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Mapping protein–DNA interactions with DiMeLo-seq

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Abstract

We recently developed directed methylation with long-read sequencing (DiMeLo-seq) to map protein–DNA interactions genome wide. DiMeLo-seq is capable of mapping multiple interaction sites on single DNA molecules, profiling protein binding in the context of endogenous DNA methylation, identifying haplotype-specific protein–DNA interactions and mapping protein–DNA interactions in repetitive regions of the genome that are difficult to study with short-read methods. With DiMeLo-seq, adenines in the vicinity of a protein of interest are methylated in situ by tethering the Hia5 methyltransferase to an antibody using protein A. Protein–DNA interactions are then detected by direct readout of adenine methylation with long-read, single-molecule DNA sequencing platforms such as Nanopore sequencing. Here we present a detailed protocol and practical guidance for performing DiMeLo-seq. This protocol can be run on nuclei from fresh, lightly fixed or frozen cells. The protocol requires 1–2 d for performing in situ targeted methylation, 1–5 d for library preparation depending on desired fragment length and 1–3 d for Nanopore sequencing depending on desired sequencing depth. The protocol requires basic

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Author contributions

A.M., N.A. and A.S. designed the study. A.M., N.A. and L.D.B. performed the experiments. A.M., R.M. and J.M. developed dimelo software package. A.M. and N.A. analyzed and interpreted the data. A.M., N.A. and R.M. made the figures. A.M. and J.M. wrote the manuscript, with input from N.A., R.M., L.D.B., K.S., G.K., A.F.S. and A.S. A.S. and N.A. supervised the study.

Competing interests

N.A., A.M., K.S., A.F.S. and A.S. are co-inventors on a patent application related to this work. The remaining authors declare no competing interests.

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molecular biology skills and equipment, as well as access to a Nanopore sequencer. We also provide a Python package, dimelo, for analysis of DiMeLo-seq data.

Introduction

Common methods for mapping protein–DNA interactions rely on selective amplification and sequencing of short DNA fragments from regions bound by the protein of interest[1-7]. These powerful short-read methods for profiling protein–DNA interactions have been used to map the binding patterns of thousands of proteins in human cells[8]. However, because the measurement uses amplified fragments of DNA, these methods dissociate joint binding information at neighboring sites, remove endogenous DNA methylation and are limited in detecting haplotype-specific interactions and interactions in repetitive DNA. Directed methylation with long-read sequencing (DiMeLo-seq) addresses these limitations by using long-read, single-molecule sequencing to measure protein–DNA interactions genome wide[9] (Fig. 1).

With DiMeLo-seq, adenines in the vicinity of a protein of interest are methylated in situ by tethering the Hia5 methyltransferase to an antibody using protein A. In this way, DiMeLo-seq uses antibody-targeted DNA methylation to record protein-binding events directly on genomic DNA. Protein–DNA interactions are then detected by direct readout of adenine methylation with long-read, single-molecule DNA sequencing platforms. Single-molecule sequencing technologies, such as Oxford Nanopore Technologies (ONT) sequencers and the Pacific Biosciences Sequel IIe, can directly detect DNA modifications, including endogenous CpG methylation and exogenous adenine methylation deposited by DiMeLo-seq to mark protein-binding events. Sequencing single, native DNA molecules, as opposed to sequencing short, amplified clones, enables simultaneous detection of endogenous CpG methylation and protein binding on the same read. Additionally, these single-molecule sequencing platforms can sequence much longer reads compared with those generated with illumina sequencing, producing reads up to a megabase in length and enabling detection of multiple protein binding events across long distances. Furthermore, long-read, single-molecule sequencing makes DiMeLo-seq capable of detecting protein-binding events in a truly genome-wide fashion, including repetitive regions of the genome where short reads cannot be accurately positioned.

Development of the protocol

DiMeLo-seq was inspired by the targeted methylation strategy used in DNA adenine methyltransferase identification sequencing (DamID-seq)[7] and builds from short-read techniques for mapping protein–DNA interactions (e.g., Cleavage Under Targets and Tagmentation (CUT&Tag)[5], Cleavage Under Targets and Release Using Nuclease (CUT&RUN)[6] and pA-DamID[10]), as well as recent work that implements long-read sequencing and detection of exogenous methylation to profile chromatin accessibility (Fiber-seq[11], single-molecule long-read accessible chromatin mapping sequencing assay (SMAC-seq)[12], single-molecule adenine methylated oligonucleosome sequencing assay (SAMOSA)[13], nanopore sequencing of nucleosome occupancy and methylome (NanoNOMe)[14], methyltransferase treatment followed by single-molecule long-read

sequencing (MeSMLR-seq)[15]). Developing DiMeLo-seq required substantial optimization, with over 100 conditions tested[9]. These optimization experiments revealed critical protocol components that improved efficiency including these key observations: (1) The methyltransferase Hia5 performs better than EcoGII in situ, (2) digitonin and Tween-20 dramatically increase methylation levels compared with other detergents for nuclear permeabilization and (3) the following protocol parameters all improve methylation levels: a low salt concentration, inclusion of BSA in all buffers, increased incubation times and replenishment of the methyl donor during activation.

Overview of the procedure

The procedure is divided into four main sections:

1. Nuclear extraction and permeabilization, with optional cell fixation (Steps 1–11).
2. In situ targeted DNA methylation, to mark interactions between a protein of interest and DNA (Steps 12–38). This involves incubation with the primary antibody (Steps 12–15), incubation with the secondary antibody/methyltransferase fusion protein (Steps 20–23) and activation of the methylation reaction (Steps 34–38), along with appropriate quality control (QC) steps.
3. DNA extraction, with protocols optimized for the desired length and quality of output DNA reads (Step 39). At this step, optional sequence enrichment procedures may be employed to enable selective sequencing of target regions with higher read depth (Step 40).
4. DNA sequencing and downstream analysis (Steps 41–43). Sequencing libraries are prepared using protocols optimized for the desired length and quality of output DNA reads, then sequenced on a long-read Nanopore sequencer (Step 41). Modified bases are called using appropriate basecalling models; the resulting data can be analyzed and visualized using the dimelo Python package (Steps 42–43).

Applications of the method

DiMeLo-seq can be used to profile the genome localization of any DNA-binding protein for which there is a specific, high-quality antibody. The protocol can be used on fresh, fixed or frozen cells from culture or from primary tissue. As DiMeLo-seq uses antibody-based targeting, it is also able to profile posttranslational modifications such as protein acetylation, methylation and phosphorylation. In our previous work[9], we demonstrated the application of DiMeLo-seq for profiling binding landscapes of LMNB1, CTCF, H3K9me3 and CENPA in cultured human cells. Here we applied DiMeLo-seq to profile H3K27ac, H3K27me3 and H3K4me3 in cultured human cells and H3K9me3 in *Drosophila melanogaster* embryos.

Comparison with other methods

The key distinguishing feature of DiMeLo-seq compared with other methods is that protein–DNA interactions are measured on long, native, single molecules of DNA. Long reads facilitate mapping of protein–DNA interactions and histone modifications in highly repetitive regions of the genome, measurement of multiple binding events on

the same chromatin fiber and detection of haplotype-specific interactions. Measurement of native DNA molecules allows for binding assessment in the context of endogenous CpG methylation. Additionally, single-molecule sensitivity enables measurement of heterogeneous binding at a given locus.

Long-read sequencing has enabled the recent development of a handful of new methods for profiling chromatin. For example, Fiber-seq[11], SMAC-seq[12], SAMOSA[13], NanoNOMe[14] and MeSMLR-seq[15] are all methods that profile chromatin accessibility on long molecules of DNA and can infer protein–DNA binding footprints, although they do not directly measure specific target proteins. Furthermore, recent studies described BIND&MODIFY[16] and nanoHiMe-seq[17], methods that use similar strategies as DiMeLo-seq to profile protein–DNA interactions, albeit with lower reported sensitivity.

Traditional methods for profiling protein–DNA interactions such as ChIP-seq, CUT&RUN, CUT&Tag and DamID-seq all rely on amplification of short DNA fragments, producing sequencing reads that are hundreds of base pairs in length. These methods use coverage as a proxy for binding and exhibit an inverse relationship between resolution and read length. Short reads often preclude haplotype phasing, mapping to repetitive regions and measuring multiple binding events on the same DNA molecule. Additionally, these short-read methods require amplification, thereby losing the endogenous mCpG marks. Joint protein binding and mCpG measurement can be done with chromatin-immunoprecipitation bisulphite sequencing (BisChIP-seq, also known as ChIP-BS-seq), but this requires lossy and harsh bisulfite conversion that degrades DNA[18,19]. ChIP-seq also requires physical separation of protein-bound DNA regions and extensive washes, which can reduce sensitivity and prohibit single-cell resolution. DiMeLo-seq is a complimentary method that provides a solution to the limitations listed above. Moreover, like other antibody-based approaches, such as ChIP-seq, CUT&Tag and CUT&RUN, DiMeLo-seq is compatible with primary cells and can be used to target posttranslational modifications.

DamID-seq is a method that reports protein–DNA interactions without the use of antibodies, instead relying on a fusion of an exogenous methyltransferase (Dam) and a protein of interest to methylate DNA in vivo. The methylated sites are then digested with a methylation-sensitive restriction enzyme to convert methyl marks into a signal that can be detected with Illumina sequencing. Dam is an adenine methyltransferase that recognizes GATC sites, limiting the resolution of this method in GATC-depleted regions of the genome. DamID-seq requires a genetically tractable system for expression of the Dam fusion protein. Further, optimizing the Dam fusion expression levels and induction times present major hurdles in adopting this method compared with antibody-based approaches. Importantly, with DamID-seq, binding patterns of the introduced Dam fusion rather than the endogenous protein are measured. Thus, DamID-seq is a powerful approach for profiling protein–DNA interactions and for capturing an integrated signal of where a protein has bound over the induction time period, but these limitations have made DamID-seq adoption slower relative to antibody-based approaches. We have demonstrated that cells expressing a methyltransferase fusion construct can also be sequenced with long-read platforms, combining the benefits of DamID-seq and DiMeLo-seq[9].

As there is no amplification step, DiMeLo-seq experiments require substantial input (typically ~1 million cells) to generate sufficient material for sequencing. With short-read methods such as CUT&Tag and DamID-seq, protein-bound regions are selectively amplified and sequenced, thereby enriching for protein-bound regions and allowing for input as low as a single cell. While DiMeLo-seq cannot provide genome-wide coverage of single cells, the single-molecule sensitivity of long-read sequencing effectively provides single-cell resolution at a given genomic site, allowing the user to measure cell-to-cell heterogeneity of protein binding at that site. We assessed the effective single-cell resolution of DiMeLo-seq in a previous study[9] showing a strong correlation between DiMeLo-seq interaction frequency and single-cell DamID-seq interaction frequency. Without enrichment for regions of interest, DiMeLo-seq will sequence the whole genome uniformly, requiring deep sequencing to achieve sufficient coverage of specific target regions. However, there are options to enrich for regions of interest with DiMeLo-seq such as alpha higher-order repeat restriction and enrichment by size (AlphaHOR-RES)[9], other restriction-enzyme-based enrichment approaches[20] and the ONT Cas9-based targeted library preparation kit (SQK-CS9109). Adaptive sampling methods could also be used, such as Readfish[21], UNCALLED[22] and the adaptive sampling that is built into ONT's MinKNOW software.

Experimental design

Cell preparation—We have applied DiMeLo-seq to profile protein–DNA interactions in human cell lines, including GM12878, HEK293T, HG002 and HAP1, and in *D. melanogaster* embryos here and in our previous study[9]. Cell lines are cultured under the recommended conditions and are collected for DiMeLo-seq typically at 75–100% confluency. To collect the cells, we perform a single wash with cold phosphate-buffered saline (PBS) and then begin nuclear permeabilization.

In this protocol, we illustrate DiMeLo-seq on GM12878 cells as follows. GM12878 cells (GM12878, Coriell Institute; mycoplasma tested; RRID: CVCL_7526) were maintained in RPMI-1640 with $_{L}$-glutamine (Gibco, 11875093) supplemented with 15% fetal bovine serum (VWR 89510-186) and 1% penicillin–streptomycin (Gibco, 15070063) at 37 °C in 5% CO_2. For each target, 3.24 M cells from fresh culture were input to DiMeLo-seq.

For details of *D. melanogaster* embryo preparation, refer to Supplementary Methods.

Cell type-specific and target-specific experimental parameters—While the standard DiMeLo-seq protocol described here has performed consistently for all cell types tested, application to other cell types and primary tissue may require tuning of digitonin concentration for efficient nuclear permeabilization. A digitonin concentration of 0.02% (wt/vol) has worked well for human GM12878, HG002, HAP1, HEK293T and *Drosophila* embryos. The optimal digitonin concentration can be determined using Trypan blue (Fig. 2a,b). Successful permeabilization allows Trypan blue to localize to the nucleus (Fig. 2b). If too little digitonin is used, Trypan blue internalization is sparse, as in Fig. 2a. If too much digitonin is used, fewer nuclei are recovered after digitonin treatment relative to the number of cells input. Recovery of ~80–90% should be expected and all nuclei should appear permeabilized as in Fig. 2b. Primary tissue also requires upstream processing for

nuclear extraction before the nuclear permeabilization step (Supplementary Methods). It is important that the nuclear extraction method does not contain NP-40, as we have found this detergent can greatly reduce methylation.

As long as a sufficient number of nuclei can be collected, DiMeLo-seq can be applied to any cell type or primary tissue. We typically start with 1–5 million cells for a single protein target. We do not see appreciable loss of nuclei during the nuclear permeabilization step for the cell types we have tested with 0.02% (wt/vol) digitonin, so it is sufficient to quantify input cell count rather than quantifying nuclei after permeabilization. However, with other cell types and detergent concentrations, there may be higher levels of nuclei loss during permeabilization. In these cases, counting nuclei after permeabilization may be advisable to assure there are 1–5 million nuclei for the primary antibody-binding step. When preparing cells for multiple experiments or targets, we often permeabilize nuclei in a single batch; we typically start with up to 20 million cells in 1 ml of Dig-wash, divide nuclei into separate tubes for each target and then continue with primary antibody binding for each experiment in parallel.

When working with new protein targets, the key variables to optimize are the antibody concentration and the extent of fixation for targets with low binding affinity. An antibody dilution of 1:50 has worked well for all targets reported here and in Altemose et al.[9]. Extensive washes are performed after antibody binding to remove any unbound antibody, making excess antibody less detrimental. We have demonstrated that light fixation is compatible with the DiMeLo-seq workflow. If targeting a protein that binds transiently, including fixation (optional Steps 3–7) may improve signal by preventing the protein from dissociating from the DNA during the DiMeLo-seq protocol. However, care should be taken to evaluate the susceptibility of any new protein targets to epitope masking by fixation[23].

Recommended controls—Typical controls include an IgG isotype control and a free pA-Hia5 control. The IgG isotype control measures nonspecific antibody binding. The free pA-Hia5 control measures chromatin accessibility, similar to Fiber-seq and related methods, and is analogous to the Dam-only control used in DamID-seq[11,24]. This control is performed by excluding the primary antibody and pA-Hia5 binding steps (Steps 12–23) and instead adding pA-Hia5 before activation (Step 34) at 200 nM. While these controls are not required, they provide a useful measure of background methylation and bias caused by variable chromatin accessibility. Excluding pA-Hia5 as a control to account for modified basecalling errors can also be included. If troubleshooting a DiMeLo-seq experiment, using one of the antibodies and cell lines validated here and in Altemose et al.[9] may also be a useful control.

Choice of commercial kits and sequencing platforms—Commercially available kits and techniques for DNA extraction, library preparation and sequencing are rapidly improving. The protocol described here produces consistent localization profiles shown below and in Altemose et al.[9], but it is important to note that after the in situ methylation steps, any DNA extraction method, library preparation kit, flow cell chemistry and sequencing device can be used as long as N^6-methyladenosine (m6A) is maintained (no amplification is performed) and the flow cell and device have basecalling models available

for calling m6A. We have also demonstrated sequencing of DiMeLo-seq samples with Pacific Biosciences' Sequel IIe[9].

Sequencing considerations—The key considerations for sequencing are fragment size and sequencing depth. The target N50 (half of sequenced bases are from a fragment size of N50 or larger) varies by application. Longer reads may be desired when mapping to repetitive regions, probing coordinated binding events at longer distances or phasing reads. For example, if interested in studying multiple binding events on single molecules, the target protein's binding site density and the number of sites the user would like to capture on a single molecule should inform the desired fragment size. With ligation-based library preparation we typically target an N50 of ~20–50 kb, which results in fragment size distributions as in Fig. 2c,d. With other library preparation kits (e.g., SQK-ULK114), much larger fragments can be sequenced; however, there is a tradeoff between fragment length and throughput. If a user targets larger fragment sizes, the total sequencing output for the flow cell, and thus total coverage, will be reduced.

Greater sequencing depth provides more accurate and sensitive recovery of binding landscapes. This target sequencing depth will also vary by application, depending on the binding footprint of the protein, the mappability of the region of interest and the biological question at hand. Final libraries can be saved and flow cells can be reloaded, so it is recommended to do an initial pilot run with shallow sequencing followed by deeper sequencing as needed. For example, for initial tests of new protein targets in human cells, we typically sequence to 0.3–1× coverage (~1–3 Gb) to validate and determine optimal experimental conditions, and then sequence more deeply to ~5–45× coverage depending on the analysis we are performing. For example, when targeting CTCF, we sequenced to ~25× coverage and detected 60% of ChIP-seq peaks with a false positive rate of 1.6% (ref. 9). See the sequencing saturation analysis with CTCF-targeted DiMeLo-seq in Altemose et al.[9].

Additional experimental considerations—The protocol will be kept up to date (for details, see Supplementary Methods). The version used for this manuscript is version 2 (ref. 25). Sequencing a single sample with the product list described in this manuscript costs roughly US$1,100. This calculation assumes an average antibody price and ligation-based library preparation without multiplexing. On a MinION flow cell, this yields ~25 Gb data, or ~8× coverage of the human genome. On a PromethION flow cell, this yields ~125 Gb data, or ~40× coverage of the human genome.

All spins are at 4 °C for 3 min at 500*g*. Spinning in a swinging bucket rotor can help pellet the nuclei. To prevent nuclei from lining the side of the tube, break all spins into two parts: 2 min with the tube hinge facing inward, followed by 1 min with the tube hinge facing outward. This two-part spin is not needed if using a swinging bucket rotor. Working with Eppendorf DNA LoBind tubes can reduce loss of material. Use wide-bore tips when working with nuclei. Do not use NP-40 or Triton-X100 for nuclear extraction, permeabilization or any other stage of the protocol, as they appear to dramatically reduce methylation activity. We use Tween-20 to reduce hydrophilic nonspecific interactions and BSA to reduce hydrophobic nonspecific interactions. We also found that including BSA at the activation step substantially increases methylation activity. For pA-incompatible

antibodies, a secondary antibody can be used as a bridging antibody, but performance is diminished; instead, we recommend using pA/G-Hia5 for pA-incompatible antibodies. See Supplementary Table 1 for a performance comparison for pA/G-Hia5 and pA-Hia5. While both achieve a comparable on-target to off-target methylation ratio, pA/G-Hia5 on-target methylation rates are slightly lower that those with pA-Hia5 when targeting LMNB1 in HEK293T cells.

We have found that the pA-Hia5 fusion protein can be stored at −80 °C for at least 6 months without decline in performance. We typically spin down the pA-Hia5 protein aliquots at 10,000*g* for 10 min at 4 °C before each experiment to remove insoluble protein and to confirm that there has been no reduction in soluble protein concentration.

Analysis—We have created a Python package called dimelo for analysis of DiMeLo-seq data (https://github.com/streetslab/dimelo). The dimelo package input format requires raw output files produced by the Nanopore sequencer to first be converted to BAM files. Recommendations for the basecalling and alignment steps, which will create an aligned BAM file with ‘Mm’ and ‘Ml’ tags that describe methylation calls, can be found in the package documentation (https://streetslab.github.io/dimelo/). For this manuscript, we have used Megalodon (v2.3.1) and Guppy (v4.5.4) with the Rerio res_dna_r941_min_modbases-all-context_v001.cfg basecalling model. An ONT account is useful for accessing user manuals and for troubleshooting. Basecalling is being rapidly improved by ONT and others, so basecalling suggestions reported here are likely to become outdated quickly. After basecalling and alignment, the resulting BAM file is the input to the QC, visualization and custom analysis functions from the dimelo software package. This analysis software can be run as an imported Python module or from the command line. Figure 3 provides a summary of the functions included in the dimelo package.

A recommended workflow is to first run qc_report to generate summary statistics and histograms for metrics such as coverage, read length, mapping quality, basecall quality and alignment quality (Fig. 4). Next, three functions are provided for visualization: plot_enrichment, plot_browser and plot_enrichment_profile. All functions take BAM file(s) as input and region(s) of interest defined as a string or BED file.

The plot_enrichment function compares methylation levels across samples or across different genomic regions. This tool is useful for looking at overall on- versus off-target methylation and for comparing methylation levels in regions of interest across samples.

The plot_browser function allows the user to view single molecules with base modifications colored according to the probability of methylation within a region of interest. This function can either produce a static PDF of the single molecules or an interactive HTML file that allows the user to zoom and pan around the browser plot, using plotting code adapted from Methplotlib[26]. Plots of aggregate coverage and the fraction of methylated bases over the window of interest are also generated with this function.

The plot_enrichment_profile function creates single-molecule plots and an aggregate plot of the fraction of methylated bases centered at features of interest defined in a BED file.

For example, one may enter a BED file with the locations of features of interest (i.e., the binding motif for a given protein, transcription start sites (TSSs), etc.) to view the methylation profiles around those features. Inputting multiple BAM files creates an overlay of the methylation profiles across samples, while inputting multiple BED files creates an overlay of methylation profiles for a given sample across the different sets of regions defined in the BED files.

The parse_bam function summarizes the base modification information from a BAM file and stores it into an easy to manipulate data format. This gives users the ability to more easily create custom figures and analyses.

For all functions, the user can specify the modification(s) of interest to extract: ‘A’, ‘CG’ or ‘A+CG’ to annotate exogenous mA, endogenous CpG methylation or both, respectively. The probability threshold for calling a base as modified is also a parameter to each function. For discussion of threshold determination see Supplementary Note 6 of Altemose et al.[9].

Expertise needed to implement the protocol—To perform DiMeLo-seq and analyze the data produced, basic molecular biology skills and basic command line skills are required. Experience working with long molecules of DNA is also beneficial as care must be taken to maintain long fragments for sequencing[27].

Limitations

The performance of DiMeLo-seq is strongly dependent on the quality of the antibody used to target the protein of interest. For proteins that do not have a specific high-quality antibody compatible with protein A, one could consider epitope tagging or perform in vivo expression of a protein–methyltransferase fusion[28]. The activity of pA-Hia5 can vary across enzyme preparations; if performing comparative studies, it may be advisable to perform all experiments with the same enzyme batch. Enzyme activity can also be evaluated before use, as described in the Supplementary Methods. DNA must be accessible for Hia5 to efficiently methylate in situ. Thus, targets in less accessible regions of the genome may require longer incubations or deeper sequencing. Additionally, this preferential methylation of open chromatin results in signal bias. Hia5 may also methylate DNA in *trans* if three-dimensional contacts bring topologically associated loci close enough to the target protein, potentially creating nonspecific background. Such artifacts should be accounted for if absolute measurement of binding frequency is desired. DiMeLo-seq experiments typically require ~1 million cells as input, although Concanavalin A beads (which we previously showed are compatible with DiMeLo-seq) and lower-input library preparation kits can reduce required input material[9]. Unlike other protein–DNA interaction mapping methods that enrich protein-bound DNA, the standard DiMeLo-seq protocol and sequencing library preparation yields uniform coverage of the entire genome. If only particular regions of the genome are of interest, the protocol can be modified with an enrichment step or targeted sequencing library preparation.

The resolution of DiMeLo-seq can be considered both at the single molecule level and in ensemble measurements. This resolution is dependent on the choice of target and antibody, the adenine density of the target locus and the local chromatin environment.

Across molecules in aggregate, we have observed a slightly larger binding footprint with DiMeLo-seq compared with other methods. For example, when targeting CTCF, DiMelo-seq measures an 88-bp footprint compared to ~50 bp for methods such as DNase I footprinting and ChIP-exo. This is probably because Hia5 cannot reach the DNA directly adjacent to the binding event within ~20 bp (ref. 9). On single molecules, DiMeLo-seq can localize the center of binding footprints to within ~200 bp; however, this may be improved with optimized peak calling algorithms. Single-molecule resolution is also related to the measurement sensitivity, which can vary according to the same parameters as resolution. Therefore, both sensitivity and resolution are reduced in heterochromatin and in GC-rich regions of the genome.

Here and in our previous study, we have benchmarked DiMeLo-seq's performance in targeting LMNB1, CTCF, H3K9me3, CENPA, H3K27ac, H3K27me3 and H3K4me3 (ref. 9). When targeting CTCF and LMNB1, we estimated 54% and 59% sensitivity (94% specificity), but this is dependent on the protein, antibody and chromatin environment and must be evaluated for new targets[9]. Transiently bound proteins may benefit from the optional fixation step at the start of the DiMeLo-seq protocol; however, as described by others[29], we have observed that fixation decreases DNA fragment sizes. High levels of fixation are also known to mask epitopes and prevent antibody binding[23]. Therefore, the user should weigh the possible benefits of fixation against the potential fragment size hit at sequencing and potential reduction in signal.

To assess the accuracy of DiMeLo-seq, we developed a simple peak calling algorithm to identify binding sites de novo and used this peak caller to identify CTCF binding sites. At ~25× coverage, 60% of ChIP-seq peaks are detected with a false positive rate of 1.6% (ref. 9). In our previous study, we found that among the peaks detected with DiMeLo-seq that were not annotated ChIP-seq peaks, 10% overlapped 1-kb marker deserts and gaps in the hg38 reference and were undetectable by ChIP-seq. Another 12% of these peaks fell within 500 bp of a known CTCF motif. While this analysis allows for comparison of sensitivity and accuracy between ChIP-seq and DiMeLo-seq, further development of peak calling algorithms, which take into account the systematic biases associated with DiMeLo-seq, will probably improve the sensitivity and accuracy of this technique.

Materials

Biological materials

▲ **CRITICAL** This protocol can be used for cell lines or for primary cells. In this section we list cells in which we have validated the use of DiMeLo-seq.

- Cell lines (the following have been validated here and by Altemose et al.[9]):
- GM12878 (GM12878, Coriell Institute; mycoplasma tested), RRID: CVCL_7526
- HEK293T (CRL-3216, American Type Culture Collection; validated by microsatellite typing and mycoplasma tested), RRID: CVCL_0063
- HG002 (GM24385, Coriell Institute; mycoplasma tested), RRID: CVCL_1C78

- HAP1 (UC Berkeley Cell Culture Facility; validated by short tandem repeat typing and tested for mycoplasma), RRID: CVCL_Y019

  ▲ **CAUTION** The cell lines used in your research should be regularly checked to ensure they are authentic and are not infected with mycoplasma.

- *D. melanogaster* embryos (for processing details, see Supplementary Methods)

Reagents

Reagents for in situ protocol

- HEPES–KOH 1 M pH 7.5 (Boston BioProducts, cat. no. BBH-75-K)
- NaCl 5 M (Sigma-Aldrich, cat. no. 59222C-500ML)
- Spermidine 6.4 M (Sigma-Aldrich, cat. no. S0266-5G)
- Roche complete ethylenediaminetetraacetic acid (EDTA)-free Protease Inhibitor Tablet (Sigma-Aldrich, cat. no. 11873580001)
- BSA (Sigma-Aldrich, cat. no. A6003-25G)
- Digitonin (Sigma-Aldrich, cat. no. 300410-250MG)

  ▲ **CAUTION** Acute toxic and health hazard; work in fume hood when making digitonin solution.

- Tween-20 (Sigma-Aldrich, cat. no. P7949-100ML)
- Tris–HCl 1 M pH 8.0 (Invitrogen, cat. no. 15568025)
- KCl (Sigma-Aldrich, cat. no. PX1405-1)
- EDTA 0.5 M pH 8.0 (Invitrogen, cat. no. 15575-038)
- Ethylene glycol-bis(2-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid (EGTA) 0.5 M pH 8.0 (Fisher, cat. no. 50-255-956)
- *S*-Adenosylmethionine (SAM) 32 mM (NEB, cat. no. B9003S)
- Paraformaldehyde (PFA), 16% (wt/vol) (if performing fixation) (EMS, cat. no. 15710)
- Glycine (if performing fixation) (Fisher, cat. no. BP381-1)
- Eppendorf DNA LoBind tubes 1.5 ml (Fisher, cat. no. 022431021)
- Wide bore 200 µl and 1,000 µl tips (e.g., USA Scientific, cat. no. 1011-8810; VWR, cat. no. 89049-168)
- pA-Hia5 aliquots, frozen at −80 °C (for expression and purification protocol, see Supplementary Methods)[30]; the pET-pA–Hia5 (pA-Hia5) plasmid is available from Addgene (cat. no. 174372)
- Primary antibody for protein target of interest, from species compatible with pA. For example, in this study we used histone H3K27ac antibody (pAb)

(Active Motif, cat. no. 39134 (also 39133), RRID: AB_2722569). For additional antibodies used in this protocol, see Supplementary Methods

- Secondary antibody for immunofluorescence QC (e.g., Abcam, cat. no. ab3554, RRID: AB_303901)
- Fluorescence imaging mounting media (e.g., Thermo Fisher, cat. no. P36930)
- IgG antibody–isotype control, from species compatible with pA (e.g., Abcam, cat. no. ab171870, RRID: AB_2687657)
- Trypan blue (Fisher, cat. no. T10282)
- Qubit dsDNA BR Assay kit (Fisher, cat. no. Q32850)
- Qubit Protein Assay kit (Fisher, cat. no. Q33211)

Reagents for extraction, library preparation and sequencing

▲ **CRITICAL** We have validated the following reagents but extraction, library preparation and sequencing reagents are improving rapidly. The important considerations are to choose a DNA extraction method that maintains long DNA molecules, to perform amplification-free library preparation, and to use ONT kit chemistry that is compatible with mA calling.

- Monarch Genomic DNA Purification kit (NEB, cat. no. T3010S)
- Monarch HMW DNA Extraction kit (NEB, cat. no. T3050L)
- Agencourt AMPure XP beads (Beckman Coulter, cat. no. A63881)
- Blunt/TA Ligase Master Mix (NEB, cat. no. M0367S)
- NEBNext quick ligation module (NEB, cat. no. E6056S)
- NEBNext End Repair dA-tailing Module (NEB, cat. no. E7546S)
- NEBNext FFPE DNA repair kit (NEB, cat. no. M6630S)
- Ligation Sequencing kit (ONT, cat. no. SQK-LSK109; ONT, cat. no. SQK-LSK110 or latest kit compatible with m6A calling)
- Native Barcoding Expansion 1–12 (ONT, cat. no. EXP-NBD104, or latest kit compatible with m6A calling)
- Native Barcoding Expansion 13–24 (ONT, cat. no. EXP-NBD114, or latest kit compatible with m6A calling)
- Circulomics Short Read Eliminator kit (cat. no. SS-100-101-01)
- Flow Cell Wash Kit (ONT, cat. no. EXP-WSH004)
- Flow cells (ONT FLO-MIN106D or ONT FLO-PRO002, or latest flow cells compatible with m6A calling)

EQUIPMENT

- Centrifuge that can hold 4 °C (e.g., Thermo Fisher Scientific, model no. 75002447)
- Rotator (e.g., Millipore Sigma, model no. Z740289) for end-over-end tube rotation
- Heat block/thermal mixer (e.g., Fisher Scientific, model no. 15-600-330)
- ONT Nanopore sequencer (e.g., MIN-101B)
- Magnetic separation rack (if targeting N50 ~20 kb) (e.g., NEB, model no. S1515S)
- Qubit (e.g., Thermo Fisher Scientific, model no. Q33238)
- Tapestation (not required; for QC) (e.g., Agilent, model no. G2992AA)
- Microscope (not required; for QC) (e.g., Leica, model no. DM IRE2)
- Microscope slides (not required; for QC) (e.g., VWR, model no. 88311-601)
- Microscope cover slips (not required; for QC) (e.g., VWR, model no. 48366-045)

Reagent setup

▲ **CRITICAL** Prepare all buffers fresh the day of the experiment, filter buffers through a 0.2-μm filter and keep buffers on ice or at 4 °C.

(For optional fixation) Glycine stock solution—Prepare a 1.25 M glycine stock solution. Solution can be stored at 2–8 °C for 6 months.

Digitonin—Solubilize digitonin in preheated 95 °C Milli-Q water to create a 5% (wt/vol) digitonin solution (e.g., 10 mg/200 μl).

Wash buffer—Prepare wash buffer according to the following table. This is sufficient for ten samples.

Component	Amount	Final concentration
HEPES–KOH, 1 M, pH 7.5	1 ml	20 mM
NaCl, 5 M	1.5 ml	150 mM
Spermidine, 6.4 M	3.91 μl	0.5 mM
Roche Complete tablet –EDTA	1 tablet	–
BSA	50 mg	0.1% (wt/vol)
H_2O (Milli-Q)	Up to 50 ml	–

Dig-wash buffer—Add 0.02% (wt/vol) digitonin to wash buffer. For example, add 20 µl of 5% (wt/vol) digitonin solution to 5 ml wash buffer. The optimal concentration of digitonin may vary by cell type.

Tween-wash buffer—Add 0.1% (vol/vol) Tween-20 to wash buffer. For example, add 50 µl Tween-20 to 50 ml wash buffer.

Activation buffer—Prepare the activation buffer but wait to add SAM until the activation step. This is sufficient for 500 samples. Extra is made to avoid pipetting small volumes.

Component	Amount	Concentration
Tris, pH 8.0 1 M	750 µl	15 mM
NaCl 5 M	150 µl	15 mM
KCl 1 M	3 ml	60 mM
EDTA, pH 8.0 0.5 M	100 µl	1 mM
Ethylene glycol-bis(2-aminoethylether)-*N,N,N´,N´*-tetraacetic acid, pH 8.0 0.5 M	50 µl	0.5 mM
Spermidine, 6.4 M	0.391 µl[a]	0.05 mM
BSA	50 mg	0.1% (wt/vol)
H_2O (Milli-Q)	up to 50 ml	–
SAM, 32 mM	(Add at activation step)	800 µM

[a]To reduce pipetting error, first perform a 1:10 dilution of Spermidine in H_2O (MiLLi-Q) by adding 1 µl of 6.4 M Spermidine to 9 µl H_2O (MiLLi-Q). Mix well and then add 3.91 µl of this dilution to the activation buffer.

Procedure

1. Prepare 1–5 million cells per condition.
2. Wash cells in PBS. Spin and remove supernatant.

(Optional) Fixation

● TIMING 10 min

3. Resuspend cells in PBS.
4. Add PFA to 0.1% (wt/vol) (e.g., 6.2 µl of 16% (wt/vol) PFA to 1 ml cells) for 2 min with gentle pipette mixing.
5. Add 1.25 M glycine (sterile; 0.938 g in 10 ml) to twice the molar concentration of PFA to stop the crosslinking (e.g., 60 µl of 1.25 M glycine to 1 ml). Pipette mix.
6. Centrifuge for 3 min at 500*g* at 4 °C and remove the supernatant.
7. Continue with nuclear permeabilization, starting with Step 8.

Nuclear permeabilization

● TIMING 15 min

8. Resuspend cells in 1 ml Dig-wash buffer in a 1.5-ml DNA LoBind tube. Incubate for 5 min on ice. Please note that appropriate permeabilization conditions may vary by cell type. Consider varying digitonin concentration or permeabilization time for optimal performance.

▲ **CRITICAL STEP** To avoid loss of DNA, use DNA LoBind tubes for all steps.

▲ **CRITICAL STEP** To avoid rupturing nuclei and losing material, use wide-bore tips when working with nuclei.

▲ **CRITICAL STEP** Do not use NP-40 or Triton-X100 for nuclear extraction, permeabilization or any other stage of the protocol, as they appear to dramatically reduce methylation activity.

◆ TROUBLESHOOTING

9. Split nuclei suspension into separate 1.5 ml DNA LoBind tubes for each condition.

10. Spin at 4 °C for 3 min at 500*g* and remove supernatant.

▲ **CRITICAL STEP** To prevent nuclei from lining the side of the tube, break all spins into two parts: 2 min with the tube hinge facing inward, followed by 1 min with the tube hinge facing outward. This two-part spin is not needed if using a swinging bucket rotor.

11. QC: check permeabilization was successful by taking 1 µl of the nuclei following the 5-min incubation on ice, diluting to 10 µl with PBS and staining with Trypan blue.

Primary antibody binding

● TIMING overnight (or 2.5 h)

12. Gently resolve each pellet in 200 µl Tween-wash containing primary antibody at 1:50 or the optimal dilution for your antibody and target.

13. Place on rotator at 4 °C overnight. Samples can be incubated for ~2 h at 4 °C instead of overnight if performing the in situ protocol in a single day.

14. Spin at 4 °C for 3 min at 500*g* and remove supernatant.

15. Wash twice with 0.95 ml Tween-wash. For each wash, gently and completely resolve the pellet. This may take pipetting up and down ~10 times. Following resuspension, place on rotator at 4 °C for 5 min before spinning down at 4 °C for 3 min at 500*g*.

Quantify pA-Hia5 concentration

● TIMING 30 min

16. Thaw pA-Hia5 protein from −80 °C at room temperature (20–25 °C) and then move to ice immediately.

17. Spin at 4 °C for 10 min at 10,000*g* or higher to remove aggregates.

18. Transfer the supernatant to a new tube and save it, discarding the previous tube.

19. Use Qubit with 2 μl sample volume to quantify protein concentration.

pA-Hia5 binding

● TIMING 2.5 h

20. Add pA-Hia5 protein to Tween-wash to a final concentration of 200 nM and then gently resolve pellet in 200 μl Tween-wash containing 200 nM pA-Hia5.

21. Place on rotator at 4 °C for ~2 h.

22. Spin at 4 °C for 3 min at 500*g* and remove supernatant.

23. Wash twice with 0.95 ml Tween-wash. For each wash, gently and completely resolve the pellet. Following resuspension, place on rotator at 4 °C for 5 min before spinning down at 4 °C for 3 min at 500*g*. (Optional) If performing the binding QC step, take out 25 μl of nuclei in Tween-wash before performing final spin.

(Optional) QC to verify primary antibody and protein A binding

● TIMING 1 h

▲ **CRITICAL** This stage is only recommended for protein targets with specific nuclear localization patterns detectable by immunofluorescence (e.g., LMNB1)

24. Add 1.6 μl of 16% (wt/vol) PFA to 25 μl of nuclei in Tween-wash (saved from previous step) for 1% (wt/vol) total PFA concentration.

25. Incubate at room temperature for 5 min.

26. Add 975 μl of Tween-wash to stop the fixation by dilution.

27. Add 1 μl fluorophore-conjugated secondary antibody.

28. Put on rotator for 30 min at room temperature, protected from light.

29. Wash two times with Tween-wash as in Step 23 (or just once). The pellet probably will not be visible.

30. Resuspend in mounting media after last wash. Use as little as possible, ideally 5 μl.

31. Put 5 μl on a slide, make sure there are no bubbles and put on a coverslip.

32. Seal with nail polish along the edges.

33. Once the nail polish has dried, image or put at −20 °C.

◆ TROUBLESHOOTING

Activation

● TIMING 2.5 h

34. Gently resolve pellet in 100 µl of activation buffer per sample. Be sure to add SAM to a final concentration of 800 µM in the activation buffer at this step. In 100 µl of activation buffer, this means adding 2.5 µl of the SAM stock that is at 32 mM.

35. Incubate at 37 °C for 2 h on a heat block. Replenish SAM by adding an additional 800 µM at 1 h. This means adding an additional 2.5 µl of the SAM stock that is at 32 mM to the 100 µl reaction. Pipette mix every 30 min. Tapping to mix also works.

36. Spin at 4 °C for 3 min at 500*g* and remove supernatant.

37. Resuspend in 100 µl cold PBS.

38. Check nuclei by Trypan blue staining to determine recovery and check integrity of nuclei if desired.

DNA extraction

39. Perform DNA extraction. Follow option A for a target N50 of ~20 kb and follow option B to prepare high molecular weight (HMW) samples for a target N50 of ~50 kb.

▲ **CRITICAL STEP** We have validated the following reagents, but extraction, library preparation and sequencing reagents are improving rapidly. The important considerations are to choose a DNA extraction method that maintains long DNA molecules, to perform amplification-free library preparation and to use ONT kit chemistry that is compatible with mA calling. These are workflows we have validated and modifications we have made.

a. Target N50 of ~20 kb

● TIMING 1 h

i. Use the Monarch Genomic DNA Purification kit. Follow the protocol for genomic DNA isolation using cell lysis buffer. Include RNase A. If fixation was performed, be sure to do the 56 °C incubation for lysis for 1 h (not just 5 min) to reverse crosslinks.

ii. Perform two elutions: 100 µl and then 35 µl.

■ **PAUSE POINT** Samples can be stored at 4 °C or −20 °C.

iii. Quantify DNA yield by the Qubit dsDNA BR Assay kit. Optionally, analyze read length distribution using a TapeStation/Bioanalyzer.

iv. Concentrate by SpeedVac if necessary for 1–3 µg DNA in 48 µl for input to library prep. Do not use heat with the SpeedVac to prevent fragmenting the DNA.

b. Target N50 ~50 kb

● TIMING 1 h

i. Use the NEB Monarch HMW DNA Extraction kit. Follow the protocol for genomic DNA isolation using cell lysis buffer. Include RNase A. Perform lysis with 2,000 r.p.m. agitation. We have validated 2,000 r.p.m. gives N50 ~50–70 kb but if longer reads are desired, we expect 300 r.p.m. would work. To reiterate, make the following changes to the protocol:

- If fixation was performed, be sure to do the 56 °C incubation for lysis for 1 h (not just 10 min) to reverse crosslinks
- Agitate for 10 min and then keep at 56 °C without agitation for 50 min

■ **PAUSE POINT** Samples can be stored at 4 °C or −20 °C.

ii. Quantify DNA yield by the Qubit dsDNA BR Assay kit. Optionally, analyze read length distribution using a TapeStation/Bioanalyzer.

iii. Concentrate by speedvac if necessary to obtain 1–3 μg DNA in 48 μl for input to library prep.

(Optional) Enrichment

● TIMING 2–72 h (depending on protocol)

40. Perform enrichment. If the sequencing cost and time for sufficient coverage becomes prohibitive, a few enrichment strategies can be used. A restriction enzyme-based approach such as AlphaHOR-RES relies on preferential digestion of DNA outside of target regions followed by size selection to maintain larger on-target fragments, and can add up to 3 d to the protocol (for enrichment protocol, see Supplementary Methods. Version 1 of the AlphaHOR-RES protocol is used for this manuscript)[9,20,31]. The ONT Cas9 Sequencing kit (SQC-CS9109) is another option to selectively ligate adapters to targeted regions during library preparation, and adds ~2 h to the protocol. Adaptive sampling methodologies can also be used with no changes to the wet laboratory protocol[21,22].

Library preparation and sequencing

41. Perform library preparation and sequencing. Follow option A for a target N50 of ~20 kb and follow option B target N50 of ~50 kb.

▲ **CRITICAL STEP** We have validated the following reagents, but extraction, library preparation and sequencing reagents are improving rapidly. The important considerations are to choose a DNA extraction method that maintains long DNA molecules, to perform amplification-free library preparation and to use a flow cell that is compatible with mA calling. These are workflows we have validated and modifications we have made.

A. Target N50 ~20 kb

● TIMING 3 h

i. If multiplexing samples on a flow cell, follow the Nanopore protocol for Native Barcoding Ligation kit 1–12 and Native Barcoding Ligation kit 13–24 with ONT SQK-LSK109. If not multiplexing, use ONT SQK-LSK110. We recommend the following modifications:

- Load ~3 μg DNA into end repair
- Incubate for 10 min at 20 °C for end repair instead of 5 min
- Load ~1 μg of end repaired DNA into barcode ligation
- Double the ligation incubation time(s) to at least 20 min
- Elute in 18 μl instead of 26 μl following barcode ligation reaction cleanup to allow for more material to be loaded into the final ligation
- Load ~3 μg of pooled barcoded material into the final ligation. If needed, concentrate using speedvac to be able to load 3 μg into the final ligation
- Perform final elution in 13 μl elution buffer (EB). Take out 1 μl to dilute 1:5 for quantification by Qubit (and size distribution analysis by TapeStation/Bioanalyzer if desired)
- Load ~1 μg of DNA onto the sequencer. Input requirements vary by sequencing kit and are becoming lower

B. Target N50 ~50 kb

● TIMING 5 d

i. Follow the Nanopore protocol for ONT SQK-LSK110 (method validated with this kit only, not with multiplexing with ONT SQK-LSK109) with the following modifications (inspired by Kim et al.[32,33]):

- Increase end preparation time to 1 h with a 30-min deactivation
- Following end preparation, perform a cleanup by combining 60 μl SRE buffer from Circulomics (SS-100-101-01) with the 60 μl end prep reaction
- Centrifuge this reaction at 10,000*g* at room temperature for 30 min (or until DNA has pelleted)

- Wash pelleted DNA with 150 µl of 70% ethanol two times, using a 2 min spin at 10,000*g* between washes
- Resuspend the pellet in 31 µl EB
- Incubate at 50 °C for 1 h. Incubate at 4 °C for at least 48 h
- For the ligation step, reduce ligation volume by half (total of 30 µl DNA in a 50 µl reaction volume). Increase the ligation incubation to 1 h
- Pellet DNA at 10,000*g* at room temperature for 30 min
- Wash the pellet twice with 100 µl long fragment buffer, using a 2 min spin at 10,000*g* between washes
- Resuspend the pellet in 31 µl EB. Take out 1 µl to dilute 1:5 for quantification by Qubit (and size distribution analysis by TapeStation/Bioanalyzer if desired)
- Incubate at least 48 h at 4 °C
- Load 500 ng of DNA onto the sequencer. Input requirements vary by sequencing kit and are becoming lower
- If you see the number of active pores has dropped considerably after 24 h, recover pore activity using the flow cell wash kit, then load additional library material

◆ TROUBLESHOOTING

Analysis

● TIMING 10 h

42. Perform modified basecalling (see the ‘Analysis’ section of ‘Experimental design’).

◆ TROUBLESHOOTING

43. (Optional) Use the dimelo Python package for QC and data visualization (see the ‘Analysis’ section of ‘Experimental design’).

◆ TROUBLESHOOTING

Troubleshooting

Troubleshooting advice can be found in Table 1.

Timing

N50 ~20 kb

Day 1, Steps 1–13, buffer preparation, nuclear permeabilization, primary antibody binding: 2 h

Day 2, Steps 14–39, primary antibody wash, pA binding, activation and DNA extraction: 8 h (9 h if fixation was performed)

Day 3, Step 41, perform library preparation and start sequencing: 3 h

Day 4, Step 41, reload sequencer if necessary: 1 h

Day 5, Step 41, reload sequencer if necessary: 1 h

Day 6, Steps 42–43, basecall and perform initial analysis with dimelo: 10 h

N50 ~50 kb

Day 1, Steps 1–13, buffer preparation, nuclear permeabilization, primary antibody binding: 2 h

Day 2, Steps 14–39, primary antibody wash, pA binding, activation and DNA extraction: 9 h (10 h if fixation was performed)

Day 3, Step 41, perform library preparation end repair and clean: 2 h

Day 5, Step 41, perform library preparation ligation and clean: 2 h

Day 7, Step 41, start sequencing

Day 8, Step 41, reload sequencer if necessary: 1 h

Day 9, Step 41, reload sequencer if necessary: 1 h

Day 10, Steps 42–43, basecall and perform initial analysis with dimelo: 10 h

Anticipated results

One person can collect and analyze sequencing data within 3–8 d of beginning the DiMeLo-seq protocol. In this section, we show representative data from DiMeLo-seq experiments targeting H3K27ac, H3K27me3 and H3K4me3 in GM12878 cells and H3K9me3 in *D. melanogaster* embryos (Table 2). We use these targets to provide example output from the dimelo package and include suggested figures to evaluate performance and to perform exploratory analysis with DiMeLo-seq data. The signal at each location is reported as the ratio of adenine methylation events to the total number of adenines at that location (mA/A). Adenine methylation calls are made based on a methylation probability threshold chosen to minimize the false-discovery rate for each experiment. Therefore, mA/A should be interpreted as a metric of relative enrichment of signal to background, rather than as an absolute measure of the number of methylated adenines.

The specificity and efficiency of methylation vary by target, depending on the antibody quality, how broad the binding domain is and the chromatin environment, among other factors. The on-target and off-target methylation levels when targeting H3K27ac, H3K27me3 and H3K4me3 with DiMeLo-seq are shown in Fig. 5a-c. These plots are generated from the plot_enrichment function. For H3K27ac, to define on-target regions, we used top ChIP-seq peaks for H3K27ac (ENCODE ENCFF218QBO[34]). For off-target, we used top ChIP-seq peaks for H3K27me3 (ENODE ENCFF119CAV[34]). We similarly analyze on- and off-target for H3K27me3 with H3K27me3 top ChIP-seq peaks for on-target and H3K27ac top ChIP-seq peaks for off-target regions. For H3K4me3, to define on-target regions, we used top ChIP-seq peaks for H3K4me3 (ENCODE ENCFF228TWF[34]); for off-target, we used TSSs of unexpressed genes where H3K4me3 is not expected to be present. The on-target methylation levels are higher for H3K27me3 compared with H3K27ac, despite H3K27me3 being a repressive mark in a less accessible genomic context. This is probably because it binds a broader genomic region, allowing a larger methylated footprint. The performance difference could also occur if the anti-H3K27me3 antibody performs better than the anti-H3K27ac antibody used in these experiments. The off-target methylation level is also higher in H3K27me3 compared with H3K27ac. This is probably because the off-target region used in this analysis is H3K27ac ChIP-seq peaks, which are in very accessible regions of the genome, and off-target methylation with DiMeLo-seq occurs preferentially within open chromatin. Again, higher off-target methylation can also be caused by differences in antibody performance.

The methylation profile centered at features of interest can be visualized using the plot_enrichment_profile function. Here, we show profiles from H3K27ac-, H3K27me3- and H3K4me3-targeted DiMeLo-seq with aggregate methylation curves from reads centered at ChIP-seq peaks of varying strength (Fig. 5d-f) (ENCODE ENCFF218QBO, ENCFF119CAV, ENCFF228TWF[34]). H3K27ac and H3K4me3 have narrow peaks, while H3K27me3 has a broader peak. Signals for all three marks track with ChIP-seq peak strength, indicating concordance between DiMeLo-seq and ChIP-seq in aggregate.

To qualitatively demonstrate the concordance between DiMeLo-seq and other methods for measuring protein–DNA interactions–here ChIP-seq and CUT&Tag–we created aggregate browser tracks across a stretch of chromosome 1 (Fig. 5g) (ENCODE ENCFF218QBO, ENCFF119CAV, ENCFF228TWF; GEO GSM5530639, GSM5530673 (refs. 34,35)). DiMeLo-seq signal for all three histone marks tracks with ChIP-seq and CUT&Tag profiles. These curves were generated using the BED file output from the plot_browser function with smoothing in a 100-bp window. DiMeLo-seq also measures endogenous CpG methylation together with protein binding. An aggregate mCpG signal from the three DiMeLo-seq samples is shown, and dips in mCpG are evident where H3K27ac and H3K4me3 signals are highest. H3K27ac and H3K4me3 are both marks of open chromatin and have peaks overlapping accumulations in ATAC-seq signal (ENCODE ENCFF603BJO[34]).

In addition to comparing DiMeLo-seq with other methods, we also evaluated methylation profiles around genomic features where our targets are expected to localize. In particular, both H3K27ac and H3K4me3 are found at TSSs[36]. Using the plot_enrichment_profile function, we created the aggregate methylation and single-molecule methylation plots

shown in Fig. 6a. As expected, both marks have enrichment at the TSSs, with the highest methylation levels at the TSSs for the genes with highest expression[36]. The periodicity from positions 0 bp to 500–1,000 bp with respect to the TSS indicate preferential methylation of linker DNA between strongly positioned nucleosomes downstream from the TSSs for both targets. For genes that are not expressed (quartile 1), no enrichment at TSSs is evident.

Using the plot_browser function, single molecules are shown from H3K4me3-targeted DiMeLo-seq in a window around a highly expressed gene in GM12878 (Fig. 6b). Methylated adenines are enriched around the TSS for the highly expressed gene ATP1A1. Together with mA, the endogenous mCpG can also be analyzed. Here, it is evident that mCpG is depleted in the regions around TSS where H3K4me3 is enriched, as has been previously reported[37]. Multiple TSSs are spanned by some of the molecules in the region from 116.38 Mbp to 116.43 Mbp on chromosome 1, highlighting DiMeLo-seq's ability to probe multiple binding events on a single molecule.

DiMeLo-seq can be used to target proteins in nuclei not only from cultured cells but also from primary tissue or intact organisms. We mapped H3K9me3 distributions in *D. melanogaster* embryos across the genome and show that averaging methylation signal from single molecules generates profiles consistent with previously published ChIP-seq data[38] (Fig. 7). DiMeLo-seq coverage is consistent across the entire *D. melanogaster* genome because DiMeLo-seq's long reads can be mapped in repetitive regions of the genome. Spikes in coverage that also appear as spikes in DiMeLo-seq and ChIP-seq signal are due to centromeric repetitive sequences that are missing from the reference. This same repetitive DNA is where H3K9me3 is abundant, resulting in corresponding spikes in DiMeLo-seq methylation signal as well. We highlight a transition on chr3L where H3K9me3 accumulates and show that the accumulation is evident on single molecules using the plot_browser function.

It is important to note that the fraction of bases reported as modified should not be interpreted as the fraction of cells or molecules that have protein bound. First, this fraction is averaged across all adenines on all reads overlapping a given window and thus is an aggregate rather than a single-molecule statistic. Second, this fraction varies considerably with the modification probability threshold used. Third, the bin size used for creating aggregate plots influences the computed fractions. To measure the single-molecule interaction frequency, analysis should be performed as described for LMNB1 in Altemose et al.[9].

The DiMeLo-seq protocol described here enables profiling of protein–DNA interactions in repetitive regions of the genome, makes phasing easier for determining haplotype-specific interactions[9], detects joint binding events on single molecules of DNA and captures protein binding together with endogenous CpG methylation. These analyses are uniquely enabled by the use of long-read, native sequencing because short, clonally amplified reads cannot map to repetitive regions, span fewer single-nucelotide polymorphisms making phasing more difficult, cannot store information about multiple binding events on a single read and lose endogenous CpG methylation information through amplification. DiMeLo-seq performance varies by protein target, antibody quality and chromatin environment; therefore,

methylation sensitivity and specificity must be evaluated for each new target. The dimelo software package provides tools for QC and data exploration for the multimodal datasets that DiMeLo-seq produces.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Data availability

Data generated for this protocol: raw sequencing data are available in the Sequence Read Archive (SRA) under BioProject accession PRJNA855257 and processed data are available on Gene Expression Omnibus (GEO) under accession GSE208125. All raw fast5 sequencing data from the accompanying Altemose et al. manuscript are available in the SRA under BioProject accession PRJNA752170. External data sources used in this protocol: H3K27ac ChIP-seq data in GM12878 available from ENCODE Project Consortium under accession ENCFF218QBO (https://www.encodeproject.org/files/ENCFF218QBO/). H3K27me3 ChIP-seq data in GM12878 available from ENCODE Project Consortium under accession ENCFF119CAV (https://www.encodeproject.org/files/ENCFF119CAV/). H3K4me3 ChIP-seq data in GM12878 available from ENCODE Project Consortium under accession ENCFF228TWF (https://www.encodeproject.org/files/ENCFF228TWF/). H3K27ac CUT&Tag data in GM12878 available on Gene Expression Omnibus (GEO) under accession GSM5530639. H3K27me3 CUT&Tag data in GM12878 available on GEO under accession GSM5530673. ATAC-seq data in GM12878 available from ENCODE Project Consortium under accession ENCFF603BJO (https://www.encodeproject.org/files/ENCFF603BJO/). TSS and gene annotations from NCBI RefSeq downloaded from UCSC Genome Browser (https://genome.ucsc.edu/cgi-bin/hgTrackUi?g=refSeqComposite&db=hg38). RNA-seq data in GM12878 available from ENCODE Project Consortium under accession ENCFF978HIY (https://www.encodeproject.org/files/ENCFF978HIY/). *D. melanogaster* H3K9me3 ChIP-seq data available on GEO under accession GSE140539. File GSE140539_H3K9me3_sorted_deepnorm_log2_smooth.bw was used.

Code availability

The dimelo Python package for analysis of DiMeLo-seq data is available on Github: https://github.com/streetslab/dimelo.

Related links

Key reference using this protocol

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Key points

- DiMeLo-seq uses long-read, single-molecule sequencing to map protein–DNA interactions genome wide, in nuclei from fresh, fixed or frozen cells, and from primary tissues or intact organisms.
- Compared with short-read methods, this enables mapping of multiple interaction sites on single DNA molecules, profiling protein binding in the context of endogenous DNA methylation, identifying haplotype-specific protein–DNA interactions and mapping protein–DNA interactions in repetitive regions of the genome.

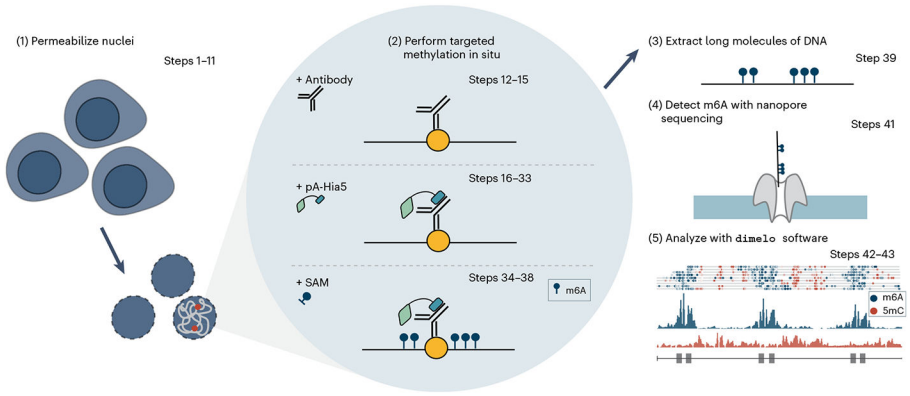


Fig. 1 |. DiMeLo-seq protocol overview.

The step numbers in the procedure are indicated. (1) Permeabilize nuclei from fresh, frozen, or fixed cells (Steps 1–11). (2) Perform a series of steps within the permeabilized nuclei: (i) bind primary antibody to the protein of interest (Steps 12–15), (ii) bind pA-Hia5 to the primary antibody (Steps 16–33), (iii) add SAM, the methyl donor, to activate methylation (Steps 34–38). (3) Extract long molecules of DNA (Step 39). Optionally, enrich for genomic sequences of interest (Step 40). (4) Sequence this DNA with a Nanopore sequencer to detect m6A directly (Step 41). (5) Analyze modified basecalls from sequencing using the dimelo software package (Steps 42–43).

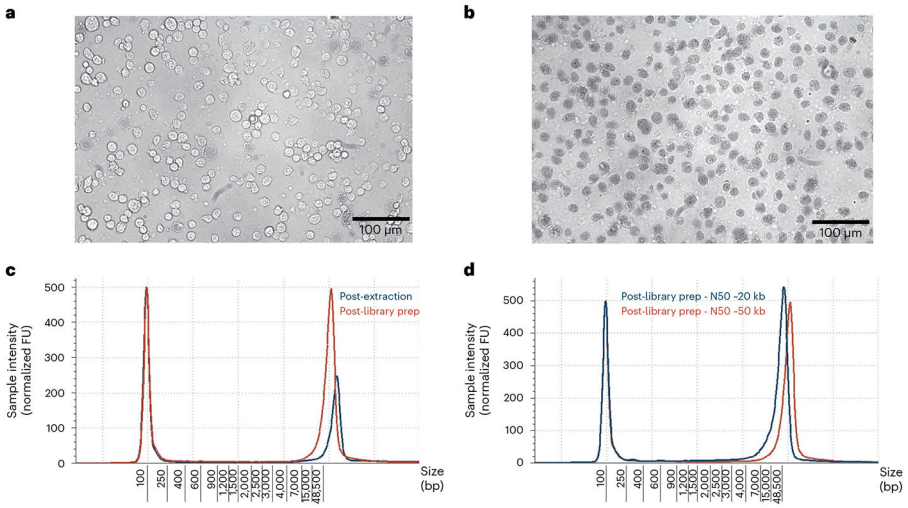


Fig. 2 |. Experimental QC. a,b,

To determine successful permeabilization, cells are stained with Trypan blue before (**a**) and after (**b**) digitonin treatment. Successful permeabilization allows Trypan blue to enter the nuclei, while still maintaining high recovery of nuclei from cells. Overpermeabilization results in lower recovery of nuclei. Underpermeabilization does not allow Trypan blue to enter the nuclei. Scale bars, 100 µm. **c,d**, TapeStation traces of DNA size distribution after the DiMeLo-seq in situ protocol and DNA extraction. Representative traces from ligation-based library preparation are shown for the fragment size distribution after extraction and after library preparation, following protocols for N50 ~50 kb (Steps 39B and 41B) (**c**) and the size distribution after library preparation for the two ligation-based methods presented in this protocol (**d**). The blue curve results in N50 ~20 kb (Steps 39A and 41A), while the red curve results in N50 ~50 kb (Steps 39B and 41B). Larger fragment sizes can be achieved with other ultralong kits.

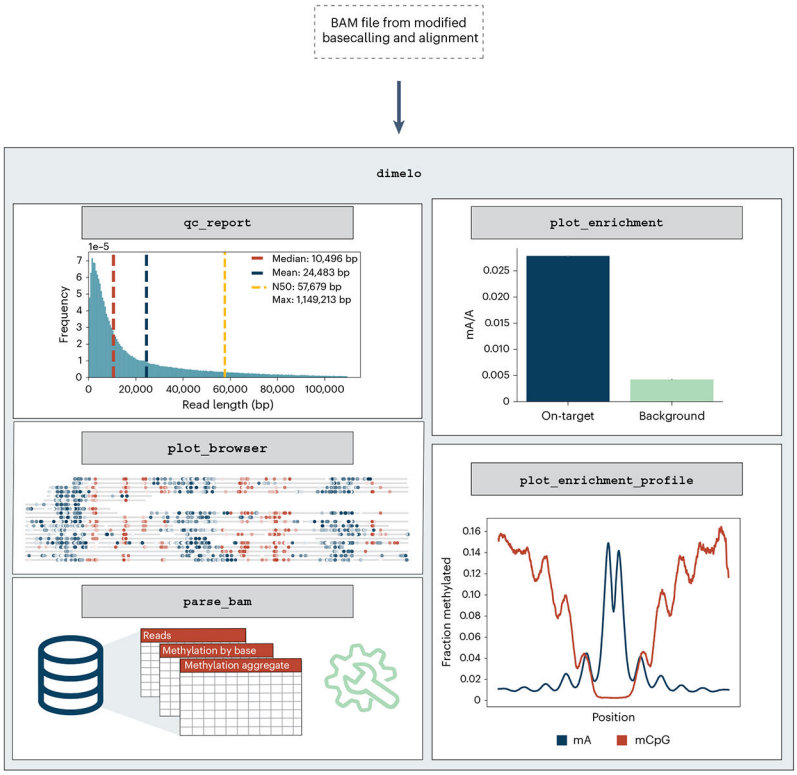
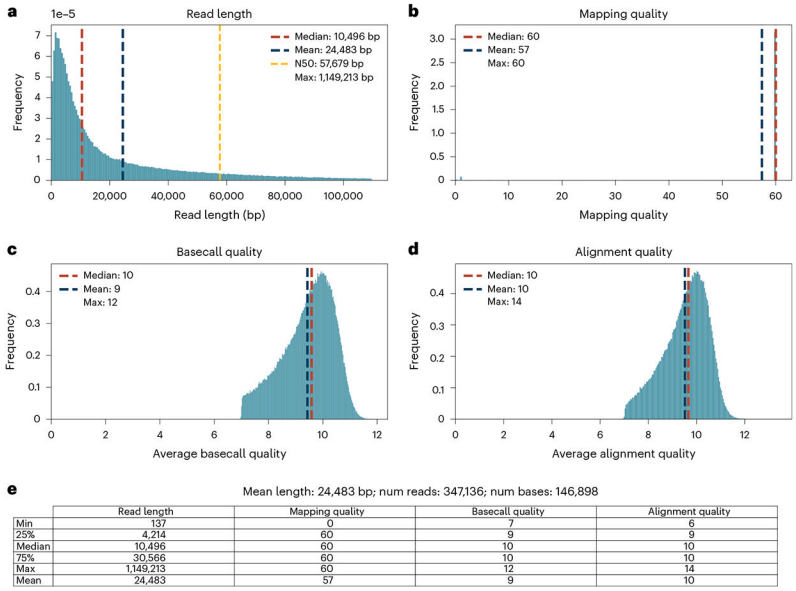


Fig. 3 |. Analysis pipeline overview.

Basecalling and alignment are performed on the FAST5 output from the Nanopore sequencer. The resulting BAM that contains the modified base information is then input to the dimelo software package. A recommended workflow involves QC with qc_report, followed by visualization with plot_browser, plot_enrichment and plot_enrichment_profile. For custom analysis, parse_bam stores base modification calls in an intermediate format that makes it easier to manipulate for downstream analysis.



	Read length	Mapping quality	Basecall quality	Alignment quality
Min	137	0	7	6
25%	4,214	60	9	9
Median	10,496	60	10	10
75%	30,566	60	10	10
Max	1,149,213	60	12	14
Mean	24,483	57	9	10

Fig. 4 |. Sequencing QC.

The qc_report function takes in one or more BAM files and for each, outputs a QC report including the following 5 features. **a**, A histogram of read lengths with the median, mean, N50, and max value annotated. **b**, A histogram of mapping quality. **c,d**, Basecall quality scores are present in BAM outputs from Guppy but not from Megalodon. Histograms of average basecall qualities per read are shown in **c** and **d**. The scores can be reported over the entirety of each read (**c**, basecall quality) or the aligned portion of each read (**d**, alignment quality). Here, the mean indicates that our sample's average basecall quality is Q10, which is equivalent to a 10%-per-base error rate. **e**, A summary table with descriptive statistics of each feature (**a–d**), in addition to highlighting important values such as mean length of reads, total number of reads, and total number of bases sequenced. Example data used in this figure are from targeting H3K9me3 in *D. melanogaster* embryos.

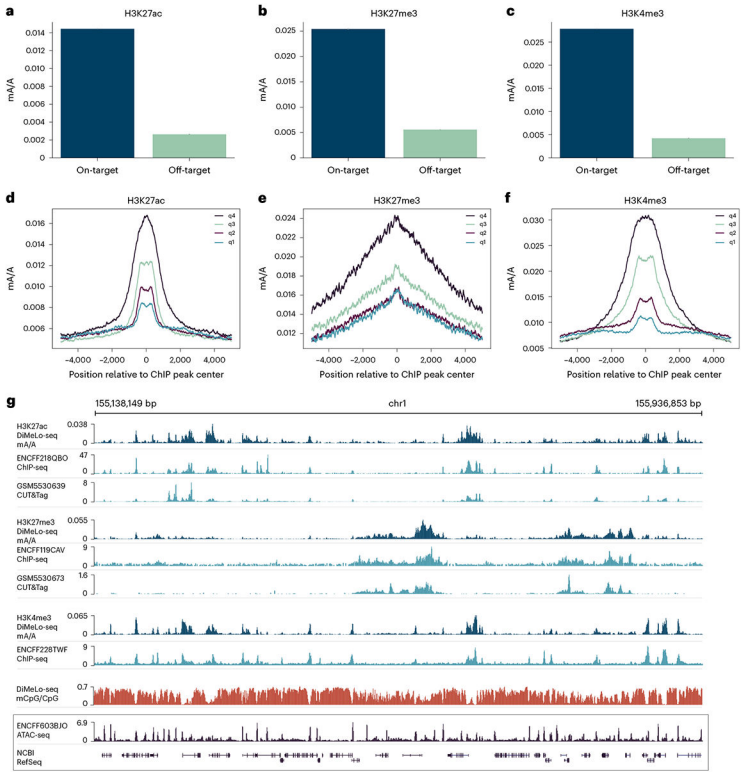


Fig. 5 |. Validation of targeted methylation in GM12878 cells.

a–c, Using BED files defining on- and off-target regions, the plot_enrichment function can be used to determine whether methylation is concentrated within expected regions. We have defined on-target regions using ChIP-seq peaks for the corresponding histone marks. We defined off-target regions when targeting H3K27ac as H3K27me3 ChIP-seq peaks (**a**) and when targeting H3K27me3 as H3K27ac ChIP-seq peaks (**b**); for off-target regions for H3K4me3 we use TSSs for unexpressed genes (**c**). A methylation probability threshold of 0.75 was used. Error bars represent 95% credible intervals determined for each ratio by sampling from posterior beta distributions computed with uninformative priors. **d–f**, Methylation profiles centered at ChIP-seq peaks for H3K27ac- (**d**), H3K27me3- (**e**) and H3K4me3-targeted (**f**) DiMeLo-seq are plotted using plot_enrichment_profile. The quartiles (quartile 4 (q4) to quartile 1 (q1)) indicate the strength of the ChIP-seq peaks which the DiMeLo-seq reads overlap. A methylation probability threshold of 0.75 was used. **g**, Aggregate browser traces comparing DiMeLo-seq signal to ChIP-seq and CUT&Tag. BED files used for creating aggregate curves are generated either from parse_bam or plot_browser. The CpG methylation signal is aggregated from the H3K27ac-, H3K27me3- and H3K4me3-targeted DiMeLo-seq experiments. A methylation probability threshold of 0.8 was used. ATAC-seq and NCBI RefSeq annotations are also shown.

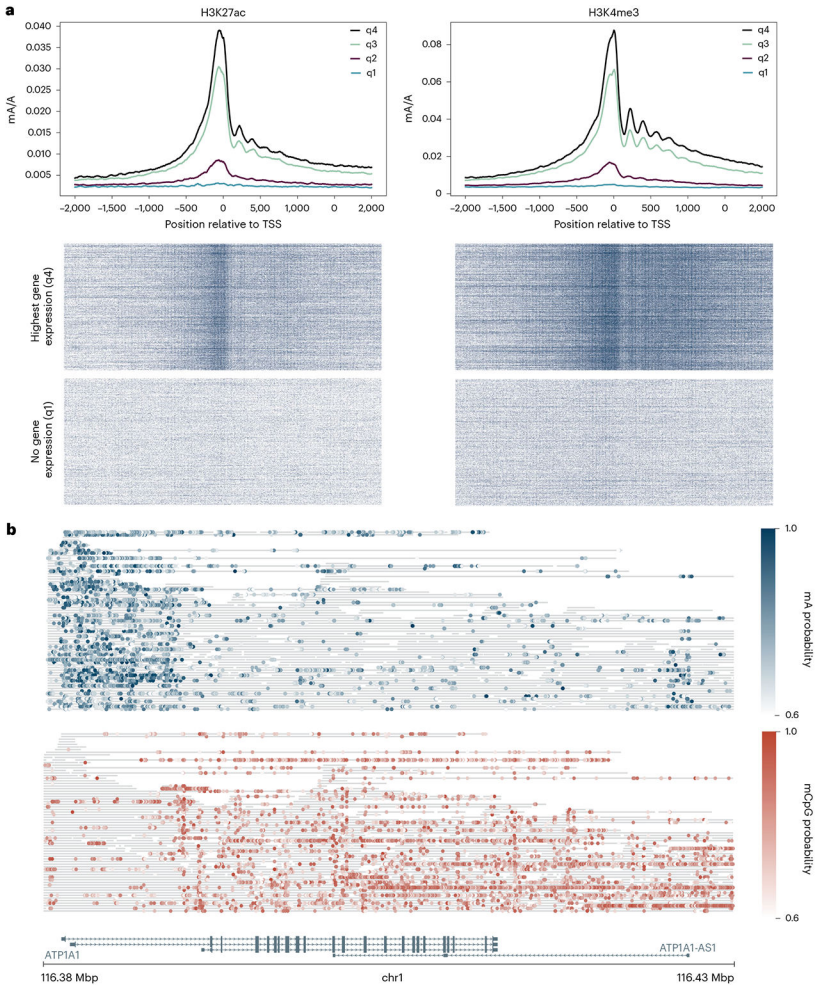


Fig. 6 |. Evaluating protein binding at regions of interest.

Both H3K27ac and H3K4me3 are found at TSSs. **a**, The signal from H3K27ac- and H3K4me3-targeted DiMeLo-seq at TSS. Reads overlapping TSS, gated by gene expression level from highest gene expression (q4) to lowest gene expression (q1). Aggregate mA/A profiles are shown for all reads spanning these TSSs. Single molecules are shown below with blue representing mA calls for TSS for the highest gene expression (q4) and for no gene expression (q1). Aggregate and single-molecule plots were produced with plot_enrichment_profile. A methylation probability threshold of 0.75 was used. **b**, Single-molecule browser plots produced from plot_browser from H3K4me3-targeted DiMeLo-seq experiment. Using a methylation probability threshold of 0.6, mA (top, blue) and mCpG (bottom, red) calls are shown for the same molecules (gray lines). NCBI RefSeq genes are shown below.

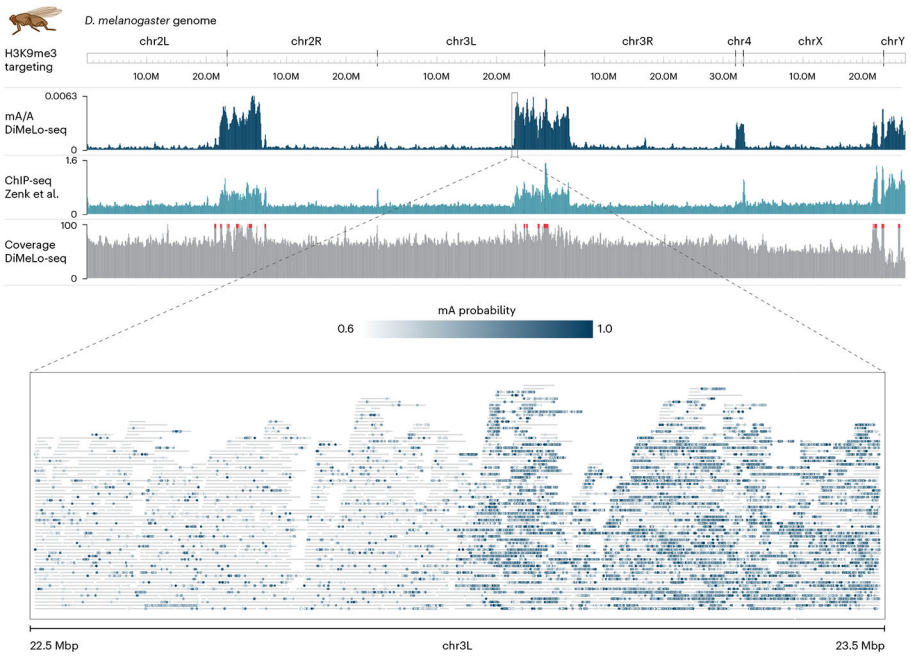


Fig. 7 |. H3K9me3-targeted DiMeLo-seq in *D. melanogaster* embryos.

Aggregate mA/A across the entire *D. melanogaster* genome from a DiMeLo-seq experiment targeting H3K9me3 is shown in dark blue. H3K9me3 ChIP-seq data in *D. melanogaster* embryos is shown in light blue[38]. Coverage from the DiMeLo-seq experiment is shown in gray. A region on chr3L where a transition from H3K9me3 depletion to H3K9me3 enrichment is highlighted with a single-molecule browser plot generated from plot_browser. Gray lines indicate reads and blue dots indicate mA calls with intensity colored by probability of methylation. An alignment length filter of 10 kb was applied. A methylation probability threshold of 0.6 was used.

Table 1 |

Troubleshooting table

Step	Problem	Possible reason	Solution
8	Few intact nuclei	Digitonin concentration is not optimal for the cell type	Try a range of digitonin concentrations and perform QC with Trypan blue stain
		Nuclei sticking to the sides of tubes	Follow the recommended two-step centrifugation procedure with a 2-min spin with the tube hinge facing inward, followed by a 1-min spin with the tube hinge facing outward. Alternatively, use a swinging bucket rotor
33	No difference in fluorescence between IgG control and targeted methylation	Target abundance is low and/or target is diffuse	Immunofluorescence may not provide sufficient QC information for your target. Try a different QC step
		Insufficient washing	Add another wash step after secondary antibody binding
		Antibody concentration is not optimal	Try a range of primary and secondary antibody concentrations
		Primary or secondary antibody is not working	Try a different antibody
		Permeabilization failure	To confirm permeabilization, perform Trypan blue QC step with varying digitonin concentrations
41	Unable to pipette viscous DNA	DNA is too long	Fragment DNA or follow library preparation protocol for presevering longer fragments (Step 41)
	Bead clumping	DNA is too long for bead-based cleanup	Fragment DNA or follow library preparation protocol for preserving longer fragments (Step 41)
	Low recovery from bead cleanup	DNA is too long for bead-based cleanup	Fragment DNA or follow library preparation protocol for preserving longer fragments (Step 41)
		DNA is too short for long fragment buffer used in bead cleanup	Handle HMW DNA carefully with wide bore tips and ensure your DNA extraction method maintains long DNA fragments
	Short reads	DNA sheared during library preparation	Handle HMW DNA carefully with wide bore tips; follow library preparation protocol for preserving longer fragments (Step 41)
		Too much DNA loaded onto sequencer	Repeat qubit of final library. For target N50 ~20 kb, load ~1 μg of library; for target N50 ~50 kb, load 300–500 ng of library
42	Low yield from sequencer	Low input and long DNA fragments cause pores to become inactive quickly	Perform flow cell wash and reload every ~24 h and/or load more DNA onto the flow cell. Washing and reloading becomes very important with larger fragment sizes
		Bubbles destroy pores	Use a new flow cell and be sure not to introduce bubbles during the flow cell loading process
42, 43	Lower than expected m6A signal observed in analysis	Defective SAM	Use a new batch of SAM. This reagent is not very stable and should not be used if expired
		Defective antibody	Perform DiMeLo-seq with an antibody you know performs well (e.g., antibodies from this manuscript and from Altemose et al.[9]) to determine if a different antibody should be used
		Defective pA-Hia5	Perform enzyme activity assays described in Supplementary Methods to determine if making a new batch of pA-Hia5 is required

Table 2 |

Summary of experimental specifications for histone modifications profiled using DiMeLo-seq

Target	Cell type	Antibody	Library prep kit	Flow cell chemistry	Device	Gb	Coverage	N50 (bp)
H3K27ac	GM12878	Active Motif 39133	SQK-LSK110	R9.4.1	PromethION	124	41×	25,536
H3K27me3		Active Motif 39055				122	41×	27,226
H3K4me3		Active Motif 39916				124	41×	25,163
H3K9me3	*D. melanogaster* embryo	Active Motif 39062			MinION	8.24	46×	27,843