Isolation of DNA by EdU-labelling for Mass Spectrometry (iDEMS)

Kathleen R. Stewart-Morgan
Novo Nordisk Foundation Center for Protein Research (CPR), Faculty of Health and Medical Sciences, University of Copenhagen, 2200 Copenhagen, Denmark https://orcid.org/0000-0001-7570-6217

Cristina E. Requena
MRC London Institute of Medical Sciences (LMS), Du Cane Road, London, W12 0NN, UK
https://orcid.org/0000-0001-5239-9162

Petra Hajkova (petra.hajkova@lms.mrc.ac.uk)
MRC London Institute of Medical Sciences (LMS), Du Cane Road, London, W12 0NN, UK
https://orcid.org/0000-0003-4145-1468

Anja Groth (anja.groth@cpr.ku.dk)
Novo Nordisk Foundation Center for Protein Research (CPR), Faculty of Health and Medical Sciences, University of Copenhagen, 2200 Copenhagen, Denmark https://orcid.org/0000-0003-0577-1771

Method Article

Keywords: DNA methylation, DNA hydroxymethylation, epigenetics, chromatin replication, mass spectrometry

Posted Date: January 12th, 2023

DOI: https://doi.org/10.21203/rs.3.pex-2074/v1

Abstract

DNA methylation and hydroxymethylation are important regulators of gene expression and genome stability in mammalian genomes. DNA replication dilutes these marks, and their restoration kinetics are important to epigenetic cell memory. We therefore developed isolation of DNA by EdU-labelling for Mass Spectrometry (iDEMS), a highly sensitive, quantitative mass spectrometry-based method for measuring DNA modifications on metabolically labelled DNA. Unlike sequencing-based methods, iDEMS does not require any amplification step, providing direct measurements from labelled DNA. Through additional methionine-isotope labelling and/ or incorporation of downstream steps such as chromatin immunoprecipitation, iDEMS facilitates novel insights into methylation metabolism and epigenetic crosstalk in chromatin. Excluding cell culture, DNA labelling and isolation requires two working days including an overnight digestion; preparation and running of samples requires three to four days.

This protocol is related to the publication “Quantifying propagation of DNA methylation and hydroxymethylation with iDEMS” in Nature Cell Biology.

Introduction

The epigenome both reflects and directs cell-specific transcription programs, and therefore is critical in defining cell identity. It comprises chemical modifications of both proteins associated with DNA, such as histones, and direct modification of DNA bases themselves. Faithful propagation of the epigenome must occur with every cell division to ensure continuity of cell identity in daughter cells. 5’-methylcytosine, or DNA methylation, is the most common DNA modification in eukaryotic genomes. It is a repressive mark, important in suppressing transcription and maintaining genome stability[1]. In mammals, DNA methylation decorates 70-80% of CpG dyads in most cell types, and symmetrically marks complementary DNA strands. DNA methylation can be oxidized to 5-hydroxymethylcytosine, or DNA hydroxymethylation[2]. Unlike DNA methylation, DNA hydroxymethylation tends to be found on only one of the two cytosines in a CpG dinucleotide. While DNA hydroxymethylation was first characterized as an intermediate in active DNA demethylation, further work has shown that it also acts as a stable epigenetic mark[3]. During S phase, the DNA modification landscape is diluted, generating hemi-methylated DNA strands and asymmetric distribution of hydroxymethlyation on sister chromatids (Figure 1). Accurate and timely restoration of these marks post-replication is critical to their roles in preserving epigenetic cell memory.

Metabolic labelling of DNA has proven a powerful approach for investigating the effects of DNA replication on chromatin[4]. A number of studies, including of DNA methylation restoration[5], have utilized the thymidine analog bromodeoxyuridine (BrdU) to label and track DNA. With the advent of 5-ethynyl-2'-deoxyuridine (EdU), which is detectable by highly sensitive click chemistry, shorter labelling times and

therefore higher resolution can be achieved. Recently, several groups have utilized EdU labelling to track DNA methylation restoration[6,7]. Whether using BrdU or EdU labelling, all of these studies employed next-generation bisulfite sequencing methods to profile DNA methylation on labelled DNA. Though bisulfite sequencing is a standard method for DNA methylation analysis that provides important positional information in addition to methylation status, it is known to be vulnerable to biases arising from DNA degradation during bisulfite conversion and subsequent differences in amplification efficiencies between DNA molecules[8]. Moreover, these studies reported different restoration times for DNA methylation, and none could explore the post-replicative restoration kinetics of DNA hydroxymethylation.

To investigate DNA methylation and hydroxymethylation post-replication using an alternative method, we developed iDEMS, or isolation of DNA by EdU-labelling for Mass Spectrometry. iDEMS employs a short (10 minute) EdU pulse to label nascent DNA, which can be chased to additionally track post-replicative maturation of DNA modifications. Genomic DNA is isolated post-labelling and sonicated. Isolation of labelled DNA is achieved through Click-IT biotinylation and streptavidin pulldown. Post-isolation, DNA can either be processed as double-stranded DNA (dsDNA) or subjected to denaturation for separate analysis of the newly replicated (EdU+ ssDNA) and parental ssDNA strands. Labelled DNA is then digested and analyzed via liquid chromatography linked to tandem mass spectrometry (LC-MS/MS). iDEMS provides quantitative measurements of absolute DNA methylation and hydroxymethylation on labelled DNA, without the need for amplification or chemical conversion. iDEMS can be combined with stable isotope labelling by amino acids in cell culture (SILAC) to provide unique insights into methionine metabolism, unobtainable by sequencing approaches. We also successfully integrated iDEMS with chromatin immunoprecipitation (ChIP) to investigate epigenetic crosstalk between DNA methylation and histone post-translational modifications post-replication.

The following protocol describes generation of matched EdU+ dsDNA, EdU+ ssDNA and parental (EdU-) ssDNA samples via iDEMS in cultured adherent cells. We include overall workflows with key steps and controls highlighted (Figures 2, 3). Options for SILAC-iDEMS and ChIP-iDEMS are also included.

Reagents

Reagents for iDEMS

Cell culture reagents (user defined; cell type-specific- ideally DMEM-based)

UltraPure Tris-HCl buffer (1 M, pH 7.5; Thermo Fisher, 15567027)

UltraPure EDTA (0.5 M, pH 8.0; Life Technologies, 15575-038)

NaCl (Sigma, S7653)

Tween 20 (Sigma, P9416)

NaOH (VWR, 28226.293)

20 mM 5-ethynyl-2’-deoxyuridine (EdU; Invitrogen, A10044) (1000x)

1X PBS, pH 7.4 (Gibco, 10010023) (cooled to 4 *°C*)

Ethanol (Sigma, 51976)

Zymo Quick-DNA Midiprep kit (Zymo, D4075)

LC/MS Grade Water (Thermo Fisher, 10777404)

Qubit dsDNA BR Assay Kit (Thermo Fisher, Q32850)

1X Click-IT buffer (Click-iT EdU Alexa Fluor 488 Imaging Kit, Thermo Fisher, C10337)

0.5 mM picolyl-azide-PEG4-biotin (Jena Bioscience, CLK-1167-100)

0.1 mM CuSO4 (Click-iT EdU Alexa Fluor 488 Imaging Kit, Thermo Fisher, C10337)

0.5 mM THPTA (Sigma, 762342)

10 mM sodium ascorbate (Click-iT EdU Alexa Fluor 488 Imaging Kit, Thermo Fisher, C10337)

Agencourt AMPure XP beads (Beckman Coulter, A63881)

Buffer EB (Qiagen, 19086)

Dynabeads MyOne Streptavidin T1 beads (Invitrogen, 65602)

Nucleoside digestion mix (NEB, M0649)

$^{13}C^{15}N$-dC (Silantes, #124603602)

$^{13}C^{15}N$-dG (Silantes, #124603603)

$d_2{}^{15}N_2$-hmdC (obtained in our study from T. Carell, Center for Integrated Protein Science at the Department of Chemistry, Ludwig-Maximilians-Universität München, Germany)

dC (Berry & ass., PY72106)

dG (Berry & ass., PR3452)

5mdC (Carbosynth, ND06242)

5hmdC (Berry & ass., PY7588)

Consumables

Cell scrapers (TPP, 9903)

CELLSTAR polypropylene tubes, conical bottom, 15 ml (Greiner Bio-One, 188271)

CELLSTAR polypropylene tubes, conical bottom, 50 ml (Greiner Bio-One, 227261)

DNA LoBind tubes, 1.5 ml (Eppendorf, 022431021)

DNA LoBind tubes, 2.0 ml (Eppendorf,. 0030108078)

Sorenson low-binding aerosol barrier tips, MicroGuard G, maximum volume 10 µl (Sigma, Z719374)

Sorenson low-binding aerosol barrier tips, MultiGuard, maximum volume 20 µl (Sigma, Z719412)

Sorenson low-binding aerosol barrier tips, MultiGuard, maximum volume 200 µl (Sigma, Z719447)

Eppendorf Dualfilter T.I.P.S. LoRetention (50–1,000 µl) (Eppendorf, EP0030078683)

Qubit Assay Tubes (Thermo Fisher, Q32856)

microTUBE AFA Fiber Pre-Slit Snap-Cap 6x16mm (Covaris, 520045)

Recipes

1X B&W buffer (5 mM Tris-HCl pH 7.5, 0.5 mM EDTA, 1 M NaCl, 0.05% Tween-20)

2X B&W buffer (10 mM Tris-HCl pH 7.5, 1 mM EDTA, 2 M NaCl, 0.1% Tween-20)

1X TE with 0.05% Tween-20 (10 mM Tris-HCl, pH 7.5, 1 mM EDTA, 0.05% Tween-20)

100 mM NaOH with 0.05% Tween-20 (freshly prepared before each use from 10 M NaOH stock)

70% ethanol (70% ethanol, 30% LC/MS grade water; cooled to -20 *°C*)

80% ethanol (80% ethanol, 20% LC/MS grade water; freshly prepared before each use)

If chasing EdU label in a timecourse:

10 mM thymidine (Sigma, T9250) (1000x)

1X PBS, pH 7.4 (Gibco, 10010023) (warmed to 37 *°C*)

Additional SILAC-iDEMS Reagents

DMEM without methionine or cysteine (Gibco, 21013-024) (note: may need to be user-defined for specific cell type)

L-methionine (*methyl*-$^{13}C,d_3$), 30 mg/L (Sigma, 299154)

cysteine hydrochloride, 63 mg/L, (Sigma, 30120)

Additional ChIP-iDEMS Reagents

BD Microlance stainless steel needles, 21G (BD, 304432)

Plastic syringe (1 ml) (Terumo, SS+01H1)

EGTA (0.5 M, sterile solution, pH 8.0; bioWorld, 40520008-2)

HEPES (Sigma, H4034)

KCl (Sigma, P9333)

MgCl2 (Sigma, M8266)

Sucrose for molecular biology (Sigma, S0389)

Glycerol (for molecular biology, >99% (vol/vol); Sigma, 56-81-5)

Triton X-100 (molecular biology grade; Sigma, T8787-100ml)

Counting chambers: KOVA Glasstic slide 10 with counting grids (KOVA International, 87144)

100 mM PMSF (Sigma, 93482-250ML) (100X)

Leupeptin (Sigma, 11034626001), 1 mg/ml (1000X)

Pepstatin (Sigma, 11524488001), 1 mg/ml (1000X)

Aprotinin (Sigma, 10981532001), 1 mg/ml (1000X)

Trichostatin A (Sigma, T8552-5MG), 1 mg/ml (1000X)

Sodium dodecyl sulfate (SDS) solution, 20% (wt/vol) (Sigma, 05030)

Liquid nitrogen

$CaCl_2$ (Sigma, 21097)

MNase (Worthington, cat. no. LS004797)

Qiagen QIAquick PCR Purification Kit (Qiagen, 28104)

ChIP grade antibody (user-specific)

IgG Dynabeads (Invitrogen, user-specific)

Qubit dsDNA HS Assay Kit (Thermo Fisher, Q32854)

Bioanalyzer High Sensitivity DNA Kit (Agilent, 5067-4626)

ChIP-iDEMS Recipes

Buffer A (10 mM HEPES pH 7.9, 10 mM KCl, 1.5 mM $MgCl_2$, 0.34M sucrose, 10% glycerol)

Buffer D (20 mM HEPES pH 7.9, 0.2 mM EDTA pH 8.0, 250 mM KCl, 20% glycerol, 0,2 Triton-X 100)

Low salt washing buffer (20 mM Tris-HCl pH 8.0, 2 mM EDTA, 150 mM NaCl, 1% Triton X-100, 0.1% SDS)

High salt washing buffer (20 mM Tris-HCl pH 8.0, 2 mM EDTA, 500 mM NaCl, 1% Triton-X 100, 0.1% SDS)

ChIP elution buffer (20 mM Tris-HCl pH 8.0, 10 mM EDTA, 1% SDS)

Equipment

Tissue culture hood

Tissue culture incubator

ThermoMixer C (Eppendorf, 5382000015)

E220- Evolution focused-ultrasonicator (Covaris, 500239)

Qubit 4 fluorometer (Thermo Fisher, Q33238)

DiaMag Rotator (Diagenode, B05000001)

DynaMag-2 magnet (Invitrogen, 12321D)

Vortex-Genie 2 (Scientific Industries, SI-A256)

Multifuge X4R Pro (Thermo Scientific, 75009515)

Centrifuge 5418 R (Eppendorf, 5401000010)

Agilent RRHD Eclipse Plus C18 2.1 × 100 mm 1.8 µm column on a HPLC 1290 system (Agilent)

Agilent 6490 triple quadrupole mass spectrometer (Agilent)

ChIP-iDEMS Equipment

Rotapure (Rotapure Lab Instruments)

2100 Bioanalyzer (Agilent, G2939BA)

Software

Agilent MassHunter Software v10.0

GraphPad Prism Software v9.2.0

Procedure

iDEMS

Cell Culture

1. Seed 4 x 15 cm dishes and grow until 70-80 % confluent in 30 ml of the appropriate media, corresponding to approximately 2 x 10^7 cells/ dish (Figure 2a).

a. NOTE: if doing a long timecourse, take the additional culturing time into account when determining the ideal seeding density at time of EdU labelling- plates should be 70-80% confluent at time of harvesting.

DNA Labelling (30 minutes)

1. Prepare needed cell culture reagents:

a. Prepare 30 ml appropriate media supplemented with 20 µM EdU/ plate and warm to 37 *°C.*

b. For liquid waste, prepare a 1 L beaker in TC hood.

c. Ensure 1X PBS at 4 *°C* and 70% EtOH at -20 *°C* are easily accessible.

2. Change media on dishes with 30 ml warmed 20 µM EdU media/ plate.

3. Incubate at 37 *°C* for 10 minutes.

4. After 10 minute incubation, retrieve plates from 37 *°C*.

5. Empty media into 1 L waste beaker by pouring. Pour the 1X PBS at 4 *°C* into each dish until covering the bottom of the dish to wash. Repeat wash, emptying 1X PBS into waste beaker.

a. NOTE: pouring liquid waste into a waste beaker is done for speed. This is crucial when working with multiple plates at a time. To keep EdU labelling to exactly 10 minutes in each plate, we recommend staggering media changes and washes by e.g. 30 seconds between plates. Be sure to dispose of liquid waste properly after cell harvesting.

6. Pour ice-cold 70 % EtOH at -20 *°C* onto plates. Continue to gDNA isolation or store dishes at -20 *°C* for up to 24 hours.

a. NOTE: pour a maximum of 15 ml 70 % EtOH onto plates - just enough to cover. In our hands, this enhances scraping efficiency and ultimately DNA yield.

If doing a timecourse: DNA Labelling and Chase

1. Prepare needed cell culture reagents:

a. Prepare 30 ml appropriate media supplemented with 20 µM EdU/ plate and warm to 37 *°C.*

b. Prepare 30 ml appropriate media supplemented with 10 µM thymidine/ plate and warm to 37 *°C.*

c. Prepare a 1 L beaker for liquid waste under TC hood.

d. Ensure 1X PBS at 4 *°C,* 1X PBS at 37 *°C* and 70 % EtOH at -20 *°C* are easily accessible.

2. Follow steps 2-4 as described in *DNA Labelling* to EdU-label dishes.

3. After 10 minute EdU incubation, pour media into 1 L waste beaker. Pour the 1X PBS at 37 *°C* into each dish until covering the bottom of the dish to wash. Repeat wash, pouring 1X PBS into waste beaker.

4. Add 30 ml warmed 10 μM thymidine media to dishes and return to incubator for the desired maturation time.

5. After the desired maturation interval, pour ice-cold 70 % EtOH at -20 *°C* onto dishes. Continue to gDNA isolation or store plates at -20 *°C* for up to 24 hours.

gDNA Isolation (1 hour, plus overnight digestion)

1. Scrape dishes to detach cells.

2. Transfer the 70 % EtOH containing cells from two dishes into a 50 ml Falcon tube. Repeat with the second two dishes (total: 2 x 50 ml Falcon tubes).

3. Spin tubes at 2,000 x g for 2 minutes at 4 *°C*.

4. Aspirate supernatant. Resuspend pellets in 1 ml 1X PBS and combine into one Falcon tube.

5. Aliquot cells into 4 x 15 ml Falcon tubes, 1 ml/ tube.

6. Spin tubes at 400 x g for 2 minutes at RT.

7. Aspirate supernatant.

8. Resuspend pellets in 1 ml 1X PBS by pipetting.

9. Add 1 ml BioFluid & Cell Buffer and 30 μl Proteinase K (both from Zymo Quick-DNA Midiprep kit) to each tube.

10. Vortex for 15 seconds. Incubate tubes at 55 *°C* with shaking at 1200 rpm on a thermomixer overnight.

11. Add 2 ml prepared Genomic Lysis Buffer (from Zymo Quick-DNA Midiprep kit) to each tube (total volume/ tube: 4 ml). Vortex for 15 seconds.

12. Prepare 4 ZymoSpin™ V-E Column/Reservoirs (from Zymo Quick-DNA Midiprep kit) by checking columns are firmly screwed into reservoirs and inserting into 50 ml Falcon tubes. Transfer lysates to columns.

13. Spin tubes at 1,000 x g for 5 minutes at RT. Discard flow through.

a. NOTE: if all the liquid does not pass through the column after 5 minutes, repeat spin until all liquid has passed through.

14. Add 9 ml DNA Pre-Wash Buffer (from Zymo Quick-DNA Midiprep kit) to column.

15. Spin tubes at 1,000 x g for 5 minutes at RT. Discard flow through.

16. Add 7 ml g-DNA Wash Buffer (from Zymo Quick-DNA Midiprep kit) to column.

17. Spin tubes at 1,000 x g for 5 minutes at RT. Discard flow through.

18. Remove and discard the reservoir and place the ZymoSpin™ V-E Column into a Collection Tube (from kit).

19. Spin tubes at 12,000 x g for 1 minute at RT.

20. Transfer columns to new Collection Tubes. Add 200 µl g-DNA Wash Buffer to columns using a 200 µl pipet tip.

21. Spin tubes at 12,000 x g for 1 minute at RT.

22. Transfer columns to 1.5 ml DNA LoBind tubes. Add 200 µl LC/MS grade water to column.

23. Incubate at RT for 5 minutes.

24. Spin tubes at 12,000 x g for 1 minute at RT.

25. Re-load the eluate onto the same column to re-elute. Repeat steps 23-24 and discard column (final volume: 200 µl).

26. Combine eluates derived from the same sample/ timepoint into one tube (final volume: 800 µl).

27. Use 1 µl eluate and the Qubit dsDNA BR Assay to quantify gDNA. Eluted gDNA can be stored at -20 *°C* or used immediately for sonication.

gDNA Sonication (15 minutes)

1. If gDNA yield was >60 µg, dilute DNA in LC/MS grade water to have 800 µl at a 77 ng/ µl concentration for sonication.

a. NOTE: gDNA yield can vary widely based on scraping and digestion efficiency. iDEMS works in our hands starting from as low as 40 µg gDNA; lower starting amounts have not been tested.

2. Aliquot gDNA into 6 x Covaris microTUBEs, 130 µl / tube (maximum 10 µg gDNA/ tube) (Figure 2b).

3. Sonicate to 300 bp-sized fragments in a prepared Covaris E220-Evolution using the following conditions:

a. Peak Incident Power (W): 140

b. Duty Factor: 10%

c. Cycles/ Burst: 200

d. Treatment Time: 80 seconds

i. NOTE: sonication conditions may have to be optimized for different cell types and/ or different sonication platforms.

4. Combine sonicated DNA from the same sample into a 1.5 ml DNA LoBind tube.

5. Transfer 5 µl sonicated DNA into a separate 1.5 ml DNA LoBind tube. This is the “total gDNA” control (Figure 2c). Store at -20 *°C* until ready for mass spectrometry analysis.

6. Take remaining sonicated DNA and proceed directly to Click-IT biotinylation.

Click-IT Biotinylation (45 minutes)

1. Aliquot sonicated DNA into 6 x 1.5 ml DNA LoBind tubes, 120 µl / tube (Figure 2d).

a. NOTE: 120 µl is used to accommodate some loss of DNA during sonication.

2. Prepare THPTA-CuSO4 premix by mixing 2 µl 50 mM THPTA and 0.2 µl 100 mM CuSO4 per sample in a separate 1.5 ml DNA LoBind tube.

3. Prepare 10X buffer additive by mixing 2 µl 100X buffer additive and 18 µl PCR-grade H2O per sample in a separate tube.

4. Set up the click reaction by adding the reagents to the purified DNA in the following order: 35.8 µl LC/MS grade water, 20 µl 10X Click-iT buffer, 2 µl 100 mM picolyl-azide-PEG4-biotin, 2.2 µl THPTA-CuSO4 premix, 20 µl 10X buffer additive (final volume: 200 µl).

5. Incubate for 30 minutes at RT, in the dark or covered in foil.

6. During incubation, equilibrate AMPure beads at RT for 30 minutes prior to use. Keep AMPure beads at RT to use during parental ssDNA purification (see below).

DNA Purification (45 minutes)

1. To purify DNA, add 100 µl equilibrated AMPure beads to each tube (0.5:1 bead ratio).
2. Mix thoroughly by vortexing.
3. Incubate the tube(s) at RT for 10 minutes to bind large, unwanted DNA fragments to the beads.

4. During incubation, prepare another 1.5 ml DNA LoBind tube with 500 µl AMPure beads.
5. During incubation, prepare 400 ul of 80 % ethanol per sample.
6. During incubation, warm a thermoblock to 37 °C.
7. Place the tube(s) on the magnet to capture the beads. Incubate until the liquid is clear, ~5 minutes.
8. 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.
9. Incubate tube(s) at RT for 10 minutes to bind the desired DNA fragments to the beads.
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. On the rack, turn the tubes 180°, forcing the beads through the ethanol to the opposite wall of the tube.
13. Incubate the tube(s) on the magnet at RT for ≥30 seconds.
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.
16. Dry the beads at RT for 1-2 minutes. Caution: Avoid over-drying of the beads, as it may result in dramatic yield loss.
17. Remove the tube(s) from the magnet. Resuspend the beads in 252 µl of buffer EB (Qiagen).
18. 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.
19. Incubate for 5-10 minutes to elute DNA and evaporate residual ethanol.
20. Place the tube(s) on the magnet to capture the beads. Incubate until the liquid is clear.
21. Carefully transfer 250 µl of the supernatant to a new low-binding tube. You now have 6 tubes per sample/ timepoint, each containing 250 µl biotinylated and purified DNA.

Streptavidin Pulldown (45 minutes)

1. Resuspend the stock of Myone T1 streptavidin beads by vortexing.
2. Pipet 10 µl of bead suspension per tube into a 1.5 ml DNA LoBind tube. Pellet the beads using a magnetic rack (≥30 seconds). Remove and discard the supernatant.
3. Remove tubes from the magnetic rack and add 200 µl of 1xB&W buffer. Mix by pipetting. Place tubes 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 250 µl 2X B&W buffer per tube.

6. Add 250 µl resuspended streptavidin beads to each tube (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.
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 9-10, waiting 1 minute off the magnetic rack between washes.
12. NOTE: Perform washes on maximum 4-6 reactions at a time to avoid overdrying the beads.
13. Place all tubes on magnetic rack. Once beads collect, aspirate supernatant and resuspend all tubes of the same **sample/ timepoint** in 100 µl 1XB&W buffer (i.e., combine the beads in the 6 tubes from each sample into 1 tube. Now you have 1 tube total (Figure 2e)).
14. Spin tubes briefly. Pellet beads on a magnetic rack. Remove supernatant.
15. Wash beads with 200 µl 1XB&W buffer and mix by pipetting.
16. Pellet the beads using a magnetic rack (≥30 seconds). Remove and discard the supernatant.
17. Repeat steps 14-15 twice (3 washes total), waiting 1 minute off the magnetic rack between washes.
18. Resuspend sample in 150 µl 1xB&W buffer.
19. Label a 1.5 ml DNA LoBind tube “EdU+ dsDNA”. Transfer 50 µl of resuspended beads (1/3 of total) to this labelled tube. This is the EdU+ dsDNA sample (Figure 2f).
20. Keep the EdU+ dsDNA sample on ice during alkaline washes of the remaining 100 µl (2/3) of beads.

Stranded Sample Generation (10 minutes)

1. Freshly prepare 100 mM NaOH with 0.05% Tween and set aside at RT.

2. Label a 1.5 ml DNA LoBind tube “Parental ssDNA”. Keep aside at RT.

3. Place tube containing the 100 µl bead suspension on the magnet to collect the beads and remove supernatant.

4. Wash beads with 200 µl 1XB&W buffer and mix by pipetting.

5. Pellet the beads using a magnetic rack (≥30 seconds). Remove and discard the supernatant.

6. Repeat steps 4-5, waiting 1 minute off the magnetic rack between washes.

7. Add 100 µl of prepared 100 mM NaOH with 0.05% Tween and mix by thorough pipetting. Keep at RT for 1 minute off the magnetic rack.

8. Place the tube on the magnet to pellet the beads.

9. Remove the supernatant with a pipette and **transfer it** to the “Parental ssDNA” tube.

10. Repeat alkaline washes (step 7-9) 2 more times, being sure to add all supernatant to the “Parental ssDNA” tube. Final volume in the “Parental ssDNA” tube should be 300 µl.

11. Put “Parental ssDNA” tube on ice.

EdU+ dsDNA/ EdU+ ssDNA Sample Washes (10 minutes)

1. Return the “EdU+ dsDNA” tube to the magnet. Now 2 tubes, 1 “EdU+ dsDNA” tube and 1 tube with beads subjected to NaOH denaturation (these are the “EdU+ ssDNA” samples) (Figure 2g), should be on the magnet.

2. Remove supernatant from the “EdU+ dsDNA” tube and perform two washes with 200 µl 1xBW buffer for both “EdU+ dsDNA” and “EdU+ ssDNA” tubes.

3. Wash twice with 200 µl 1X TE with 0.05% Tween-20.

4. Wash once with 200 µl 10 mM Tris-HCl pH 7.5.

5. Wash twice with 200 µl LC/MS water.

6. Resuspend beads in 10 µl LC/MS water. Store at -20 *°C* until ready for mass spectrometry analysis.

Parental ssDNA Purification (30 minutes)

1. To purify DNA, add 540 µl equilibrated AMPure beads to each tube (1.8:1 bead ratio).
2. Mix thoroughly by vortexing.
3. Incubate the tube at RT for 10 minutes to bind the desired DNA fragments to the beads.
4. During incubation, prepare 400 µl of 80 % ethanol per sample.
5. During incubation, warm a thermoblock to 37 °C.
6. Place the tube on the magnet to capture the beads. Incubate until the liquid is clear.
7. Carefully remove and discard supernatant.
8. Keeping the tube on the magnet, add 200 µl of freshly prepared 80 % ethanol. On the rack, turn the tube 180°, forcing the beads through the ethanol to the opposite wall of the tube.
9. Incubate the tube on the magnet at RT for ≥30 seconds.
10. Carefully remove and discard the ethanol.
11. Repeat steps 8-10 once. Try to remove all residual ethanol without disturbing the beads, using a P10 pipette if necessary.

12. Dry the beads at RT for 1-2 minutes. Caution: Avoid over-drying of the beads, as it may result in dramatic yield loss.
13. Remove the tube from the magnet. Resuspend the beads in 12 µl of LC/MS grade water.
14. Put the tube with lid 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.
15. Incubate for 5-10 minutes to elute DNA and evaporate residual ethanol.
16. Place the tube on the magnet to capture the beads. Incubate until the liquid is clear.

8. Carefully transfer 10 µl of the supernatant to a new low-binding tube. This is the “Parental ssDNA” sample (Figure 2h). Store at -20 *°C* until ready for mass spectrometry analysis.

SILAC-iDEMS

Cell Culture

1. Prepare both standard (“light”) media containing unlabelled amino acids, and “heavy” media containing labelled methionine.

a. NOTE: media will be cell type-specific.

2. Seed 4 x 15 cm dishes and grow until 70-80% confluent in 30 ml of media containing standard amino acids, corresponding to approximately 2 x 10^7 cells/ dish (Figure 2a).

a. NOTE: if doing a long timecourse, take the additional culturing time into account when determining the ideal seeding density at time of EdU labelling- plates should be 70-80% confluent at time of harvesting.

3. Prepare needed cell culture reagents:

a. For liquid waste, prepare a 1 L beaker in TC hood.

b. Ensure 1X PBS at 37 *°C* is easily accessible.

4. 4 hours prior to EdU labelling, retrieve dishes and pour ”light” media into 1 L waste beaker. Pour the 1X PBS at 37 °C into each dish until covering the bottom of the dish to wash. Repeat wash, pouring 1X PBS into waste beaker.

5. Add warmed “heavy” media to dishes and return to incubator for 4 hours.

6. Proceed with iDEMS protocol from *DNA Labelling* step.

a. NOTE: once you have switched from “light” to “heavy” media, maintain cells in heavy media, including during the EdU label and any maturation time, until cell harvesting (Figure 3a).

ChIP-iDEMS

NOTE: the following protocol has been adapted from[9], which describes native chromatin immunoprecipitation from EdU-labelled samples in detail.

1. Seed 2 x 15 cm dishes/ ChIP and grow until 70-80% confluent in 30 ml of the appropriate media, corresponding to approximately 2 x 10^7 cells/ dish.

a. NOTE: if doing a long timecourse, take the additional culturing time into account when determining the ideal seeding density at time of EdU labelling- plates should be 70-80% confluent at time of harvesting.

DNA Labelling (15 minutes)

2. Prepare needed cell culture reagents:

a. Prepare appropriate media with 20 μM EdU and warm to 37 *°C.*

b. For liquid waste, prepare a 1 L beaker in TC hood.

c. Ensure 1X PBS at 4 *°C* is easily accessible.

3. Change media on dishes with warmed 20 μM EdU media.

4. Incubate at 37 *°C* for 10 minutes.

5. After 10 minute incubation, retrieve plates from 37 *°C*.

6. Empty media into 1 L waste beaker by pouring. Pour the 1X PBS at 4 *°C* into each dish until covering the bottom of the dish to wash. Repeat wash, emptying 1X PBS into waste beaker.

a. NOTE: pouring liquid waste into a waste beaker is done for speed. This is crucial when working with multiple plates at a time. To keep EdU labelling to exactly 10 minutes in each plate, we recommend staggering media changes and washes by e.g. 30 seconds between plates. Be sure to dispose of liquid waste properly after cell harvesting.

7. Proceed immediately to nuclei isolation.

Nuclei Isolation (1.5 hours)

1. Collect the cells by scraping with a clean cell scrapper in a cold room and transfer the cell suspension to 50 ml Falcon tubes, 1 tube/ dish.

2. To collect the remaining cells, rinse each plate with 10 ml of ice-cold 1X PBS and collect in the same 50 ml Falcon tubes. Keep on ice.

3. Centrifuge for 10 minutes at 4 °C, 300 x g. Discard the supernatant.

a. During spin: prepare 10 ml of Buffer A + inhibitors. Keep on ice.

i. NOTE: inhibitors needed can vary based on ChIP of interest. As standard, we use 1 mM PMSF, 1 µg/ml leupeptin, 1 µg/ml pepstatin, and 1 µg/ml aprotinin as our inhibitor cocktail.

4. Resuspend the pellet from 2 plates in 1.5 ml of Buffer A + inhibitors.

5. Transfer pellet to a 1.5 ml DNA LoBind tube.

6. Centrifuge for 5 minutes at 4 °C, 1,300 × g. Discard the supernatant.

7. Resuspend the pellet in 1 ml of Buffer A + inhibitors.

8. Add 10 µl 10 % Triton X-100 (final concentration: 0.1 %) and mix by inverting gently.

9. Lay the tube horizontally on ice in a cold room for 7 minutes.

10. Centrifuge for 5 minutes at 4 °C, 1,300 × g. Discard the supernatant.

11. Resuspend the pellet in 1 ml of Buffer A + inhibitors using a wide-orifice pipette tip. You can make the wide-orifice tips yourself by cutting the tips with a clean scalpel or scissors prior to use.

12. Centrifuge for 5 minutes at 4 °C, 1,300 × g. Discard the supernatant.

13. Resuspend the pellet in 1 ml of Buffer A + inhibitors using a wide-orifice tip.

14. Pipet 1 µl resuspended nuclei into a new 1.5 ml DNA LoBind tube containing 99 µl Buffer A + inhibitors (1:100 dilution). Keep the dilution on ice.

15. Distribute remaining suspension between 2 x 500 µl aliquots in 2 x 1.5 ml DNA LoBind tubes.

16. Snap-freeze the 500 µl aliquots in liquid nitrogen. Store at -80 °C until ready to proceed with MNase digestion.

17. To count nuclei, load 10 µl of the diluted nuclei suspension in the chamber of a Kova glasstic slide and count nuclei manually.

MNase Digestion and Chromatin Preparation (4-6 hours)

1. Thaw nuclei on ice.

2. Pre-warm the nuclei for 5 minutes, at 30 °C, 300 rpm in a thermomixer.

3. Add 5 µl of 100 mM $CaCl_2$ to 500 µl nuclei suspension and mix by inverting the tube.

4. Add MNase and mix by inverting the tube. Use 1 µl of Worthington MNase (50 U/µl) per 2.5 x 10^7 of nuclei (= 0.2 µl/ 5e6 nuclei).

5. Incubate in a thermomixer for 20 minutes, at 30 °C, 300 rpm in a thermomixer.

a. NOTE: to keep the exact same digestion time for every sample, we recommend adding MNase and stopping digestion in the same order and to allow a fixed interval of time (e.g. 30 seconds to 1 minute) between tubes. It is critical to store MNase in small aliquots and use a new aliquot each time.

6. Place the tube on ice and stop digestion by adding 10 µl of a premixed 1:1 solution of 0.1 M EGTA, pH 8.0 and 0.5 M EDTA, pH 8.0. Mix immediately by inverting the tube several times.

7. Add 5 µl 10% Triton X-100 and 75 µl 2 M KCl. Mix immediately by inverting the tube several times.

8. Supply the digested chromatin with protease inhibitors. Mix immediately by inverting the tube.

9. Elutriate digested chromatin through a 21 Gauge needle attached to a 1 or 2 ml syringe (up and down 10 times) in a cold room.

10. Incubate the tubes rotating at 20 rpm in a cold room for 2-4 hours to release the chromatin.

11. Centrifuge for 10 minutes, at 4 °C, 14,000 x g.

12. Transfer the supernatant containing the soluble chromatin fraction to a new 1.5 ml DNA LoBind tube and keep on ice. Keep the pellet for quality control (next step).

a. NOTE: The native chromatin cannot be stored or frozen. Proceed immediately to quantitating chromatin.

Quality Control of MNase-Digested Chromatin and Isolation of Total gDNA Sample (1.5 hours)

1. Resuspend the pellet saved from Step 12 (above) using 100 µl TE, pH 8.0 and 2.5 µl 20% SDS.

2. Transfer 10 µl of the supernatant from Step 12 (above) to a new 1.5 ml DNA LoBind tube and add 90 µl TE, pH 8.0 and 2.5 µl 20% SDS.

3. Incubate both pellet and aliquot of supernatant for 15 minutes, at 37 °C, 300 rpm in a thermomixer.

4. Purify using a Qiagen QiaQuick PCR purification column following the manufacturer's instructions, except performing the final elution in 50 µl of LC/MS H2O.

5. Check the fragment size distribution by running 1 µl purified DNA on an Agilent Bioanalyzer chip, an equivalent fragment analyzer or a 1.8 % agarose gel in 1x TBE buffer.

6. Use 1 µl purified DNA to measure the DNA concentration of the pellet and the supernatant fractions, using a Qubit fluorometer and following the manufacturer's instructions for the Qubit dsDNA BR Assay Kit.

a. NOTE: Typically, about 90 % of chromatin (measured as amount of DNA) is released into the supernatant, while about 10 % will remain in the pellet.

b. NOTE: The MNase digestion should result in predominantly mononucleosome-sized fragments. MNase digestion conditions must be calibrated for each cell type and each batch of MNase.

7. Keep DNA isolated from the supernatant fraction. Add 40 µl LC/MS grade water to purified DNA (new volume: 90 µl).

8. Aliquot 10 µl into a new 1.5 ml DNA LoBind tube. This is the "total gDNA" control (Figure 3b). Store at -20 °C until ready for mass spectrometry analysis.

9. Keep remaining 80 µl at -20 °C for eventual processing as the "EdU+ ssDNA" control (described in *Click-IT Biotinylation*, below).

Native ChIP: Antibody Incubation (30 minutes, plus overnight incubation)

13. Aliquot the needed amount of Buffer D and add appropriate protein inhibitors.

14. For each immunoprecipitation take an equivalent of 50 µg of DNA of the soluble chromatin isolated post-MNAse digestion.

15. Adjust the volume to 500 µl with Buffer D. For the 'no antibody' control, take 20 µg of chromatin and adjust to 200µl with Buffer D.

16. Add the desired amount of antibody per 50 µg of chromatin. Do not add antibody to “no antibody “control.

a. NOTE: The amount of antibody may need to be optimized depending on the antibody and the protein of interest.

17. Incubate overnight on a rotating platform (Rotapure) in a cold room.

Native ChIP: Antibody Capture, Washes and Elution (3.5 hours)

1. Aliquot the needed amounts of Buffer D, Low Salt Wash Buffer and High Salt Wash Buffer and add appropriate protein inhibitors. Keep all at 4 °C or on ice to keep cold.

2. Aliquot 150 µl of the appropriate IgG Dynabeads (depending on the primary antibody used) per ChIP reaction and 50 µl for the ‘no antibody’ control into a new 1.5 ml DNA LoBind tube.

3. Wash beads 3 times with 500 µl of Buffer D. For each wash, incubate for 1 minute on a magnet on ice by turning the tube in the rack. Collect beads on the magnet and discard the supernatant. Resuspend beads in Buffer D (150 µl per ChIP reaction plus 50 µl for each ‘no antibody’ control).

4. Add 150 µl of washed IgG Dynabeads to each ChIP reaction and 50 µl to each ‘no antibody’ control.

5. Incubate for 2-3 hours on a rotating platform at 20 rpm at 4 °C.

a. During incubation, place 1 x 1.5 ml DNA LoBind tube/ sample on ice to get cold.

6. Put the tubes on a magnetic rack placed on ice to collect the beads. Remove and discard the supernatant.

7. Working on ice, add 500 µl of prepared, ice-cold Low-Salt Wash buffer and transfer sample to the pre-chilled 1.5 ml DNA LoBind tube.

8. Bring the tubes to a cold room and incubate on a rotating platform for 5 minutes. Collect beads on the magnet and discard the supernatant. Place the tubes on ice.

9. Working in the cold room, repeat steps 7-8 two more times, except for the transfer step to a new tube.

10. Add 500 µl of prepared, ice-cold High-Salt Wash buffer to the tubes. Bring the tubes to the cold room, invert several times to resuspend gently the beads and incubate on a rotating platform for 5 minutes.

11. Collect beads on the magnet and discard the supernatant. Place the tubes on ice.

12. Working in the cold room, repeat steps 10-11 two more times.

13. Add 100 µl of ChIP elution buffer to each tube to elute the immunoprecipitated chromatin.

14. Incubate in a thermomixer for 15 minutes, at 37 °C, 1400 rpm.

15. Collect beads on the magnet and transfer supernatant to a new 1.5 mL tube.

16. Repeat steps 13-15 and combine the supernatants from the two elutions in one tube (final volume: 200 µl).

17. Purify DNA using the Qiaquick PCR Purification kit following the manufacturer's protocol. Spin the PB buffer + eluate in two rounds, 600 µl/ round.

18. After washing and spinning to dry, place the column to a 1.5 ml DNA LoBind tube and elute the purified DNA by adding 50 µl of Buffer EB to the center of the column membrane. Let the column stand for 1 minute and centrifuge for 30 seconds at 14,000 x g at RT.

19. Samples can be stored at -20 °C at this point for up to one year.

Quality Control of Immunoprecipitated DNA (1 hour)

1. Use 0.5 µl ChIP DNA and 20 µl of 'no antibody' control to measure DNA concentration with a Qubit fluorometer and following the manufacturer's instructions for the Qubit dsDNA HS assay kit. No DNA should be detected in the 'no antibody' control.

2. Use 1 µl ChIP DNA to check the size distribution of the immunoprecipitated material using a Agilent Bioanalyzer or an equivalent fragment analyzer.

DNA Purification and Isolation of ChIP dsDNA Sample (45 minutes)

1. To size-select immunoprecipitated DNA, add 40 µl equilibrated AMPure beads to the 50 µl DNA in each tube (0.8:1 bead ratio).
2. Mix thoroughly by vortexing.
3. Incubate the tube(s) at RT for 10 minutes to bind large, unwanted DNA fragments to the beads.
4. During incubation, prepare another 1.5 ml DNA LoBind tube with 110 ul AMPure beads.
5. During incubation, prepare 400 µl of 80 % ethanol per sample.
6. During incubation, warm a thermoblock to 37 °C.
7. Place the tube(s) on the magnet to capture the beads. Incubate until the liquid is clear.
8. 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.

9. Incubate tube(s) at RT for 10 minutes to bind the desired DNA fragments to the beads.
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. On the rack, turn the tubes 180°, forcing the beads through the ethanol to the opposite wall of the tube.
13. Incubate the tube(s) on the magnet at RT for ≥30 seconds.
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.
16. Dry the beads at RT for 1-2 minutes. Caution: Avoid over-drying of the beads, as it may result in dramatic yield loss.
17. Remove the tube(s) from the magnet. Resuspend the beads in 88 µl LC/MS water.
18. 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.
19. Incubate for 5-10 minutes to elute DNA and evaporate residual ethanol.
20. Place the tube(s) on the magnet to capture the beads. Incubate until the liquid is clear.
21. Carefully transfer 86 µl of the supernatant to a new low-binding tube.

22. Use 1 µL to measure the DNA concentration using a Qubit fluorometer and following the manufacturer's instructions for the Qubit dsDNA HS Assay Kit.

23. Aliquot 5 µl into a new 1.5 ml DNA LoBind tube. This is the "ChIP dsDNA" sample (Figure 3c). Store at -20 °C until ready for mass spectrometry analysis.

24. Take remaining 80 µl DNA and proceed directly to Click-IT. Keep equilibrated AMPure beads at RT for post-Click-IT purification.

Click-IT Biotinylation (45 minutes)

1. Thaw 80 µl DNA aliquot saved from MNase digestion and perform Click-IT on this sample in parallel with the size-selected ChIP DNA sample(s) to generate the "EdU+ ssDNA" control.

2. 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 DNA LoBind tube.

3. Prepare 10X buffer additive by mixing 1 µl 100X buffer additive and 9 µl PCR-grade H2O per sample in a separate tube.

4. Set up the click reaction by adding the reagents to the purified DNA in the following order: 10 µl 10X Click-iT buffer, 1 µl 100 mM picolyl-azide-PEG4-biotin, 1.1 µl THPTA-CuSO4 premix, 10 µl 10X buffer additive (final volume: 100 µl).

5. Incubate for 30 minutes at RT.

DNA Purification (30 minutes)

1. To purify DNA, add 200 µl equilibrated AMPure beads to each tube (2:1 bead ratio).
2. Mix thoroughly by vortexing.
3. Incubate the tube at RT for 10 minutes to bind the desired DNA fragments to the beads.
4. During incubation, prepare 400 µl of 80 % ethanol per sample.
5. During incubation, warm a thermoblock to 37 °C.
6. Place the tube on the magnet to capture the beads. Incubate until the liquid is clear.
7. Carefully remove and discard supernatant.
8. Keeping the tube on the magnet, add 200 µl of freshly prepared 80 % ethanol. On the rack, turn the tube 180°, forcing the beads through the ethanol to the opposite wall of the tube.
9. Incubate the tube on the magnet at RT for ≥30 seconds.
10. Carefully remove and discard the ethanol.
11. Repeat steps 8-10 once. Try to remove all residual ethanol without disturbing the beads, using a P10 pipette if necessary.
12. Dry the beads at RT for 1-2 minutes. Caution: Avoid over-drying of the beads, as it may result in dramatic yield loss.
13. Remove the tube from the magnet. Resuspend the beads in 52 µl Buffer EB.
14. Put the tube with lid 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.
15. Incubate for 5-10 minutes to elute DNA and evaporate residual ethanol.
16. Place the tube on the magnet to capture the beads. Incubate until the liquid is clear.
17. Carefully transfer 50 µl of the supernatant to a new low-binding tube.

Streptavidin Pulldown (45 minutes)

1. Perform streptavidin pulldown as described in the main iDEMS protocol, with the following modifications: use 20 µl Myone T1 streptavidin beads/ tube; resuspend in 50 µl 2X B&W Buffer/ tube (final volume in each tube: 100 µl); do not set aside “EdU+ dsDNA” sample (unless desired).

Stranded Sample Generation (10 minutes)

1. Perform stranded sample generation as described in the main iDEMS protocol, with the following modifications: do not set aside “Parental ssDNA” sample (unless desired).

2. Following stranded sample generation, you now have generated an “EdU+ ssDNA” sample and “ChIP EdU+ ssDNA” sample(s) (Figures 3d-e). Store at -20 °C until mass spectrometry analysis.

5mdC/5hmdC quantification by LC-MS/MS

Sample preparation (2-4 hours, plus overnight incubation)

1. Digest a minimum of 1 ng of DNA to nucleosides overnight at 37 °C using nucleoside digestion mix (NEB, M0649).

a. NOTE: For EdU labeled samples, the digestion will be on beads. Following overnight digestion, place tube on magnetic rack and transfer the sample to a new 1.5 ml DNA LoBind tube.

2. Prepare heavy labelled nucleoside mix as a spike –in containing: 50 fmol of $^{13}C^{15}N$-dC, 50 fmol of $^{13}C^{15}N$-dG and 0.25 fmol of $d_2{}^{15}N_2$-hmdC). Prepare enough to use 10 µl per sample and standard curve point.

3. Prepare samples by mixing the digested DNA with the isotope-labelled synthetic nucleoside spike-in in a 1:1 volume ratio.

a. NOTE: The amount of digested DNA injected to LC-MS/MS for quantification must be experimentally tested. This ensures that a similar amount of nucleosides is injected between samples and that measurements fall within the linear range of quantification (calculated by running standards in parallel).

b. NOTE: Use digestion mix only (without DNA) at the same dilution as a control for potential external nucleic acid contamination.

4. Prepare standard curve samples using the commercial nucleosides at range of dilutions (dC and dG: 0.1 - 1000 fmol; 5mdC and 5hmdC: 0.05 – 50 fmol, in 10 µl) with the same spike-in mix as in Step 2. Mix the standards with the heavy labelled spike-ins in a 1:1 volume ratio.

Mass spectrometry (1 hour, plus 20 minutes per sample run)

1. Inject samples (20 µl, containing 1-3 ng DNA) into an Agilent HPLC 1290 system connected to an Agilent 6490 triple quadrupole mass spectrometer. For the liquid chromatography, use an Agilent RRHD Eclipse Plus C18 2.1 × 100 mm 1.8 µm column. Mobile phase A: 0.1% formic acid in 100% water and B: 0.1% formic acid in 80% methanol. Chromatographic details are in Table 1.
2. Run two technical replicates per sample, plus blanks (water) every five runs and quality controls (QCs) every 10 runs. Prepare QCs from standard mix (50 fmol of dC and dG; 2.5 fmol of 5mdC and 5hmdC).
3. Analyze the nucleosides separated on the LC column using multiple reaction monitoring (MRM) mode. Set specific parameters and transition pairs to be monitored (details in Tables 2 and 3) using the Agilent MassHunter Acquisition Software.

Data analysis

1. Calculate the area of each peak by integrating the peaks found according to their mass, using the Agilent MassHunter Quantitative Analysis Software.
2. To calculate the concentration of nucleosides in each sample, generate a standard curve for each individual nucleoside species by dividing the peak area of the standard by the peak area of the matching isotope-labelled synthetic nucleoside used as a spike-in. Use a statistics software (e.g., GraphPad Prism) to obtain a linear regression with a weighing factor 1/x. Consider only the data points that 1) do not diverge from the projected linear regression curve by more than 20% and 2) satisfy the signal-to-noise ratio > 10. A new standard curve must be included and the parameters of accuracy checked within each sample run.
3. For each injected experimental sample, determine the nucleoside peak ratio (sample/relevant spike-in) and calculate the absolute concentration using the standard curve.
4. Present the measured dC derivatives' concentrations as normalized to dG levels (measured in the same samples – for originally dsDNA samples) or by total dC (dC(t)) levels (dC + 5mdC + 5hmdC - for originally ssDNA samples).

Troubleshooting

Please see notes provided in relevant Procedure sections.

Time Taken

Excluding cell culture, DNA labelling and isolation requires two working days including an overnight digestion; preparation and running of samples requires three to four days. Timing for each step is provided in the Procedure.

Anticipated Results

References

1. Allshire, R. C. & Madhani, H. D. Ten principles of heterochromatin formation and function. *Nat Rev Mol Cell Bio* 19, 229–244 (2018).

2. Wu, X. & Zhang, Y. TET-mediated active DNA demethylation: mechanism, function and beyond. *Nat Rev Genet* 18, 517–534 (2017).

3. Bachman, M. *et al.* 5-Hydroxymethylcytosine is a predominantly stable DNA modification. *Nat Chem* 6, 1049–1055 (2014).

4. Stewart-Morgan, K. R., Petryk, N. & Groth, A. Chromatin replication and epigenetic cell memory. *Nat Cell Biol* 22, 361–371 (2020).

5. Charlton, J. *et al.* Global delay in nascent strand DNA methylation. *Nat Struct Mol Biol* 25, 327–332 (2018).

6. Ming, X. *et al.* Kinetics and mechanisms of mitotic inheritance of DNA methylation and their roles in aging-associated methylome deterioration. *Cell Res* 30, 980–996 (2020).

7. Xu, C. & Corces, V. G. Nascent DNA methylome mapping reveals inheritance of hemimethylation at CTCF/cohesin sites. *Science* 359, 1166–1170 (2018).

8. Olova, N. *et al.* Comparison of whole-genome bisulfite sequencing library preparation strategies identifies sources of biases affecting DNA methylation data. *Genome Biol* 19, 33 (2018).

9. Petryk, N. *et al.* Genome-wide and sister chromatid-resolved profiling of protein occupancy in replicated chromatin with ChOR-seq and SCAR-seq. *Nat Protoc* 16, 4446–4493 (2021).

Acknowledgements

We are grateful to members of the Groth and Hajkova labs for providing fruitful discussion and input. This project has received funding from the European Union's Horizon 2020 research and innovation programme under the Marie Skłodowska-Curie grant agreement no. 747332 and from the Lundbeck Foundation (R219-2016-394) as grants to K.R.S.-M. Research in the Groth laboratory is supported by grants from the Lundbeck Foundation (R198-2015-269; R313-2019-448), the European Research Council (ERC CoG no. 724436), the Independent Research Fund Denmark (7016-00042B), and the Novo Nordisk Foundation (NNF21OC0067425) to A.G. Research at CPR is supported by the Novo Nordisk Foundation (NNF14CC0001). Work in the Hajkova laboratory is supported by MRC funding (MC_US_A652_5PY70) and an ERC grant (ERC-CoG no. 648879–dynamicmodifications) to P.H.

Figures

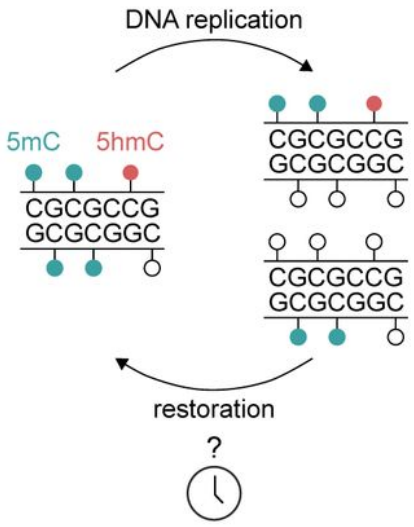

Figure 1

iDEMS quantifies DNA methylation and hydroxymethylation levels on replicated DNA, allowing accurate measurements of post-replication restoration kinetics.

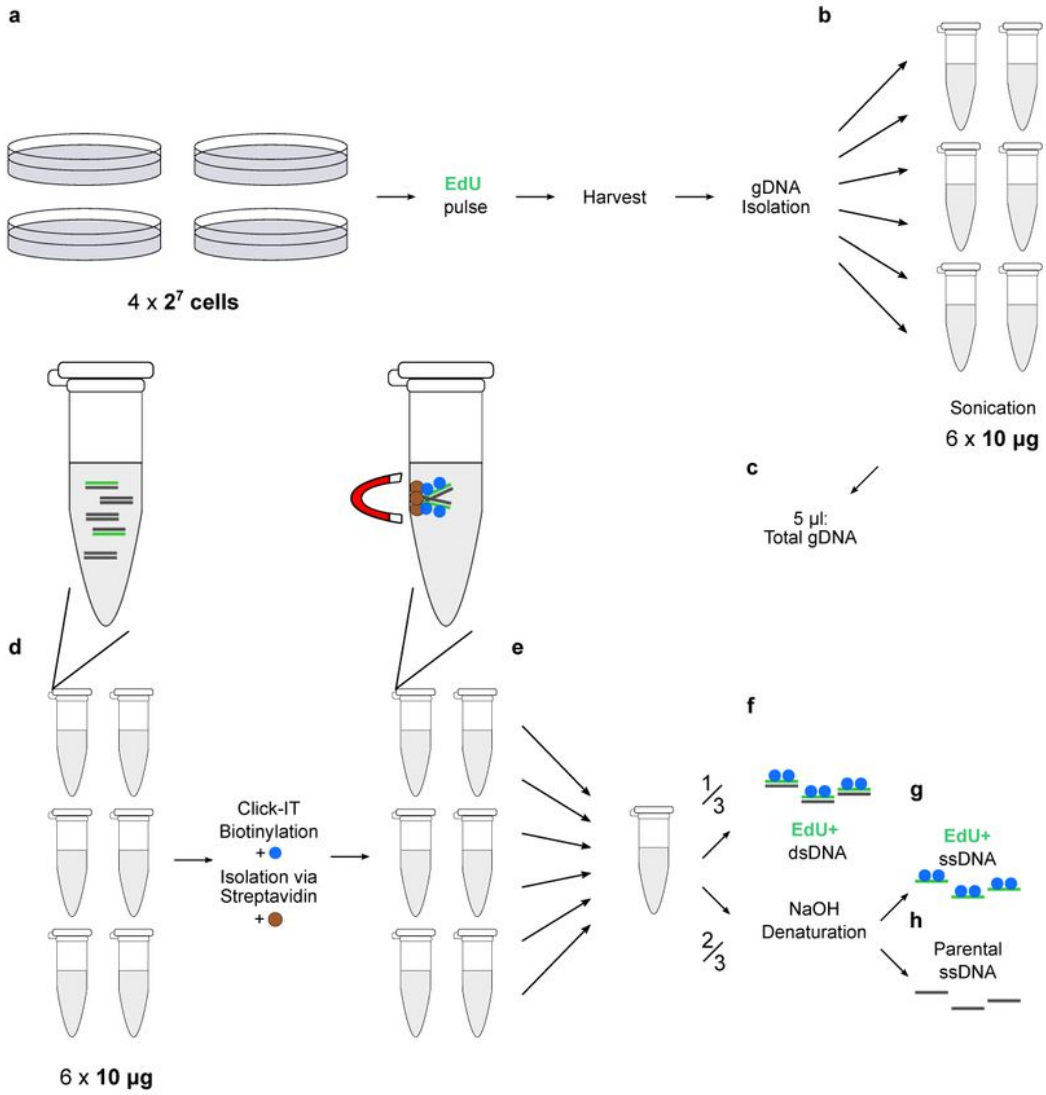

Figure 2

Key steps in iDEMS protocol. a) Grow adherent cells to confluency in 4 x 15 cm dishes. b) After isolating gDNA, split sample into 6 tubes for sonication. c) Pool the 6 sonicated samples and take the “total gDNA” control. d) After taking the “total gDNA” control, again split sample into 6 tubes for Click-IT, DNA purification and streptavidin pulldown. e) After isolating biotinylated, EdU+ dsDNA via streptavidin pulldown, consolidate tubes originating from the same DNA sample into one. f) Aliquot 1/3 of the DNA

on beads and purify as the “EdU+ dsDNA” sample. g) Perform strand separation with NaOH and purify the DNA fraction remaining on beads as the “EdU+ ssDNA” sample. h) Following strand separation, purify the DNA fraction in the alkaline wash supernatant as the “Parental ssDNA” sample.

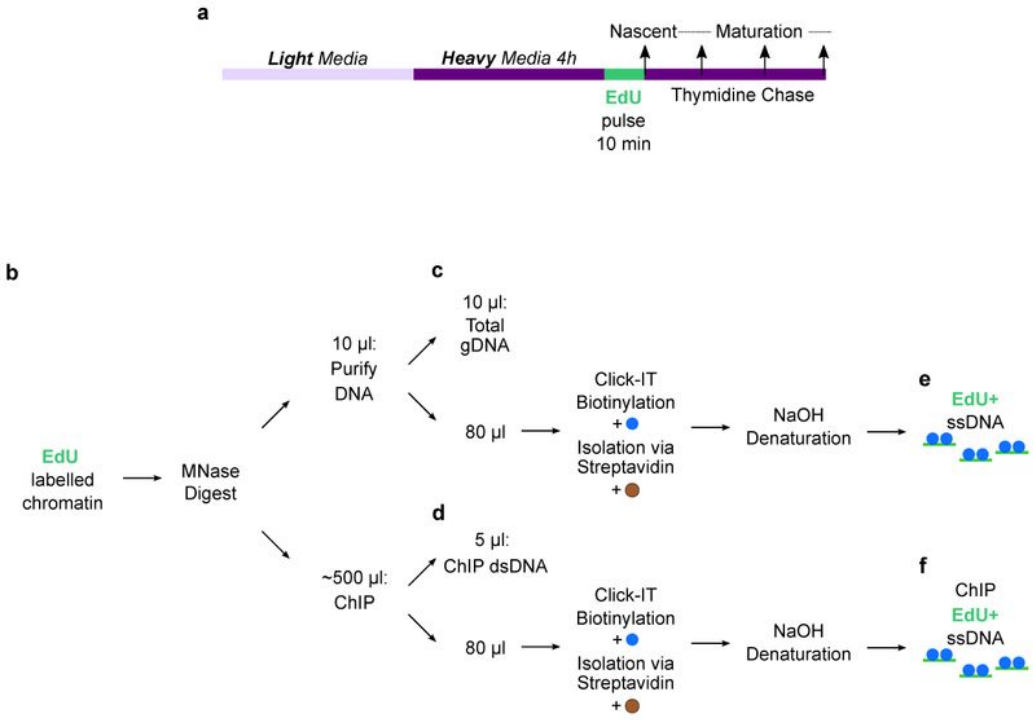

Figure 3

Key steps in SILAC-iDEMS (a) and ChIP-iDEMS (b-e) protocols. a) Perform all subsequent culturing steps in heavy media post-media swap. b) ChIP-iDEMS workflow. c) Take the “total gDNA” control from purified DNA after MNase chromatin digestion. d) Take the “ChIP dsDNA” control from purified DNA after ChIP. e) Perform Click-IT, streptavidin pulldown and strand separation on purified DNA after MNase chromatin digestion to generate “EdU+ ssDNA” sample. f) Perform Click-IT, streptavidin pulldown and strand separation on purified DNA after ChIP to generate “ChIP EdU+ ssDNA” sample. a) is adapted from Stewart-Morgan, Requena et al., Nature Cell Biology 2022.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- ProtocolExchangeTable1.xlsx
- ProtocolExchangeTable2.xlsx
- ProtocolExchangeTable3.xlsx