, 15 mL (Greiner Bio-One, Cat. No. 188271)
- CELLSTAR Polypropylene tubes, conical-bottom, 50 mL (Greiner Bio-One, Cat. No. 227261)

- Centrifuge 5418 R (Eppendorf, Cat. No. 5401000010)
- DiaMag Rotator (Diagenode, Cat. No. B05000001)
- DNA LoBind® Tubes, 1.5 mL (Eppendorf, Cat. No. 022431021)
- DNA LoBind® Tubes, 2.0 mL (Eppendorf, Cat. No. 0030108078)
- DynaMag™-2 Magnet (Invitrogen, Cat. No. 12321D)
- Eppendorf Dualfilter T.I.P.S.® LoRetention (50-1000 µL) (Eppendorf, Cat. No. EP0030078683)
- Eppendorf ThermoMixer® C (Eppendorf, Cat. No. 5382000015)
- Heracell™ 150i CO Incubator with Copper Chambers (Thermo Scientific, Cat. No. 11636250)
- Integra Biosciences™ Pipetboy ACCU 2 Pipette Controller (Fisher Scientific, Cat. No. 10798252)
- Multifuge X4R Pro (Thermo Scientific, Cat. No. 75009515)
- NextSeq 500/550 High Output Kit v2.5 (75 Cycles) (Illumina, Cat. No. 20024906)
- NextSeq 550 Sequencing System (Illumina, Cat. No. SY-415-1002)
- PCR tubes, 0.2 mL, flat cap (Thermo Fisher Scientific, Cat. No. AB0620)
- Plastic Syringe (1mL) (Terumo, Cat. No. SS+01H1)
- ProFlex PCR System (Thermo Scientific, Cat. No. 4484073)
- Qubit™ 4 Fluorometer (Thermo Fisher Scientific, Cat. No. Q33238)
- Rotapure (Rotapure Lab Instruments)
- Sorenson™ low binding aerosol barrier tips, MicroGuard G, maximum volume 10 µL (Sigma-Aldrich, Cat. No. Z719374)
- Sorenson™ low binding aerosol barrier tips, MultiGuard, maximum volume 200 µL (Sigma-Aldrich, Cat. No. Z719447)
- Sorenson™ low binding aerosol barrier tips, MultiGuard, maximum volume 20 µL (Sigma-Aldrich, Cat. No. Z719412)
- Thermostatically controlled cabinet TC 140 G (Lovibond, Cat. No. 2438210)

- Unimax 1010 Orbital Shaker (Heidolph, Cat. No. 543-12310-00)
- Vortex-Genie 2 (Scientific Industries, Cat. No. SI-A256)

**Reagent setup**

- **DMEM medium for HeLa**

  Mix 500 mL of DMEM media with 50 mL of FBS and 5 mL of Penicillin-Streptomycin.

  Store at 4°C for up to 3 weeks. Prewarm to 37°C before use.

- **1M Glycine**

  Dissolve 3.75g of Glycine in 50 mL of $ddH_2O$. Store at 4°C for up to 1 year.

- **Synchronization solution (10X)**

  Dissolve 242.24 mg of thymidine in 50 mL of culture medium under a LAF bench to obtain a concentration of 20 mM and incubate for at least 2 h in a rocker at RT. Sterilize by filtration. Store at 4 °C for up to 1 day.

- **24 mM deoxycytidine (1000X)**

  Dissolve 31.64 mg of deoxycytidine in 5 mL $ddH_2O$. Sterilize by filtration. Aliquot and store at -20°C for up to 1 year.

- **Crosslinking solution**

  Prepare just before use. Dilute 3.125 mL of 16% (wt/vol) formaldehyde in 50 mL of 1X PBS.

- **Lysis buffer**

  Prepare Lysis buffer by mixing 10 mL of SDS Buffer with 5 mL of Triton dilution buffer. Can be stored at RT for up to 1 month.

- **PBS-PMSF solution:**

  Prepare freshly 1 mM PMSF solution in 1X PBS. Keep on ice.

- **SDS Buffer**

  Prepare as outlined below. Can be stored at RT for up to 1 month.

| Final concentration | Stock concentration | Volume (mL) for 50 mL |
|---|---|---|

| | | |
|---|---|---|
| 50 mM Tris-HCl pH 8.0 | 1M Tris-Cl pH 8.0 | 2.5 |
| 5 mM EDTA pH 8.0 | 0.5 M EDTA pH 8.0 | 0.5 |
| 100 mM NaCl | 5M NaCl | 1 |
| 0.5% SDS (wt/vol) | 20% SDS (wt/vol) | 1.25 |
| dd$H_2O$ | | Up to 50 mL |

- **Triton dilution buffer**

  Prepare as outlined below. Can be stored at RT for up to 1 month.

| Final concentration | Stock concentration | Volume (mL) for 50 mL |
|---|---|---|
| 100 mM Tris-HCl pH8.0 | 1M Tris-HCl pH 8.0 | 5 |
| 5 mM EDTA | 0.5M EDTA pH 8.0 | 0.5 |
| 100 mM NaCl | 5M NaCl | 1 |
| 5 % Triton X-100 (vol/vol) | 10 % Triton X-100 (vol/vol) | 25 |
| dd$H_2O$ | | Up to 50 mL |

For the following reagents refer to the main protocol:

- **10 mM thymidine (1000X)**
- **20 mM EdU**

# PROCEDURE

## Culture of suspension HeLa S3 cells

1. Culture HeLa S3 cells in DMEM supplemented with 10 % (vol/vol) FBS as previously described[15].

   **CRITICAL STEP.** This protocol describes the procedure to perform ChOR-seq on HeLa S3. However, we anticipate that it applies to any other proliferating cell line growing in suspension.

2. A day before synchronization, seed cells in spinner flasks at 0.6 x $10^6$ cells per mL of medium and incubate at 37 °C, 5 % CO2 for 14 h until reaching a concentration of 1-1.2 x $10^6$ cells per mL. Seed enough volume of cells to get at least 5 x $10^7$ cells per condition.

   **CRITICAL STEP.** We recommend growing cells in spinner flasks some days before the

experiment to make sure that cells grow exponentially.

## Synchronization of HeLa S3 cells (optional for cell lines with a low fraction of S-phase cells in asynchronous populations) •TIMING 24 h

3. Freshly prepare a 10X stock of thymidine dissolved in the culture medium. (see **Reagent setup** in the main protocol).
4. Count the cells and adjust the concentration to 1.1 x $10^6$ cells per mL.
5. *(Optional)* For quality control of synchronization: Transfer 2 mL of asynchronous culture to a 15 mL conical tube. Keep on ice until proceeding to FACS cell cycle analysis (Box 1).
6. Add one-tenth of the final volume of the 10X Synchronization solution. (For example, add 40 mL of 10X Synchronization solution to 360 mL of culture to make 400 mL of culture at $10^6$ cells per mL)
7. Incubate for 17h at 37ºC.
8. *(Optional)* For quality control of synchronization: Right before releasing cells from synchronization transfer 2 mL of synchronized cell culture to a 15 mL conical tube. Keep on ice until proceeding to FACS cell cycle analysis (Box 1).
9. Distribute the synchronized cell culture from the spinner flask to 50 mL conical tubes (50 mL ≈ 5 x $10^7$ cells per tube).
10. Centrifuge for 5 min at RT, 300 x g. Discard the supernatant.
11. Wash the spinner flask thoroughly with 1X PBS.
12. To release cells from thymidine block, wash thoroughly with an equivalent volume of pre-warmed 1X PBS. Resuspend the cells in the same volume of pre-warmed DMEM medium containing 24 µM deoxycytidine and transfer back to the spinner flask.
13. Return the spinner flask to the incubator and allow cells to progress into early S-phase (2 h and 15 min) or mid-S-phase (3 h and 15 min).

    **CRITICAL STEP.** Cell cycle length must be determined empirically for each cell line before synchronization. It is important to consider replication timing[58] for the regions bound by the protein/modification of interest.
14. Procced to EdU labeling.

**CRITICAL STEP.** We recommend controlling for synchronization efficiency by performing flow cytometry analysis of the cell cycle comparing the asynchronous and synchronized cells. For time-course analysis, after releasing from synchronization, we recommend performing FACS cell cycle analysis of the different time points as well.

## EdU labeling of HeLa S3 cells ● TIMING 30 - 45 min

15. (*Optional*) For quality control of the thymidine release: Before labeling, transfer 2 mL of culture to a 15 mL conical tube for FACS analysis. Keep on ice until proceeding to Quality control for EdU incorporation efficiency (Box. 1)

16. Transfer 10 mL of medium from the culture to a 15 mL conical tube and add EdU (20 mM stock solution) to have a final concentration of 10 µM. (for example, 100 µL for 200 mL of culture). Mix by inverting the tube multiple times and pour 10 mL of containing EdU medium back to the spinner flask. Incubate at 37 °C for the desired amount of time.

    **CRITICAL STEP** Labelling time must be optimized, as it will determine the resolution and yield of the experiment. See **Experimental design**.

    **? TROUBLESHOOTING**

17. (*Optional*) For quality control of EdU incorporation by FACS: Transfer 2 mL of culture to a 15 mL conical tube for FACS analysis. Keep on ice until proceeding to Quality control for EdU incorporation efficiency (Box. 1).

18. Distribute the labeled cell culture from the spinner flask to 50 mL conical tubes (50 mL ≈ 5 x $10^7$ cells per tube).

19. Centrifuge for 5 min at RT, 300 x g. Discard the supernatant.

20. To analyze newly replicated chromatin, follow Option A. To perform a time course of chromatin maturation, follow Option B.

    **A. For analysis of newly replicated chromatin**:

    i. Wash cells once with an equivalent volume of ice-cold 1X PBS to stop the EdU pulse. Proceed to Fixation (step 21).

    **B. For time-course analyses**:

    i. Wash twice with warm 1X PBS and resuspend in fresh prewarmed medium containing 24 µM deoxycytidine and 10 µM thymidine (Add 1 µL of 10 mM

thymidine per mL of culture medium) to chase the EdU pulse.

ii. Incubate at 37 °C and chase for the desired amount of time before proceeding to fixation (step 21).

**CRITICAL STEP.** For long-time courses, we recommend replacing a medium containing 10 µM thymidine with a fresh prewarmed medium containing 24 µM deoxycytidine after 1 or 2 h.

## Fixation of HeLa S3 cells ● TIMING 1.5-2 h

21. Wash 5 x $10^7$ cells one time with 50 mL of ice-cold 1X PBS. Centrifuge for 5 min RT, 300 x g. Discard the supernatant.
22. Resuspend in 30 mL of crosslinking solution. We recommend resuspending the cell pellet in 5 mL and then adding the remaining 25 mL. Start counting the time for the crosslinking reaction after adding the initial 5 mL. **CRITICAL STEP**. We recommend using fresh methanol-free formaldehyde to achieve reproducible cross-linking conditions.
23. Incubate immediately on a shaking platform at RT and incubate for the recommended time. See the experimental design for advice on fixation time.
24. Quench the crosslinking reaction by adding 3.75 mL of 1M Glycine to obtain a final concentration of 0.125 M. Incubate for 5 min on a shaking platform at RT.
25. Centrifuge for 5 min at 4 °C, 300 x g. Discard the supernatant.
26. Wash each cell pellet twice in 50 mL of ice-cold PBS-PMSF solution. We recommend resuspending in 5 mL and then add the remaining 25 mL.
27. Centrifuge for 5 min at 4 °C, 300 x g. Discard the supernatant.
28. Resuspend each cell pellet in 1 mL of cold SDS Buffer containing protease inhibitors.
29. Centrifuge for 5 min at 4 °C, 300 x g. Discard the supernatant.

**PAUSE POINT**: Cell pellets can be snap-frozen in liquid nitrogen and stored at -80 °C for up to one year.

## Cell lysis and chromatin shearing of HeLa S3 cells ● TIMING 1.5-2 h

30. Resuspend each cell pellet (≈5x$10^7$cells) in 3.6 mL of Lysis buffer containing protease inhibitors and incubate for 20 min at RT.

31. Run the lysates three times through a needle.

32. Distribute the lysate in 300 µL aliquots in 1.5 mL tubes and sonicate on a Bioruptor Plus with the following settings: "20 cycles, 30s ON / 30s OFF, High". Keep tubes on ice until all the samples have been processed.

    **CRITICAL STEP.** These settings were applied to HeLa S3 cells and *D. melanogaster* S2 cells. The shearing conditions may need to be adjusted for each cell line.

33. Centrifuge for 10 min at 4 °C, 14000 x g.

34. Combine all supernatants in one 15 mL tube and distribute them again into 300 µL aliquots.

    **PAUSE POINT.** Sheared chromatin can be stored at 4 ºC for up to 2 days or snap-frozen in liquid nitrogen and stored at -80 °C for one year. Avoid repeated freezing and thawing of chromatin as this can reduce IP efficiency and reproducibility.

35. Transfer 25 µL of sheared chromatin to a PCR tube and proceed to Quality control of chromatin shearing (Box 2).

36. Transfer another 25 µL of sheared chromatin from step 34 to a 1.5 mL tube and keep it at -20 ºC until proceeding to library preparation (steps 66 -153 of Procedure 1 in the main protocol). This is the EdU-Input.

    **? TROUBLESHOOTING**

37. Continue with chromatin immunoprecipitation at step 40 of the main ChOR-seq protocol. All subsequent steps are equally applicable to adherent and suspension cell lines.

## Chromatin preparation from *D. melanogaster* S2 for spike-in ●TIMING 39 h

38. Please refer to Procedure 1 of the main protocol (steps 1-4) for culture conditions and EdU labeling of *D. melanogaster* S2 cells.

## Fixation of *D. melanogaster* S2 cells ●TIMING 1.5-2 h

39. Wash 8 x $10^7$ cells one time with 50 mL of ice-cold 1X PBS. Centrifuge for 5 min RT, 300 x g. Discard the supernatant.

40. Refer to steps 22-29 of this Supplementary procedure.

## Cell lysis and chromatin shearing of *D. melanogaster* S2 cells ●TIMING 1.5-2 h

41. Resuspend each cell pellet (≈8 x $10^7$cells) in 1.2 mL of Lysis buffer containing protease inhibitors and incubate for 20 min at RT.

42. Refer to steps 31-35 of this Supplementary procedure.

## Library preparation

**Consideration for synchronized cultures:**

Since the cell cultures are synchronized in a specific stage of the S-phase, only a fraction of the genome will undergo replication during the EdU pulse. For this reason, it is important to include an EdU-Input sample prepared from labeled and sheared chromatin (step 36 of the Supplementary procedure). The EdU-Input sample is processed in parallel with immunoprecipitated samples and equally subjected to library preparation, click biotinylation, and streptavidin pull-down as in steps 73-153 of Procedure 1. This sample will account for the fraction of regions that have been replicated during the EdU pulse at the chosen time during the S-phase at step 16 of this Supplementary procedure and will be used for the data analysis (steps 43-44 of the Supplementary procedure).

## Sequencing and data analysis

Only replicated regions can be used for data analysis. To this end, an additional filtering step must be added to the procedure described on steps 154-164 of Procedure 1 of the main protocol.

43. Define replicated regions: Run MACS2[72] with --broad parameters to identify peaks on the BAM files from EdU-INPUT, using total INPUT as the control file.

44. Filter histone PTM peaks overlapping replicated regions by using BEDtools intersect function with -wa option[71].

| **SUPPLEMENTARY TABLE 1 \| TROUBLESHOOTING** | | | | |
|---|---|---|---|---|
| **Section** | **Step** | **Problem** | **Possible reason** | **Solution** |
| *Labeling* | 16 | A low number of EdU positive cells | Click reaction did not work well | Check EdU concentration (10 μM) |
| | | | | Prepare fresh reagents for the click reaction |
| | | | Not optimal synchronization | Check by FACS-PI staining |
| *Chromatin fragmentation* | *36* | Unexpected size distribution of DNA fragments | Not optimal sonication | Optimize the sonication by changing cell number, time and eventually adjusting the intensity of energy transferred to the water bath. |
| | | | Overfixation of chromatin | Reduce fixation time. |

**? TROUBLESHOOTING:** Additional troubleshooting advice can be found in Table 2.