



DNA Methylation in Ctenophores

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Abstract

Epigenomic regulation and dynamic DNA methylation, in particular, are widespread mechanisms orchestrating the genome operation across time and species. Whole-genome bisulfite sequencing (WGBS) is currently the only method for unbiasedly capturing the presence of 5-methylcytosine (5-mC) DNA methylation patterns across an entire genome with single-nucleotide resolution. Bisulfite treatment converts unmethylated cytosines to uracils but leaves methylated cytosines intact, thereby creating a map of all methylated cytosines across a genome also known as a methylome. These epigenomic patterns of DNA methylation have been found to regulate gene expression and influence gene evolution rates between species. While protocols have been optimized for vertebrate methylome production, little adaptation has been done for invertebrates. Creating a methylome reference allows comparisons to be made between rates of transcription and epigenomic patterning in animals. Here we present a method of library construction for bisulfite sequencing optimized for non-bilateral metazoans such as the ctenophore, *Mnemiopsis leidyi*. We have improved upon our previously published method by including spike-in genomic DNA controls to measure methylation conversion rates. By pooling two bisulfite conversion reactions from the same individual, we also produced sequencing libraries that yielded a higher percentage of sequenced reads uniquely mapping to the reference genome. We successfully detected 5-mC in whole-animal methylomes at CpG, CHG, and CHH sites and visualized datasets using circos diagrams. The proof-of-concept tests were performed both under control conditions and following injury tests with changes in methylation patterns of genes encoding innexins, toxins and neuropeptides. Our approach can be easily adapted to produce epigenomes from other fragile marine animals.

Key words Ctenophora, Genome, DNA methylation, 5-mC, innexins, neuropeptides, *Mnemiopsis*

1 Introduction

A broad spectrum of epigenetic modifications control gene expression without altering the primary DNA sequence. Epigenomic regulation and dynamic DNA methylation, in particular, are widespread mechanisms orchestrating the genome operation across time and species [1–6]. Initial studies indicated that these mechanisms contribute to the development and specification of different ctenophore species (*Pleurobrachia*, *Mnemiopsis*, and *Beroë* [7, 8]), with the potential involvement of two types of genomic DNA methylation in ctenophores, 5-methyl cytosine (5-mC) and the

unconventional form of methylation 6-methyl adenine (6-mA), including 6-mA genome modifications [7] in the aboral organ. Reversible 5-mC methylation and demethylation (by TET enzymes) were primarily detected during the early cleavage stages and in combs of *Pleurobrachia bachei* [8]. Notably, 5-mC methylation occurred both at the CpG sites and other locations within promoters, exons, and introns [7]. Thus, single-nucleotide resolution in mapping and quantifying DNA methylation is imperative. Bisulfite sequencing was the first technique developed to study epigenetic changes to DNA [9]. We sought to improve upon our previously published *Mnemiopsis leidyi* methylome to better study the evolution of epigenomic regulation in basal metazoans [7]. Our approach can also be easily adapted to produce epigenomes from other fragile marine animals.

Originally established in 1992, whole-genome bisulfite sequencing (BS-seq) is the only method currently available to measure DNA methylation at a single base resolution [9, 10]. This method's primary mechanism is converting all cytosine residues to uracil residues using bisulfite (HSO_3^-). The 5-methylcytosine (5-mC) residues are unaffected. Bisulfite reacts with unmethylated cytosine residues, which are converted to uracil in a three-step reaction: (1) a sulfonation reaction adds HSO_3^- to create cytosine sulfonate, (2) deamination creates uracil sulfonate, and finally, (3) alkali desulfonation finishes the conversion of cytosine to uracil [11]. Meanwhile, methylated cytosines are protected from this bisulfite conversion, so preserved cytosine residues in sequenced BS-seq samples are recognized as methylated sites [9]. Bisulfite conversion is a harsh process that causes DNA to become single-stranded and highly fragmented. Adding a "spike-in" at a low concentration with known cytosine methylation states to samples before the bisulfite conversion reaction allows for downstream assessment of how much degradation occurred and to calculate the percentage of unmethylated cytosine residues converted per sample [12]. This protocol provides an optimized method for adding methylation spike-in controls for *Mnemiopsis*.

Many protocols exist for bisulfite converting and creating sequencing libraries for mammalian or insect samples [10, 13, 14]; however, bisulfite conversion to genomic DNA from ctenophores and *Mnemiopsis*, in particular, poses additional challenges due to the propensity of isolated genomic DNA samples to rapidly degrade if not stored at deep freezer temperatures, ideally -80°C . In this method, we compensate for the high rate of fragmentation or degradation caused by bisulfite conversion by performing multiple reactions per *M. leidyi* gDNA sample and combining these reactions during DNA methylation library construction.

2 Materials

2.1 Reagents

Library construction and quantification reagents.

1. Qubit[®] DNA assay kit (Cat # Q32850, Thermo Fisher Scientific).
2. RNase-free and DNase-free water (Cat # 10977-015, Thermo Fisher Scientific).
3. EZ methyl direct bisulfite (Cat # D5030, Zymo Research).
4. Qiagen genomic Tip 500/G (Cat # 10262) or 100/G (Cat # 10243).
5. Illumina TruSeq DNA methylation library kit 24 reactions Part # 15066014 Rev A.
6. Illumina TruSeq barcoded adapters (Cat # EGIDX81312, Epicentre/Illumina).
7. WiseGene 5-mC lambda spike-in control Cat # S001.
8. Promega D1501 lambda DNA, 250 µg.
9. 1.5 mL microcentrifuge tubes.
10. 0.2 or 0.5 mL microcentrifuge tubes.
11. FailSafe PCR Enzyme Epicentre, Catalog No. FSE51100.
12. Optional: TruSeq Index PCR Primers Illumina, Catalog No. EGIDX81312 (12 Indexes).
13. EZ DNA methyl direct 25 rxns Zymo Research, Catalog No. D5020.
14. AMPure XP System Beckman Coulter Genomics, Catalog No. A63880.
Freshly prepared 80% (v/v) ethanol diluted with nuclease-free water.
15. Qubit broad-range dsDNA assay kit Q32850.
16. Qubit reader.
17. Qubit 0.5 mL tubes.
18. Qubit high-sensitivity dsDNA assay kit Q32851.
19. 2% agarose E-gel (fits 8 samples + 1 ladder) G401002.
20. Agilent D1000 DNA screen tapes (5067–5582).
21. Agilent D100 screen tape reagents (5067–5583, Agilent).
22. Qubit 0.5 mL assay tubes (Q32856, Thermo Fisher Scientific).
23. FailSafe PCR Enzyme Epicentre, Catalog No. FSE51100.
24. Agilent D1000 DNA screen tapes.

2.2 Equipment

1. Qubit[®] 2.0 Fluorometer (Cat # Q32866, Life Technologies).
2. Magnetic rack or stand for 1.5 mL tubes (Bangs Laboratories, Inc., Catalog No. LS001, MS002, MS003 or Life Technologies, Catalog No.12321D) or magnetic plate (Life Technologies, Catalog No.123310) for 1.5 mL microcentrifuge.
3. Thermal cycler, water bath, heating block, or another temperature-controlled device.
4. E-gel electronic gel reader or alternatively gel electrophoresis equipment and imager.
5. Vortex mixer.
6. NanoDrop UV-Vis Spectrophotometer Thermo Fisher.
7. Agilent TapeStation.

3 Methods

Day 1

Since genomic DNA was already collected (*see* Table 1), the protocol begins with EZ DNA methyl direct protocol bisulfite conversion step, skipping the proteinase K treatment step.

Bisulfite conversion of DNA

Genomic DNA preparation

1. For whole-animal ctenophore genomic DNA isolations, utilize Qiagen Genomic Tip 100/G or 500/G kits, depending on animal size. Quantify genomic DNA using Qubit BR dsDNA kit.
2. For each sample, if 20 μ L of sample contains at least 100 ng but less than 500 ng of genomic DNA, *two* separate reactions for bisulfite conversion should be prepared for the sample which will be combined during the bisulfite library protocol before final PCR amplification. Failure to do this will result in final library concentrations being too low for sequencing without over-amplification during the final PCR step.

Preparation and dilution of 5-mC methylation spike-in control.

The goal is to add $\sim$ 1 μ L of methylation spike-in control to the gDNA sample before bisulfite conversion so that only 1% of starting material is spike-in control. *Note:* These instructions are assuming that 20 μ L of your gDNA sample will only be around 100–200 ng. If higher than this range, dilution of spike-in control is not needed.

1. Perform a 1:5 dilution on the WiseGene 5-mC lambda spike-in control with nuclease-free water to create a 1.25 ng/ μ L final concentration.

Table 1
Genomic DNA isolations used for *Mnemiopsis leidyi* library construction

Final #	Species	Animal #	Tissue	Concentration ($\mu\text{g}/\mu\text{L}$)	Volume
737	<i>Mnemiopsis leidyi</i>	<i>Mnemiopsis</i> -2	Tip 500	0.173	700
739	<i>Mnemiopsis leidyi</i>	<i>Mnemiopsis</i> -3-2	Tip 500	0.0642	500
743	<i>Mnemiopsis leidyi</i>	<i>Mnemiopsis</i> -5-2	Tip 500	0.0874	500
773	<i>Mnemiopsis leidyi</i>	<i>Mnemiopsis</i> -1 2 h injury	Tip 100G	0.00828	500
774	<i>Mnemiopsis leidyi</i>	<i>Mnemiopsis</i> -2 2 h injury	Tip 100G	0.00568	500
775	<i>Mnemiopsis leidyi</i>	<i>Mnemiopsis</i> -3 2 h injury	Tip 100G	0.00444	500

2. Store dilution at +4 °C or on ice until ready to use.
3. Calculate total nanograms of ctenophore gDNA in 20 μL of sample, and add appropriate volume of lambda spike-in (not exceeding 2 μL).

When proceeding directly from isolated genomic DNA, we used 20 μL of sample isolated from the genomic tip. However, pilot testing revealed that this yielded bisulfite sequencing libraries with concentrations too low to be sequenced on NextSeq Illumina platforms. To compensate for this, and especially since these samples were whole-animal methylomes, we performed two separate bisulfite conversion reactions on 20 μL each per sample and combined the samples together during the final adapter cleanup step before PCR amplification. Since bisulfite conversion will generally cause breakages, no size selections were performed. Here we outline the Zymo methyl direct bisulfite kit and TruSeq DNA library construction kit used.

1. Add 21 μL of sample + spike-in control to 130 μL of CT Conversion Reagent solution in a PCR tube. Mix the sample, and then centrifuge briefly to ensure no droplets are in the cap or sides of the tube.
2. Proceed with the EZ DNA methyl direct protocol:
 Place the PCR tube(s) in a thermal cycler, and perform the following steps:
 - (a) 98 °C for 8 min
 - (b) 64 °C for 3.5 h
 - (c) 4 °C storage for up to 20 h
 Note: The 4 °C storage step is optional.
3. Add 600 μL of M-Binding Buffer into a Zymo-Spin™ IC Column, and place the column into a provided collection tube.

- (a) Note: The capacity of the collection tube with the column inserted is 800 μL . Empty the collection tube whenever necessary to prevent contamination of the column contents by the flow-through.
4. Load the sample (from **Step 3**) into the Zymo-Spin™ IC Column containing the M-Binding Buffer. Close the cap, and mix by inverting the column several times.
5. Centrifuge at full speed ($>10,000 \times g$) for 30 s. Discard the flow-through.
6. Add 100 μL of M-Wash Buffer to the column. Centrifuge at full speed for 30 s.
7. Add 200 μL of M-Desulfonation Buffer to the column, and let stand at room temperature (20–30 °C) for 15–20 min. After the incubation, centrifuge at full speed for 30 s.
8. Add 200 μL of M-Wash Buffer to the column. Centrifuge at full speed for 30 s.
 - (a) Add another 200 μL of M-Wash Buffer, and centrifuge for an additional 30 s.
9. Place the column into a 1.5 mL microcentrifuge tube.
 - (a) Add 11 μL of nuclease-free water directly to the column matrix. Centrifuge for 30 s at full speed to elute the DNA. Check columns for clogs, and centrifuge for an additional 30 s to get full elution.
 - (b) Note: Even with the second spin, usually only ~9–9.5 μL of liquid will come through a column.
10. The DNA is ready for immediate library production or should be stored at or below –70 °C as ctenophore DNA is notorious for degradation at higher temperatures.

Day 2

Library preparation:

Two TruSeq DNA methylation library reactions will be needed to produce one ctenophore DNA methylation sequencing library to have sufficient concentration, sequence diversity, and coverage due to heavy gDNA fragmentation that occurs in *Mnemiopsis*.

Preparation:

1. Take all consumables for “Anneal the DNA Synthesis Primer” and “Synthesize DNA” sections of the protocol out of –20 °C storage and thaw on ice; keep all bisulfite converted samples at –80 °C until these reagents have completely thawed. Plus, vortex primers and buffers before creating master mixes. Do not pulse vortex mix enzymes.

2. While reagents are thawing, program the following programs into a thermocycler with a heated lid:

Program 1 annealing primer.

- (a) 95 °C for 5 min in a thermal cycler with a heated lid

Synthesize DNA program.

- (b) 25 °C, 5 min
- (c) 42 °C, 30 min
- (d) 37 °C, 2 min

Manually pause thermocycler.

- (e) 37 °C for 10 min
- (f) 95 °C for 3 min
- (g) 25 °C for 2 min

Terminal tagging program

- (a) 25 °C for 30 min
- (b) 95 °C for 3 min
- (c) Hold at 4 °C

3. Create enough master mix for all bisulfite reaction tubes to be processed for “Synthesize DNA.” Pulse vortex the master mix before adding enzyme.
4. Prepare ice/cold water bath.
5. Take out AMPure beads, and place them at room temperature.

Producing DNA methylation library through tagmentation:

1. Proceed with TruSeq DNA methylation library protocol until the end of the “Tag DNA,” and leave both sample reactions at 4 °C held in thermocycler or on ice until ready to proceed to post-tagmentation bead cleaning step.

Post-tagmentation bead cleaning alternative “Purify the Tagged DNA” protocol:

1. The DNA must be purified before PCR. Each reaction is now in a volume of 25 µL. Combine the two reactions from the same gDNA sample into a fresh 1.5 mL tube (50 µL).
Illumina recommends using a 1.6× AMPure XP bead purification. Warm the AMPure XP beads to room temperature.
2. While the beads warm, prepare 800 µL of fresh 80% ethanol at room temperature for each sample. Vortex the AMPure XP beads until they are a homogenous suspension.
 - (a) Caution: Ensure the AMPure XP beads are in a homogenous suspension before continuing.

3. Add 80 μL of the beads to each well of the plate or to each microcentrifuge tube containing ditagged DNA from “Tag the DNA.”
4. Mix thoroughly by gently pipetting the entire volume of each well/tube for 10 times. If using microcentrifuge tubes, transfer each 130 μL volume to a separate 1.5 mL tube.
5. Incubate the plate/tubes at room temperature for 5 min.
6. Place the plate/tubes in a magnetic stand at room temperature for at least 5 min until the liquid appears clear.
7. Remove and discard the supernatant from each well/tube using a pipette (P200 or smaller). Some liquid can remain in each well/tube. Take care not to disturb the beads.
 - (a) Note: To remove some residual liquid, use a smaller tipped P10 or P20 pipette.
8. With the plate/tubes remaining on the magnetic stand, slowly add 300–400 μL of 80% ethanol to each well/tube without disturbing the beads. Make sure that the beads are covered with 80% ethanol.
9. Incubate the plate/tubes at room temperature for at least 30 s, doing at least a quarter turn of the tube in the magnetic stand and a quarter turn back, then remove and discard all of the supernatants. Take care not to disturb the beads.
10. Repeat **Steps 9** and **10** one more time for a total of two 80% ethanol washes.
11. After the second wash, remove the ethanol by pipetting (as much as possible without disturbing the beads) with the plate/tubes still on the magnetic stand. Remove the plate/tubes, centrifuge for 10–30 s, and place the plate/tubes back on the magnetic stand for 1 min. Use a fine pipette tip to remove all the residual ethanol.
12. Let the wells/tubes air-dry for 3 min on the magnetic stand.
 - (a) Note: Waving tubes with lids open for part of the incubation time is fine. If wafted smell of ethanol is still very strong after 3 min, let dry for 4 min total.
13. Add 25 μL of nuclease-free water to each well/tube, and remove the plate/tubes from the magnetic stand.
14. Thoroughly resuspend the beads by gently pipetting 20 times.
15. Incubate the plate/tubes at room temperature for 3 min.
16. Place the plate/tubes on the magnetic stand at room temperature for at least 5 min or until the liquid appears clear.
17. Carefully transfer 22.5 μL of the clear supernatant, which contains the ditagged DNA, from each well/tube to a new PCR

plate/tube. There should now be only one tube per library sample.

18. Place the plate/tubes on ice, and proceed to “Amplify the Library” and “Add an Index (Barcode),” or keep at -20°C for longer-term storage.

Changes to the final PCR “Amplify library” protocol:

1. The recommended number of PCR cycles in the TruSeq DNA methylation protocol is ten cycles. This cycle number is based on the presumption that library construction begins with a starting material amount of 200–500 ng post-bisulfite treatment. However, the best pretreatment starting material for *Mnemiopsis* achieved is usually 100–200 ng per reaction before bisulfite conversion and when the two reactions are combined ~100 ng post-conversion.
2. Follow the PCR amplification protocol as described in the TruSeq DNA methylation library protocol for 14 cycles, and pause the thermocycler. Do not run the final thermocycler step (7 min at 68°C). Take 1 μL of each library sample, and perform a 1:5 dilution. Prepare a 2% agarose E-gel on an E-gel reader. Load 2 μL of diluted library sample + 23 μL of nuclease-free water. Load 2 μL of diluted 50 bp ladder (1:40 dilution) into the ladder lane + 8 μL nuclease-free water. Fill all unloaded lanes with 25 μL of nuclease-free water. Run the 2% gel forward run program on the E-gel reader using the E-gel light button to check the run progress. Run until the reference ladder begins to separate (Fig. 1).
 - (a) Note 1: If E-gel reader is unavailable, this can also be done by loading all 5 μL diluted library samples into a 2% agarose gel with appropriate loading dye.
 - (b) Note 2: It is always advised to use TapeStation to visualize bisulfite sequencing libraries and obtain quantitative information about these libraries (Fig. 2).
3. Library bands should be both visible and bright. Continue with thermocycler library amplification for a total of 16–20 cycles.
4. Proceed to the final library bead cleaning step as instructed in the protocol. However, adjust elution volume to 30 μL as at least ~2–5 μL is lost since they cannot be recovered without also pipetting out residual magnetic beads, and ~5 μL may be needed for Qubit, TapeStation, and other concentration measurements pre-sequencing. This allows for ~10 μL to be used for sequencing and ~10 μL to be stored for backup/potential resequencing.

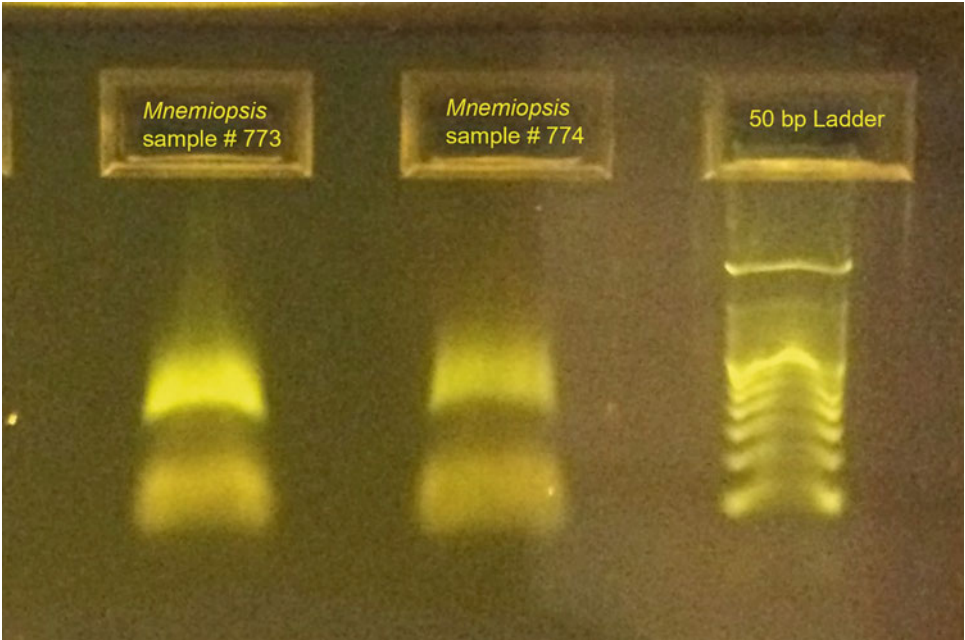


Fig. 1 Example 2% E-gel of amplified *Mnemiopsis leidyi* whole-animal bisulfite sequencing libraries. 2 μ L of sample diluted 1:5 was loaded with a 50 bp ladder after 17 cycles of PCR (Lane 1, sample # 773; Lane 2, sample # 774; Lane 3, 50 bp ladder). The orange-colored band at the bottom of each sample lane is residual sequencing adapters and adapter dimers that will be cleaned out and removed during the final AMPure bead cleaning

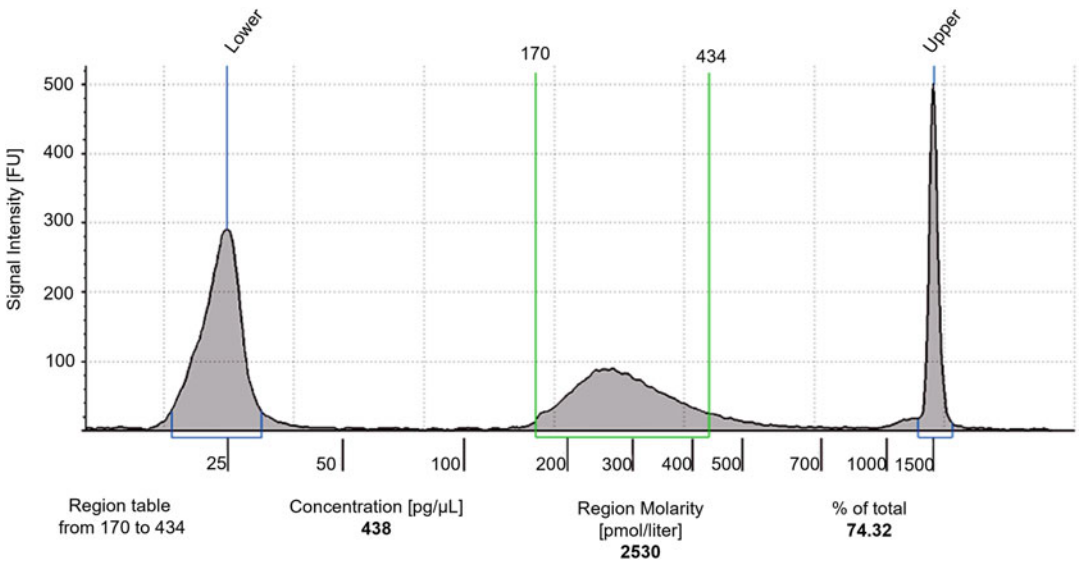


Fig. 2 Example TapeStation sample trace of *Mnemiopsis leidyi* whole-animal size distribution for bisulfite sequencing libraries. Control whole-animal bisulfite sequencing library (M37) diluted 1:5. The peak size and final concentration of this library are presented in Table 2

Table 2
Library sequencing statistics

	Barcode sequence	Amount (μL)	Sample name	Tissue	Library size	Library concentration nM	Number of reads	Number of bases
6	5'-GCCAAT-3'	10	<i>Mnemiopsis</i> an3 2 h injury	Whole animal	265	6.85	41,692,352	4,169,235,200
7	5'-CAGATC-3'	10	<i>Mnemiopsis</i> an1 2 h injury	Whole animal	261	3.255	33,478,600	3,347,860,000
8	5'-ACTTGA-3'	10	<i>Mnemiopsis</i> an2 2 h injury	Whole animal	270	3.535	35,043,424	3,504,342,400
6	5'-GCCAAT-3'	10	<i>Mnemiopsis</i> -737	Whole animal	246	4.8	71,595,718	7,159,571,800
7	5'-CAGATC-3'	10	<i>Mnemiopsis</i> -739	Whole animal	247	4.1	68,139,908	6,813,990,800
8	5'-ACTTGA-3'	10	<i>Mnemiopsis</i> -743	Whole animal	253	4.2	75,031,470	7,503,147,000

4 Downstream Bioinformatic Analysis

We used the *Mnemiopsis* Genome Project Portal as a key reference for mapping and annotation [15]. Our *Mnemiopsis* DNA methylation library sequencing reads were processed on the UF HiPerGator cloud computing system using the dmap2 methylation pipeline based on MOABS with the novel cscall methylation calling function [16]. The software can be downloaded using <http://compbio.ufl.edu/software/cscall/>.

5 Conclusions

The presented BS-seq library protocol described produced high-quality data for downstream analysis. Under two different states (control vs injury), we successfully detected 5-mC in whole-animal methylomes at CpG, CHG, and CHH sites with detection of changes in methylation of genes encoding gap junction proteins (innexins), pore-forming toxins and neuropeptides (Fig. 3). These new *Mnemiopsis* methylomes covered a higher percentage of the *Mnemiopsis leidyi* genome with a deeper depth of coverage than our previously published methylomes (Table 3). Differences were detected in the average gene body and average exonic DNA methylation in treatment and control groups. Although the overall gDNA methylation is lower than some previously reported methylation levels [7], the additional sequence coverage of these new samples also covered at least 300,000 additional CpG sites. Our methylation percentages are also consistent with levels reported in *Mnemiopsis* developmental larval methylomes [6]. Overall, these methods produced robust methylomes with novel insights into

Table 3
Coverage and percentages of CpG DNA methylation

Sample	Total CpGs in genome	Total CpGs called	% Sites called	Total methylated	Total methylated adjusted	% Methylated	% Methylated adjusted
Control	4,405,646	3,455,804	0.78	616,220	260,001	0.18	0.08
Injury	4,405,646	2,302,863	0.52	445,124	198,509	0.19	0.09

Mnemiopsis leidyi methylomes, the samples produced by these libraries had a higher percentage of genomic CpGs with sufficient sequencing coverage (78% of all CpGs for the control sample with 1,481,304 additional CpGs called than previous methylomes, and 52% injury with 328,363 additional CpGs called than previously reported) than our previously published methylome (45%). “Methylated sites” refer to any site where the number of called cytosines was greater than 0. However, when the presence of mCpG was adjusted to a binary based on false detection rates from spike-in controls, a lower overall rate of methylated CpG sites was detected than previously reported

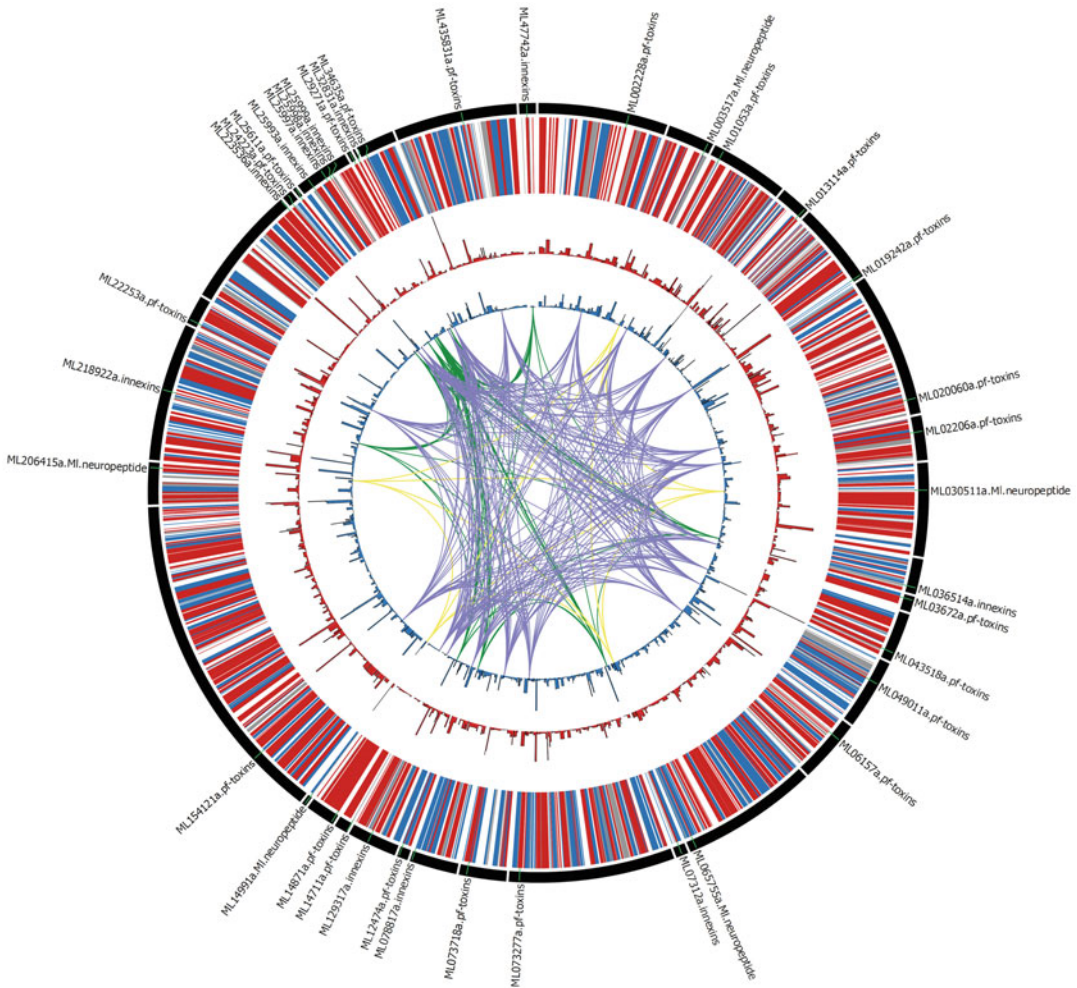


Fig. 3 Differential gene body CpG DNA methylation is detectable between whole-animal control and body-wall injury methylomes produced with the described technique. Circos genomic summary diagram for all genomic scaffolds that contain innexins (dark green linker lines), pore-forming toxins (dark purple linker lines), and neuropeptides (yellow linker lines). Genes of interest are labeled with their unique *Mnemiopsis* gene model identifiers and the gene type that is mirrored in the linker colors. The two inner barplot rings display the mean percentage of gene body methylation per gene for control (dark blue track) and 2 h injury methylomes (red track). The outer heat map plots whether the mean CpG methylation percentage—for just sites covered in both conditions—is higher in control (dark blue), 2 h post-injury (red), neither group (gray), or no overlapping cytosines were covered in the gene body between groups (white). Genes of interest are labeled with unique *Mnemiopsis* gene identifiers, and also gene groups are provided

the role of DNA methylation of innexins and neuropeptides in the regenerative abilities of *Mnemiopsis leidyi* (Fig. 3).

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