Profiling Chromatin Accessibility on Replicated DNA with repli-ATAC-seq

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i. Chapter Title – Profiling Chromatin Accessibility on Replicated DNA with repli-ATAC-seq

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ii. Summary/Abstract

Open or accessible chromatin typifies euchromatic regions and helps define cell type-specific transcription programs. DNA replication massively disorders chromatin composition and structure, and how accessible regions are affected by and recover from this disruption has been unclear. Here, we present repli-ATAC-seq, a protocol to profile accessible chromatin genome-wide on replicated DNA starting from 100,000 cells. In this method, replicated DNA is labelled with a short 5-ethynyl-2′-deoxyuridine (EdU) pulse in cultured cells and isolated from a population of tagmented fragments for amplification and next-generation sequencing. Repli-ATAC-seq provides high-resolution information on chromatin dynamics after DNA replication and reveals new insights into the interplay between DNA replication, transcription, and the chromatin landscape.

iii. Key Words

repli-ATAC-seq, DNA replication, transcription, accessible chromatin, chromatin assembly, nucleosome-free regions,

1. Introduction

Transcriptionally active chromatin is characterized by an open structure that permits entry of transcription factors and RNA polymerases to sites of transcription initiation, such as promoters and enhancers [1]. Such sites are characterized by nucleosome depletion, which renders DNA more accessible to transcription machinery [2]. These regions are important regulatory features of the chromatin landscape and, like the transcription programs they reflect, are cell type-specific [3]. These “accessible” regions can be profiled genome-wide using a number of strategies, including DNase-seq, MNaseq-seq, and FAIRE-seq [4]. An attractive alternative to these methods, which are labor-intensive and require large

amounts of input material, is ATAC-seq, or assay for transposase-accessible chromatin [5]. This method profiles accessible regions in high-resolution from low amounts of starting material, and with deep sequencing can also inform on nucleosome positioning and occupancy, especially in organisms with smaller genomes.

DNA replication compromises the chromatin landscape by evicting proteins from DNA, temporarily disrupting chromatin structure [6]. This includes nucleosomes, which are evicted prior to replication fork passage and rapidly re-assembled on the new DNA strands. Recycled parental histones are distributed largely symmetrically between daughter strands [7, 8], and in parallel newly synthesized histones are deposited to restore nucleosome density [9]. Following replication, the chromatin landscape is extensively remodelled and modified to restore the pre-replicative state [6].

DNA replication proceeds in a regulated, cell type-specific manner, with euchromatic regions replicated early in S phase and heterochromatic regions replicated late in S phase [10]. However, this process is inherently heterogeneous, driven by stochastic replication initiation from a large number of possible initiation zones (themselves found in accessible regions). This heterogeneity in the replication program, and its duration across many hours in most cell types, makes it impossible to use standard cell synchronization methods, including cell sorting and drug-based approaches, to address the transient changes in chromatin dynamics that occur in the wake of replication in high-resolution.

To investigate how these local effects manifest genome-wide, multiple groups have developed methods that combine metabolic labelling of DNA with next-generation

sequencing approaches [6]. Additionally, by chasing the DNA label, restoration of chromatin accessibility after replication can be investigated.

These novel methods have mainly adapted MNase-seq to investigate nucleosome positioning and occupancy in organisms with small genomes, including *S. cerevisiae* [11-13] and *D. melanogaster* [14]. In addition, analysis of sub-nucleosomal MNase fragments was used to assess TF occupancy post-replication in *D. melanogaster* [14].

Here we describe replication-coupled ATAC-seq, or repli-ATAC-seq [15] (Figure 1), a novel method established in mammalian cells to profile accessible chromatin genome-wide on replicated DNA. Repli-ATAC-seq produces genome-wide accessibility profiles with thorough coverage of open chromatin sites, which show high signal and generate clear peaks even in large genomes. By filtering *in silico* specifically for subnucleosomal-length fragments, repli-ATAC-seq can inform on transcription factor occupancy in replicated chromatin. The protocol described here was developed in mouse embryonic stem cells cultured in serum; cell culturing and lysis conditions may require testing in other cell types. This procedure, which can be completed in one day, starts from 100,000 asynchronous, cultured cells labelled with the thymidine analog 5-ethynyl-2′-deoxyuridine (EdU) for as little as ten minutes, enabling high-resolution profiling of the nascent chromatin landscape. We recommend using low-binding plastic ware throughout the protocol to minimize sample loss. To follow chromatin accessibility throughout chromatin maturation, we include a pulse/chase EdU labelling strategy. To compare baseline accessibility between samples, an option to include native *D. melanogaster* S2 cell chromatin as an internal spike-in control is described. Although classic ATAC-seq in asynchronous

cells can be informative for comparison, we recommend that analyses compare nascent repli-ATAC-seq datasets to fully mature datasets, to control for any technical differences introduced in the click biotinylation and streptavidin pulldown steps. Repli-ATAC-seq offers a novel approach to address the interplay between DNA replication and transcription, and provides the means to profile DNA replication-induced changes in rare and dynamic cell populations.

2. Materials

Equipment

Cell culture hood

Hemacytometer or automated cell counter such as Countess (ThermoFisher)

Microcentrifuge

Thermomixer

Magnetic 1.5 mL tube rack

Rotator with side movement

Qubit 2.0 Fluorometer (Life Technologies).

BioAnalyzer (Agilent)

Thermocycler

Reagents

Cell culture reagents (cell type-specific)

1X Phosphate-buffered saline (PBS)

Trypsin

PCR-grade H2O

100% ethanol

1.5 mL lo-binding tubes

Lo-binding pipette tips

Illumina Tagment DNA Enzyme and Buffer Kit (Illumina)

Qiagen MinElute PCR Purification kit (Qiagen)

EdU, 20 mM stock dissolved in DMSO and aliquoted at -20° C (Thermo Fisher)

Click-IT cell reaction buffer kit (Thermo Fisher)

- Click-IT cell buffer additive (Component C) (80 mg): Dissolve in 400 µL deionized H2O (100X) and store in 200 µL aliquots at -20° C for up to 1 year

THPTA 50mM stock in PCR-grade H2O (Sigma)

Picolyl-Azide-PEG4-Biotin 100 mM stock dissolved in DMSO and stored at 4° C (Jena Bioscience)

Qiagen MinElute Reaction Cleanup kit (Qiagen) (optional, only for making unbound libraries)

Agencourt AMPure XP beads (Beckman Coulter)

Myone T1 streptavidin beads (Thermo Fisher)

NEBNext High-Fidelity 2x PCR Master Mix (NEB)

Qubit HS assay (ThermoFisher)

Agilent HS DNA kit (Agilent)

Triton-X 100

Tween 20

10 mM Tris-HCl pH 7.5

For thymidine chase to study chromatin maturation:

Thymidine, 10 mM stock dissolved in deionized H2O and aliquoted at -20° C (Sigma)

For D. melanogaster spike-in:

D. melanogaster S2 cells

Shields and Sang M3 Insect Medium (Sigma)

KHCO3 (Sigma)

Yeast Extract (Sigma)

Bactopeptone (BD)

Fetal Calf Serum (GE Hyclone)

Penicillin/ Streptomycin (GIBCO)

Cell incubator at 25 °C

Buffers

Buffer A without protease inhibitors: 10 mM HEPES pH 7.9, 10 mM KCl, 1.5 mM $MgCl_2$, 0.34 M sucrose, 10 % glycerol, 0.05 % Triton-X. Store filtered and without Triton-X at 4° C for long-term use, add Triton-X just before use.

Lysis buffer: 10 mM Tris-HCl pH 7.4, 10 mM NaCl, 3 mM $MgCl_2$, 0.3% Igepal. Store without Igepal at 4° C for up to 24 hours before use, add Igepal just before use. Discard any remaining buffer after using.

Tagmentation Stop Buffer (TSB): 50 mM Tris pH 8, 10 mM EDTA pH 8, 1 % SDS. Store at RT.

2x B&W buffer: 10 mM Tris-HCl pH 7.5, 1 mM EDTA, 2 M NaCl, 0.1 % Tween

Elution Buffer: 10 mM Tris-HCl pH 8.5.

EBT Buffer: 10 mM Tris-HCl pH 8.5, 0.05% Tween 20.

3. Methods

3.1 EdU Labelling

1. Seed 2 x 10^6 mESCs per sample on a 10 cm gelatinized dish in 10 mL of appropriate media and grow them for 24h at 37 °C, 5 % CO_2. (Note 1)
2. After preparing reagents (see Note 2), begin EdU labelling.

Option 1: Nascent repli-ATAC-seq

3. Replace culturing media with 10 mL EdU media (final concentration: 20 µM) in the corresponding dish, swirl to disperse, and incubate at 37 °C for 10 minutes (see Notes 3 and 4).
4. After 10 minutes, aspirate media and wash twice in 10 mL warmed 1X PBS.
5. Add 2 mL trypsin and return to 37 °C for 1-2 minutes. Take the cold media out of the fridge and put it under the hood.
6. Check under a microscope for cell detachment. When cells are detached add 6 mL of COLD media to stop trypsin digestion.
7. Transfer cells to a 15 mL Falcon tube.
8. Take 10 µL of the cell suspension for counting using a hemacytometer or an automated cell counter.
    1. Goal : 100,000 cells for repli-ATAC-seq per sample.
7. Transfer 100,000 cells into a low-binding 1.5 mL tube. Spin cells at 500 g, 5 minutes at 4° C . From this step keep the tubes on ice. (see Note 5)

Option 2: Mature repli-ATAC-seq

3. Replace culturing media with 10 mL EdU media (final concentration: 20 µM) in the corresponding dish, swirl to disperse, and incubate at 37 °C for 10 minutes (see Notes 3 and 4).
4. After 10 minutes, aspirate media and wash twice in 10 mL warmed PBS.
5. Add 10 mL warmed media with 10 µM thymidine to the plate. Return to incubator for the desired maturation time.
6. After the desired maturation interval (see Note 6), aspirate media and wash twice in 10 mL warmed PBS.
7. Add 2 mL trypsin and return to 37 °C for 1-2 minutes. Take the cold media out of the fridge and put it under the hood.
8. Check under a microscope for cell detachment. When cells are detached add 6 mL of COLD media to stop trypsin digestion.
9. Transfer cells to a 15 mL Falcon tube.
10. Take 10 µL of the cell suspension for counting using a hemacytometer or an automated cell counter.
    a. Goal : 100,000 cells for ATAC-seq per sample.
6. Transfer 100,000 cells into a low-binding 1.5 mL tube. Spin cells at 500 *g* for 5 minutes, 4° C From this step keep the tubes on ice. (see Note 5).

Option 3: *D. melanogaster* spike-in

It may be useful to include an internal control of accessibility on replicated DNA. This can be done by EdU-labelling cultured *D. melanogaster* S2 cells for 40 hours (see Note 7) prior to labelling repli-ATAC-seq dishes and adding 100 labelled cells to trypsinized and aliquoted mESCs, prior to lysis:

1. Culture *D. melanogaster* S2 cells in suspension following general procedures as described by the Drosophila Genomics Resource Center [16].
2. Replace culturing media with EdU media (final concentration: 10 µM) in the corresponding dish, swirl to disperse, and incubate at 25 °C for 40 hours. Time the EdU labelling such that S2 cells will be fully labelled at the time of labelling nascent and any mature repli-ATAC-seq samples.
3. Transfer cells to 50 mL tubes and spin at 300 *g* for 5 minutes.
4. Aspirate supernatant and wash cells in an equivalent volume of ice-cold 1X PBS.
5. Repeat wash.
6. Take 10 µL of the cell suspension for counting using a hemacytometer or an automated cell counter.
    2. Goal : 100 cells per repli-ATAC-seq sample.
7. Transfer 100 fresh, EdU-labelled S2 cells into tubes containing cells prepared in parallel for repli-ATAC-seq. Place tubes on ice and proceed to cell lysis.

3.2 Cell Lysis

1. To prepare, aliquot 995 µL of Buffer A into a 1.5 mL tube and add 5 µL of 10 % Triton-X 100 to the aliquot. Invert to mix. For Steps 2-3, work in a 4° C room.
2. Carefully remove media (see Note 8) from labelled cells and add 200 µL of cold 1X Buffer A with Triton-X. Pipet up and down 5 times to resuspend, being careful to avoid creating bubbles.

3. Incubate on ice for 7 minutes. Lay tube on ice in the cold room, but avoid burying the tube in the ice (see Note 9).
4. Pellet nuclei by spinning at 1300 *g* for 5 minutes, 4° C and carefully remove lysis buffer as in Step 3, here using a P200 set to 198 µL and gel-loading tips to aspirate all supernatant.
5. Add 100 µL of cold 1X lysis buffer and pipet up and down 10 times to resuspend, being careful to avoid creating bubbles.
6. Split sample into 2 1.5 mL low-binding tubes each containing 50 µL lysate (equivalent to 50,000 cells) each.
7. Vortex samples for 10 seconds on medium-high strength.
8. Incubate on ice for 15 minutes at room temperature (bury tubes in ice).
9. After incubation, vortex tubes for 10 seconds at medium-high strength again. Pellet nuclei by spinning at 600 *g* for 10 minutes, 4° C and carefully remove lysis buffer as in Steps 3 and 5, here using a P200 set at 48 µL and gel-loading tips to aspirate all supernatant.

3.3 Transposase Digestion

1. Combine 2.5 µL 2XTD buffer and 2.5 µL transposase (TDE1) (from Illumina kit) per tube or as a master mix and aliquot into tubes on ice.
2. Pipet up and down 10 times to resuspend. Vortex on medium-high strength for 10 seconds.
3. Incubate for exactly 30 minutes at 37 °C in a thermocycler shaking at 1200 rpm.
4. After incubation, combine the two transposase digestions from each sample into one 1.5 mL low-binding tube (final volume: 10 ul).

5. Add 90 µL TSB to the combined digestions (final volume: 100 ul).
6. Purify with Qiagen MinElute PCR Purification Kit. To elute, add 80 µL PCR-grade H2O to columns and incubate for 5 minutes at RT. Spin at max. speed for 1 minute and then re-elute samples by adding the eluate back onto its respective column and incubating for a further 5 minutes at RT. Spin at max. speed for 1 minute and proceed to Click Biotinylation (total volume, accounting for loss: approximately 78 ul) (see Note 10)

3.4 Click Biotinylation

7. Prepare THPTA-CuSO4 premix by mixing 1 µL 50 mM THPTA and 0.1 µL 100 mM CuSO4 per sample in a separate 1.5 mL low-binding tube.
8. Prepare 10X buffer additive by mixing 1 µL 100X buffer additive and 9 µL PCR-grade H2O per sample in a separate tube.
9. Set up the click reaction by adding the reagents to the purified DNA in the following order: 10 µL 10X Click-iT buffer, 0.5 µL 100 mM picolyl-azide-PEG4-biotin, 1.1 µL THPTA-CuSO4 premix, 10 µL 10X buffer additive (see Note 11).
10. Incubate for 30 minutes at RT.
11. During incubation, equilibrate AMPure beads at RT for 30 minutes prior to use. Keep AMPure beads at RT to use after Library Amplification (Section 3.6).
12. To purify DNA, add 55 µL equilibrated AMPure beads to each sample (0.55:1 bead ratio).
13. Mix thoroughly by vortexing.
14. Incubate the tube(s) at room temperature for 10 minutes to bind large, unwanted DNA fragments to the beads.

15. During incubation, prepare another silconised 1.5 mL tube with 245 ul AMPure beads.
16. During incubation, prepare 400 ul of 80 % ethanol per sample.
17. During incubation, warm a thermoblock to 37°C.
18. Place the tube(s) on the magnet to capture the beads. Incubate until the liquid is clear.
19. Carefully remove the supernatant and **transfer** it to the corresponding prepared tube containing AMPure beads (3:1 final ratio). Discard tube(s) containing used beads (see Note 12).
20. Incubate tube(s) at RT for 10 minutes to bind the desired DNA fragments to the beads.
21. Place the tube(s) on the magnet to capture the beads. Incubate until the liquid is clear.
22. Carefully remove and discard supernatant.
23. Keeping the tube(s) on the magnet, add 200 µL of freshly prepared 80 % ethanol. On the rack, turn the tubes 180°, forcing the beads through the ethanol to the opposite wall of the tube.
24. Incubate the tube(s) on the magnet at room temperature for ≥30 seconds.
25. Carefully remove and discard the ethanol.
26. Repeat steps 23-25 once. Try to remove all residual ethanol without disturbing the beads, using a P10 pipette if necessary.
27. Dry the beads at room temperature for 1-2 minutes. Caution: Avoid over-drying of the beads, as it may result in dramatic yield loss.

28. Remove the tube(s) from the magnet. Resuspend the beads in 52 µL of Elution Buffer.
29. Put the tube(s) with lid(s) open to the warmed thermoblock at 37°C. Cover with a top of a tip box or a piece of aluminium foil to prevent contamination of open tubes.
30. Incubate for 5-10 minutes to elute DNA and evaporate residual ethanol.
31. Place the tube(s) on the magnet to capture the beads. Incubate until the liquid is clear.
32. Carefully transfer 50 µl of the supernatant to a new low-binding tube.

3.5 Streptavidin Pulldown

1. Resuspend the stock of Myone T1 streptavidin beads by vortexing.
2. Pipet 20 µL of bead suspension per sample into a 1.5 mL DNA low binding tube. Pellet the beads using a magnetic rack (≥30 seconds). Remove and discard the supernatant.
3. Remove the tube from the magnetic rack and add 200 µL of 1xB&W buffer. Mix by pipetting. Place the tube back to the magnetic rack to pellet the beads. Remove and discard the supernatant.
4. Repeat 1x B&W wash 3 times.
5. Resuspend washed streptavidin beads in 50 µL 2X B&W buffer per sample.
6. Add 50 µL resuspended streptavidin beads to each sample (final B&W concentration 1X). Mix by pipetting.
7. Incubate tubes 30 minutes at RT on a tube rotator. Ensure beads are continually in suspension.

8. Spin tubes briefly. Pellet beads on a magnetic rack. Remove supernatant (see Note 13).
9. Wash beads with 200 µL 1XB&W buffer and mix by pipetting.
10. Pellet the beads using a magnetic rack (≥30 seconds). Remove and discard the supernatant.
11. Repeat steps 4-5 3 times, waiting 1 minute off the magnetic rack between washes. Perform washes on 4-6 reactions at a time to avoid overdrying the beads.
12. Wash beads as in steps 9-10 twice with 200 µL EBT Buffer.
13. Wash beads as in steps 9-10 once with 200 µL 10 mM Tris-HCl pH 7.5
14. Pellet the beads on a magnetic rack and carefully remove all supernatant.
15. Resuspend beads in 10 µL PCR-grade H2O, transfer to a 0.2 mL low-binding tube, and keep on ice. Proceed to Library Amplification (see Note 14).

3.6 Library Amplification (on beads)

1. Set up the PCR reaction by adding the following reagents to bead-bound DNA in 0.2 mL tubes: 1.25 µL 25 µM Primer 1, 1.25 µL 25 µM Primer 2 (see Note 15), 12.5 µL NEBNext High-Fidelity 2x PCR Master-Mix.
2. Vortex to mix and spin down briefly.
3. Amplify libraries using the following conditions: 72 °C, 5 minutes; 98 °C, 30 seconds; 12 cycles of: 98 °C, 10 seconds; 63 °C, 30 seconds; 72 °C, 30 seconds; 4 °C hold.
4. Add 25 µL PCR-grade H2O to each library (final volume: 50 µL).
5. To purify libraries, add 80 µL equilibrated AMPure beads to each sample (1.6:1 bead ratio).
6. Mix thoroughly by vortexing.

7. Incubate the tube(s) at room temperature for 10 minutes to bind DNA fragments to the beads.
8. During incubation, prepare 400 µL of 80% ethanol per sample.
9. During incubation, warm a thermoblock to 37°C.
10. Place the tube(s) on the magnet to capture the beads. Incubate until the liquid is clear.
11. Carefully remove and discard supernatant.
12. Keeping the tube(s) on the magnet, add 200 µL of freshly prepared 80 % ethanol.
13. Incubate the tube(s) on the magnet at room temperature for ≥30 seconds, turning the tubes 180° to ensure all beads pass through the ethanol.
14. Carefully remove and discard the ethanol.
15. Repeat steps 12-14 once. Try to remove all residual ethanol without disturbing the beads, using a P10 pipette if necessary.
33. Dry the beads at room temperature for 1-2 minutes. Caution: Avoid over-drying of the beads, as it may result in dramatic yield loss.
16. Remove the tube(s) from the magnet. Resuspend the beads in 12 µL of Elution Buffer.
17. Put the tube(s) with open lids to the warmed thermoblock at 37°C. Cover with a top of a tip box or a piece of aluminium foil to prevent anything from falling into the open tubes.
18. Incubate for 5-10 minutes to elute DNA and evaporate residual ethanol.
19. Place the tube(s) on the magnet to capture the beads. Incubate until the liquid is clear.
20. Carefully transfer 10 µl of the supernatant to a new 1.5 mL low-binding tube.

3.7 Quality Control

Prior to sequencing, perform quality control of repli-ATAC-seq libraries by quantifying library concentration with Qubit and checking library fragment length distribution using an Agilent Bioanalyzer or an equivalent fragment analyzer (Figure 2).

3.8 Sequencing and Analysis

Libraries can be sequenced on an appropriate sequencing platform (repli-ATAC-seq was developed using Illumina NextSeq 500). Paired-end sequencing will provide both positional information and fragment length for all reads; single-end sequencing will provide positional information only. To enable analysis specifically of subnucleosomal fragments we therefore prefer paired-end sequencing, though this may not be necessary for all research questions. Post-sequencing, assessment of sequencing quality using FastQC and adaptor trimming using TrimGalore! or similar software is recommended. PCR duplicates, reads mapping to the mitochondrial genome, and reads mapping to any relevant sequencing blacklists (e.g., the mm10 sequencing blacklist, [17]) should be discarded, as should reads with a MAPQ score <20. Remaining reads can be processed using peak-calling and other standard bioinformatic analyses. If *D. melanogaster* spike-in was employed, map to both the target and *D. melanogaster* genomes. From the *D. melanogaster* Binary Alignment Map (BAM) file, calculate the total number of unique reads. Calculate a spike-in normalization factor for each sample by dividing 10^6 by the total number of unique reads from *D. melanogaster*. To quantify spiked-in samples by reference-adjusted reads per million (RRPM), calculate the coverage of each bin or region of interest in the target genome by computing the number of

unique reads per bin. Then, multiply by the spike-in normalization factor, prior to any log transformation or further manipulation of the data.

4. Notes

1. When setting up repli-ATAC-seq experiments, inclusion of an unlabelled, EdU- dish processed in parallel can be useful to ensure the experiment is free from any contamination from unlabelled DNA fragments in repli-ATAC-seq libraries.

2. Prior to beginning EdU labelling, ensure the following reagents are ready and at the appropriate temperature:

- Microcentrifuge is cooled to 4 °C
- Thermomixer is warmed to 37 °C
- 6 mL of appropriate media per dish is at 4 °C
- 2 mL trypsin per dish is warmed to 37 °C
- 20 mL 1X sterile PBS per dish is warmed to 37 °C
- Lysis buffer is freshly prepared and kept cold on ice
- EdU media is prepared: 10 mL appropriate media with 20 µM EdU per 10 cm dish, prepared and warmed to 37 °C
- If generating mature samples (Option 2), have 10 mL appropriate media with 10 µM thymidine per dish prepared and warmed to 37 °C

3. For pulse/chase experiments, it is recommended to stagger your EdU labelling such that all dishes are ready for collection at the same time because the first pause point following EdU labelling is after 2 hours of processing.

4. The length of EdU labelling may need to be optimized depending on the proliferation rate of the cell type of interest.

5. The remaining cells can be kept short-term on ice and processed for e.g. FACS controls during the transposase digestion.

6. One cell cycle post-pulse, EdU-labelled loci will replicate again; maturation times should therefore be well below one cell cycle length for the cell type of interest.

7. Labelling S2 cells with EdU for 40 hours will ensure genome-wide labelling.

8. It is critical to avoid disturbing or losing the cell pellet. To do this, first pipet out 900 µL supernatant using a P1000, then switch to a P200 set to 98 µL and, using gel-loading tips to pipet from the bottom of the sample while not disturbing the pellet, aspirate the remaining supernatant.

9. During incubation, the remaining, saved cells can be spun 500 x g for 5 min, and resuspended in 300 µL PBS. To fix, add 700 µL ice-cold 100% EtOH drop-wise while vortexing on low and save at 4° C for FACS labelling.

10. Purified DNA can be quantitated for quality control at this stage. It can be kept short-term (1-2 days) at 4° C or frozen at -20° C for longer term storage, but library quality seems poorer when this is done. Recommendation is to continue immediately to Click biotinylation

11. The order in which the reagents for click biotinylation are added to samples is important because addition of the buffer additive starts the click reaction. It is not recommended to make a master mix of Click-iT reagents.

12. This double size-selection removes fragments larger than approximately 600 bp, since longer fragments are both more difficult to sequence on Illumina platforms and generally less informative for accessibility studies. If these fragments would be informative in specific experiments, a 1.8:1 ratio could be used to preserve and purify fragments of all lengths.

13. If desired, the supernatant can be removed and saved in a new 1.5 mL tube. This contains tagmented, non-EdU-labelled DNA fragments and can be used to generate "unbound" libraries. Unbound libraries are virtually interchangeable in terms of coverage with standard bulk ATAC-seq libraries, and can complement repli-ATAC-seq libraries. To create unbound libraries, purify supernatant using the MinElute Reaction Cleanup Kit, eluting in 12 µL PCR-grade H2O or EB buffer. Amplify and purify libraries as described in Section 3.6, except use 8 cycles of PCR instead of 12 to amplify libraries.

14. Streptavidin-captured DNA can be stored short-term at 4° C, but it is recommended to proceed directly to PCR after streptavidin capture.

15. Primer sequences are from [5], except that in repli-ATAC-seq the final two nucleotides in each primer are joined by a phosphorothioate bond. Primer 2 is indexed for multiplexing sequencing lanes.

Figure Legends

Figure 1. Schematic of repli-ATAC-seq protocol. The cell type of interest is pulsed with EdU and harvested. If using spike-in, freshly harvested, 100% EdU-labelled *D. melanogaster* S2 cells are mixed with the cells of interest prior to nuclei isolation and lysis. DNA is digested with Tn5 transposase and EdU+ DNA fragments are isolated through click biotinylation and streptavidin conjugation. These fragments are then amplified and sequenced using next-generation sequencing. Adapted from Stewart-Morgan et al., Mol Cell 2019.

Figure 2. Representative BioAnalyzer profiles of a repli-ATAC-seq library (top) and its matched, unbound control library (bottom).

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