Single-molecule simultaneous profiling of DNA methylation and DNA-protein interactions with Nanopore-DamID

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Abstract

DNA-protein interactions and cytosine methylation control eukaryotic gene expression. Here, we present an approach to simultaneously detect cytosine methylation and DNA-protein interactions from single molecules, through selective sequencing of adenine-labelled DNA. Applying this approach to LaminB1-associated heterochromatin domains, we identify strict CpG methylation maintenance at transcriptional start sites amidst a generalised relaxation of methylation, potentially to prevent ectopic and aberrant heterochromatic gene expression.

Main

Cytosine methylation modulates the interactions of chromatin-associated proteins with DNA¹. DNA adenine methylase identification (DamID) is an approach to profile DNA-protein interactions^{2,3}. In DamID, the *E. coli* adenine methylase Dam is fused to a chromatin-associated protein (**Fig. 1a**). The Dam-fusion protein methylates adenines in GATC motifs proximal to the site of chromatin-association. Methylated GATC sites are then cleaved by the methylation-specific restriction enzyme DpnI, followed by adaptor ligation, amplification and Illumina-sequencing⁴. DamID has been adapted to detect chromatin-accessibility⁵, RNA-chromatin interactions⁶,

chromatin topology^{7,8}, nuclear lamina interactions⁹, and has been successfully implemented in various model organisms^{2,10–14}.

Despite the utility of DamID, the approach has drawbacks. Each molecule must have GATC methylation on both ends to be amplified, reducing signal and rendering DamID incompatible with bisulphite sequencing. The protocol is labour-intensive (~five days⁴) and the extensive use of PCR has the potential to introduce biases.

Oxford Nanopore Technologies (ONT) long-read sequencing can directly detect both cytosine and adenine methylation, including in regions refractory to short-read analysis^{15–17}. Exogenous promiscuous adenine methylases have recently been used to profile chromatin-accessibility¹⁸ and, by fusion to protein-A, regions of transcription factor occupancy bound by a cognate antibody^{19,20}. However, direct detection of adenine methylation requires high sequencing depth, which is prohibitively expensive in mammals. Here, we develop a Nanopore-based DamID which enables rapid and cost-effective profiling of single-molecular transcription factor-cytosine methylation interactions.

Nanopore sequencing can be used to selectively analyse adaptor-ligated DNA without purification or amplification²¹. Thus, if adaptors are only ligated to DNA cleaved by the methylated-GATC-specific enzyme DpnI these fragments can be selectively sequenced from a heterogeneous mixture of largely unligated fragments (**Fig. 1a**). To enable selective sequencing of Dam-methylated fragments, we stably transduced the mouse neuroblastoma cell line, N2A, with a doxycycline-inducible Dam-LaminB1 fusion protein expression cassette (**Supplementary figure 1a**) and constitutively expressed m6A-tracer²² (GFP fused to catalytically inactive DpnI, **Supplementary figure 1b**). Characteristic fluorescent rings at nuclear lamina were evident in cells upon doxycycline treatment, confirming that Dam-LaminB1 can efficiently modify GATC sites in lamin-associated chromatin (**Fig. 1b**). To simultaneously detect DNA-protein interactions and CpG methylation on single molecules, we expressed Dam-LaminB1 in N2A cells and extracted genomic DNA. We dephosphorylated double-strand breaks and incubated with DpnI to cleave methylated

GATC fragments. Blunt ends produced by DpnI were A-tailed and ligated to Nanopore adapters. Library preparation here takes <1.5 day, compared to 4-5 days for Illumina DamID⁴. From sequencing on a single MinION flow cell, we generated 12.8 million reads, enriched in the broad domains characteristic of LaminB1^{9,23} (**Fig. 1c**). The nanopore reads are expected to terminate at GATC motifs (**Fig. 1c**). Indeed, *de novo* motif enrichment analysis on read termini identified profound enrichment of GATC ($p=3.3e-713$, **Fig. 1d**). At least one end in 97% of reads terminated within 10bp of a GATC motif (**Fig. 1e**) and were thus derived from a DpnI-cleaved Dam-methylated site. These results provide proof-of-principle confirmation that selective sequencing of adenine methylated DNA, without amplification or purification, is rapid, efficient and cost-effective.

Figure 1: Nanopore-DamID identifies Lamin-associated domains. *a*, In Nanopore-DamID, a fusion Dam-chromatin-associated protein is expressed in cells and methylates adenines in GATC motifs proximal to sites of DNA-protein interaction. Genomic DNA is isolated from cells, dephosphorylated and digested with DpnI. Digested DNA ends are A-tailed and then Nanopore sequencing adaptors are ligated. Nanopore sequencing identifies DNA-protein

contacts and CpG methylation on single DNA molecules. **b**, Upon doxycycline treatment, the m6A-tracer (an inactive DpnI fragment fused to GFP) localises to the nuclear lamina in cells transfected with TetON-Dam-LaminB1. **c**, Sequencing of LaminB1-Dam-expressing cells shows enrichment in domains identified by RSEG²⁴ characteristic of LaminB1-DamID. Raw nanopore reads terminate at GATC sites. **d**, De novo motif discovery at the ends of Nanopore reads using MEME-ChIP²⁵ revealed the motif GATC was strongly enriched. **e**, Most reads from Nanopore-DamID terminate at a GATC motif (± 10 bp).

To compare Nanopore and Illumina-based approaches we sequenced conventional LaminB1-DamID libraries from N2A cells. LaminB1-occupancy profiles were comparable between technologies (**Fig. 2a**). Lamin-associated domains (LADs) identified by RSEG²⁴ largely overlap. 79% of bases in Illumina LADs fall within Nanopore-DamID LADs (**Supplementary tables 1 and 2**). Nanopore-detected domains were significantly broader and more contiguous than those detected by Illumina (**Fig. 2b**). The higher length of Nanopore reads (median length 996bp vs 75bp for Illumina) enables alignment of >4.5 x more bases to regions of low mappability (**Supplementary figure 2**).

Figure 2: Nanopore-DamID identifies widespread CpG hypomethylation in lamin-associated DNA. *a*, Comparison of LaminB1-DamID signal from Illumina and Nanopore-based approaches on chromosome 10. *b*, Illumina-DamID identifies more numerous, smaller Lamin-associated domains (LADs), compared to the large domains identified by Nanopore-DamID. *c*, Analysis of CpG-methylation from the Nanopore-DamID data with MethyIartist²⁶ reveals pronounced hypomethylation within a LAD on chromosome 1. *d*, Genome-wide analysis of methylation within LADs or between LADs (iLADs) reveals hypomethylation of LADs, other than at TSSs, which maintain higher methylation. *e*, Example of the interaction of LaminB1 occupancy with DNA-methylation on chromosome 3.

To determine the interactions between LaminB1 and DNA methylation we identified CpG methylation from the LaminB1-Nanopore-DamID reads. Consistent with previous reports^{27,28} we found that, despite the heterochromatic structure of LADs, CpG methylation was globally depleted in these regions compared to non-Lamin-associated DNA, (inter-LADs, iLADs) (**Fig. 2c-d, Supplementary figure 3**). However, CpG islands and, most strikingly, the regions surrounding (± 1 kb) transcriptional start sites are resistant to LAD hypomethylation (**Fig 2c-d**). The maintenance of high methylation at LAD TSSs suggests that despite tethering to the nuclear periphery, DNA methylation at the proximal promoter region may prevent aberrant heterochromatic gene expression in LADs.

Overall, Nanopore-DamID efficiently selectively sequences Dam-labelled native DNA enabling co-detection of DNA-protein contacts and DNA methylation. This selectively sequencing approach can be extended to other adenine methylase-based approaches^{18–20} to increase sequencing depth over methylated regions. Nanopore-DamID is less labour-intensive, enables superior coverage of regions with low mappability, and identifies more contiguous binding domains than short-read approaches. Nanopore-DamID facilitates elucidation of the interactions between CpG methylation and chromatin-associated proteins that control gene regulation.

Methods

Transgenic cell line preparation

1x10⁵ N2A (ATCC) cells were seeded in a well of a 6-well plate in 2ml of DMEM complete media (Gibco) supplemented with 10% Fetal Bovine Serum, 1% PenStrep and 1% L-glutamine and

maintained at 37°C with 5% CO₂ in a humidified incubator. 0.2µg Super piggyBac Transposase Expression Vector, 0.25µg m6A-tracer and 0.25µg XLONE-Dam-LamB1 (BlastR) were diluted in 100µl of Opti-MEM (Thermo Fisher). 2.1µl of FuGENE-HD® (Promega) was added and the reaction was briefly vortexed and incubated at room temperature for 10min prior to addition to the cells. The transfection mix was added dropwise to the well and incubated for 24hr. After 24hr the media was changed and the cells were selected for seven days in media containing 2.5µg/ml blasticidin.

Imaging

1x10⁵ transgenic N2A cells were seeded in two 35mm glass bottom dishes (Cellvis™). Dam-LaminB1 fusion protein expression was induced with 2µg/ml doxycycline in one of the dishes. The m6A tracer in cells of both dishes were visualised and imaged at 488nm on the Olympus FV3000 microscope at 120X magnification with a 60X oil immersion objective followed by a 2X digital zoom. Images were analysed via Fiji²⁹.

Nanopore DamID

Dam-LaminB1 fusion protein expression was induced with 2µg/ml doxycycline and genomic DNA (gDNA) was extracted from Nunc™ T75 using the NEB Monarch® gDNA extraction kit according to manufacturer's instructions with the exception of using inversion rather than vortexing for mixing. 2.5µg of gDNA was dephosphorylated by the addition of 5µl of 10X NEB Cutsmart® buffer and 1µl of QuickCIP (NEB) in a total volume of 48µl. The reaction was mixed by gentle flicking and incubated at 37°C for 30 minutes. The QuickCIP was then heat inactivated at 80°C for 5 min. 2µl of DpnI (NEB) was added to the reaction, mixed by gently flicking and incubated at 37°C overnight. DNA was A-tailed by addition of 1µl 10mM dATPs and 1µl of Klenow exo- (NEB) and incubation at 37°C for 1 hr. The digested DNA was purified using the Qiagen QiaQuick™ PCR Purification kit (which purifies fragments <10kb) and eluted in 60µl of H₂O. The DNA was

prepared for ONT sequencing with the DNA Ligation Sequencing Kit (LSK110), starting from the adaptor ligation step and using the Short Fragment Buffer.

Illumina DamID

Illumina-DamID-seq was performed as previously described with minor modifications⁴. Briefly, gDNA was extracted using the NEB Monarch gDNA extraction kit and digested overnight with DpnI (NEB). DamID adaptors were then ligated to DpnI-digested DNA. The adaptor-ligated DNA was digested with DpnII (NEB) to cleave fragments containing internal unmethylated GATC sites. Dam-methylated DNA was then amplified by PCR with MyTaq polymerase (Bioline®), sonicated to an average size of 300bp and the DamID adaptors were removed by AlwI (NEB) digestion. 1µg of sonicated DNA was prepared for sequencing using the NEBNext® Ultra™ II DNA Library Prep Kit for Illumina® without size selection and amplified with 3 cycles of PCR. Libraries were sequenced on the Illumina® NextSeq platform using single-end 75bp chemistry.

DamID analysis

Nanopore reads were aligned to the mouse genome (mm10) using Minimap2³⁰ with the options -a --MD --cs=long -x map-ont. Mapped reads were filtered for map quality using SAMtools³¹ view -q 10 and converted to bed format using bedtools bamtobed and bigwig format using deepTools³² bamCoverage. The coordinates of the ends of reads were extracted using awk, extended to 100bp and converted to FASTA using bedtools getfasta. The sequences surrounding read ends were surveyed for enriched 4bp motifs using MEME-ChIP²⁵. GATC motifs in the mouse genome (mm10) were extracted using the damidseq_pipeline³³. The intersection of Nanopore read ends with GATC motifs was calculated using bedtools intersect. Lamin-associated domains were identified using Rseg²⁴ with the option -i 20 and no correction for deadzones. Short-read DamID was mapped using bwa mem³⁴ and filtered for alignment quality using samtools view -q 10. CpG methylation was called from Nanopore-DamID data using megalodon v2.2.9 with the model

dna_r9.4.1_450bps_modbases_5mc_hac_prom.cfg. DNA methylation was visualised using methylartist²⁶.

Code availability

CpG methylation analysis was performed using megalodon available from <https://github.com/nanoporetech/megalodon> and methylartist²⁶ available from <https://github.com/adamewing/methylartist>.

Data availability

All sequencing data is deposited in the NCBI Short Read Archive (SRA) repository as BioProject PRJNA751437.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

S.W.C. designed the study. Y.M.A.J, S.B.A. and S.W.C. performed the experiments. A.D.E., and S.W.C. performed the analysis. S.W.C and G.J.F. funded the study. S.W.C., Y.M.A.J., N.J. and G.J.F. wrote the manuscript. All authors read and approved the final manuscript.

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Supplementary figure 1: Constructs used for Nanopore-DamID. a, An all-in-one TetON-3G piggyBac construct based on XLONE³⁵ enables generation of a stable cell line with an integrated doxycycline-inducible Dam-LaminB1. **b,** A piggyBac construct enables generation of a stable cell line constitutively expressing a catalytically inactivate DpnI fragment (Dpn7) fused to eGFP. Dpn7-eGFP binds to methylated GATC sites enabling visualisation of Dam-LaminB1 activity.

Supplementary figure 2: Superior alignment of Nanopore-DamID reads to regions of low mappability. Nanopore reads span regions that are difficult to align with 75bp Illumina reads (deadzones identified by RSEG).

Supplementary figure 3: Resistance of a TSS on chr3 to LAD hypomethylation. Example of the interaction of LaminB1 occupancy with DNA-methylation.