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Single-Neuron Transcriptome and Methylome Sequencing for Epigenomic Analysis of Aging

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Abstract

Enormous heterogeneity in transcription and signaling is the feature that slows down progress in our understanding of the mechanisms of normal aging and age-related diseases. This is critical for neurobiology of aging where the enormous diversity of neuronal populations presents a significant challenge in experimental design. Here, we introduce *Aplysia* as a model for genomic analysis of aging at the single-cell level and provide protocols for integrated transcriptome and methylome profiling of individually identified neurons during the aging process. These single-cell RNA-seq and DNA methylation assays (methyl-capture/methyl enrichment) are compatible with all major next generation sequencing platforms (we used Roche/454 and SOLiD/Life Technologies as illustrative examples) and can be used to integrate an epigenetic signature with transcriptional output. The described sequencing library construction protocol provides both quantitative and directional information from transcriptional profiling of individual cells. Our results also confirm that different copies of DNA in polyploid *Aplysia* neurons behave similarly with respect to their DNA methylation.

Keywords

Single-cell RNA-seq; DNA methylation; Transcriptome; *Aplysia*; Epigenomics; Epigenetics; Aging; Memory; Next generation sequencing

1 Introduction

Enormous and highly dynamic cell heterogeneity in transcription and signaling is the feature that slows down progress in our understanding of the mechanisms of normal aging and age-related diseases. This is particularly important in neuroscience and neurobiology of aging where the enormous diversity of neuronal populations present a significant challenge in experimental designs. Thus, it is highly desirable to *directly measure molecular signatures and identify mechanisms of aging in neural circuits at single-cell resolution*. Although it is technically difficult, if not impossible, from mammalian preparations, such single-cell resolution is achievable using some invertebrate model organisms.

In this chapter, we will first introduce a model for genomic analysis at the single-cell level—the marine mollusk, *Aplysia californica* (Fig. 1). Second, using individual identified neurons of *Aplysia* as illustrated examples, we will provide protocols for single-cell transcriptome

(RNA-seq) and methylome profiling. Importantly, recent single-cell microarray and RNA-seq studies demonstrated that different neurons indeed age differently [1], Fig. 2. The discovered cell-type specificity in expression of more than several thousand genes suggests that a preexisting epigenetic landscape might significantly determine *how neurons would age*. In other words, conceptually both aging and/or memory formation at the single-cell level can be viewed as a Waddington landscape diagram (Fig. 2d).

We are only beginning to realize that at the very core of many neuronal integrative mechanisms is the evolutionarily conserved process of DNA methylation/demethylation [2–9]—i.e., the covalent modification of cytosine in animal genomes to form a fifth base (5-methylcytosine; (5mC)) or undoing this modification via a sixth base [10, 11] (5-hydroxymethyl cytosine; (5hmC)). Although the levels of 5hmC/5mC have recently been shown to change in the hippocampus with normal aging [12, 13], neither the mechanisms nor cell specificity is known.

It was proposed that age-associated decay of synaptic functions is due to selective, sometimes neuron-specific, downregulation of genes controlling polarized transport of protein-RNA cargos to distant synapses and DNA methylation [1]. Testing these hypotheses at the single-cell level should facilitate identification of molecular markers of aging in general and mechanisms of age sensitivity for certain neuronal classes in particular. The implementation of single-cell epigenomic protocols combined with RNA-seq profiling will also advance our understanding of the logic of gene regulation during normal physiological functions such as loss associative forms of memory. Further progress is also needed in generating efficient model systems to bring together all levels of information, from *genome to cell to circuit to behavior*.

2 *Aplysia* as the Emerging Model for Single-Cell Genomics of Aging

Biology has often advanced by making use of relatively simple model systems. Arguably, *Aplysia* and its simple memory-forming circuit [14–23] is one of only a few currently available model systems [17, 19, 24] that will efficiently advance single-cell studies and encourage new directions in the biology of aging. To stress this point, single-neuron methylation profiling in mammals is currently impossible due to the very small amount of DNA (5–6 pg) and the fact that any required amplification leads to loss of methylation signatures. Thus, a mouse model (e.g., the well-recognized trisynaptic circuit of the hippocampus) of aging *cannot* be used for combined single-cell methylome/RNA-seq profiling. Even reproducible single-cell RNA-seq has not been achieved for any functionally characterized mammalian neuron and initial attempts revealed unprecedented molecular heterogeneity within apparently similar neuronal populations [13, 25, 26]. In contrast, an *Aplysia* neuron yields up to 260 ng of DNA, allowing us to perform single-neuron methylome profiling efficiently (the current limitation is 1 ng of starting material or ~200 mammalian neurons) together with gene expression profiling (RNA-seq) *from the very same cell*, and follows aging at the level of single identified neurons over the entire life span. As such, the *Aplysia* model offers unprecedented advantages to address fundamental questions in aging research.

2.1 The Phenomenology of Aging

Dynamics in *Aplysia* has been studied in sufficient detail (Fig. 1) both in nature and under controlled aquaculture environments [27, 28]. Sexual maturity is generally completed within 2–3 months (~40 % of their life spans) when animals start copulative and egg-laying behaviors (usually in 80–200 g animals). It is easy to culture *Aplysia* and inbred lines can be developed (e.g., in NIH-supported *Aplysia* facilities) (Miami, <http://aplysia.miami.edu/>).

Life expectancy for *Aplysia* is about 150–350 days. *Aplysia* grow linearly without approaching a limiting size (sometimes reaching more than 1–2 kg); thus, the existence of senescence indicates that aging is not a by-product of the cessation of growth [29]. Mean lifespan of animals fed ad libitum was approximately 228 day. In contrast, animals fed standard but limited rations lived much longer (an average of 375 day) and showed a lower initial mortality rate, suggesting that caloric restriction on a single-species diet prolongs lifespan in *Aplysia* [27]. Importantly, animals reared at 13 °C or 15 °C grew as much as four times as large, lived twice as long, matured later, and spawned longer than did animals reared at 18 °C or 21 °C. Aging rate was highest for animals reared at 21 °C—as expected for the accelerated lifecycle at higher temperatures [28].

2.2 Functionally Identified Neural Circuits and Accessible Neurons in *Aplysia*

Neural circuits controlling both stereotyped and learned behaviors have been identified in the CNS of *Aplysia* [22, 23], providing a solid background to understand cellular bases of behavior at the level of a small number of individually identified neurons. The most significant progress has been made in characterizing memory-forming networks, performed in the laboratory of Eric Kandel, his collaborators, and many other groups [14–23]. The cumulative data obtained during more than 40 years of behavioral, neuronal, and molecular analysis of a network involved in defensive reactions has yielded what is now one of the best understood experimental systems for cellular studies of simple forms of learning and memory [19]. Surprisingly, a large number of critical phenomena associated with long-term synaptic plasticity and memory can be reproduced in a simpler system consisting of only 2–3 neuronal subtypes. This simplest memory-forming network (i.e., a glutamatergic sensory cell, a cholinergic motor neuron, and a serotonergic or FMRFamide-containing interneuron) can also be reconstructed in cell culture. Importantly, this network can be even further reduced to two cells where the action of the facilitatory serotonergic interneurons can be substituted by local application of serotonin (5-HT) [19] or nitric oxide (NO) [30], the neurotransmitters that induce long-term facilitation. Signal transduction pathways underlying 5-HT-induced learning mechanisms are well described [17, 19, 31, 32] and mediated by canonical cAMP/PKA/MAPK/CREB pathways, whereas NO signaling is partially coupled with cGMP pathways [30, 33]. Both of these facilitatory transmitters and aging result in large-scale changes in gene expression, synaptic reconfiguration, and long-term plasticity within individual neurons and are paralleled from flies to humans and rodents [19].

From a methodological point of view, the most remarkable feature of *Aplysia* is the presence of large, easily identified neurons [22–24]. As we recently measured, these neurons yield from 30 ng to 1.9 µg of total RNA per single neuron [1, 24]. They can also provide from 3 to

250 ng of gDNA depending upon cell size [24]. The accessibility and size of *Aplysia* neurons (ranging from 30 to 1,100 μm) are ideal for development and integration of genomic/ epigenomic profiling at a single-cell level. Both single cells and their giant nuclei can be isolated within minutes, making single-cell genomic profiling (RNA-Seq/ChIP-Seq) entirely feasible [1, 34] (Fig. 3). It was also shown that <1 % of the cytoplasm from a single cell is needed to directly characterize and quantify hundreds of intracellular metabolites, signal molecules, and various peptides using capillary electrophoresis and mass spectrometry [35–39]. These posttranscriptional and posttranslational assay protocols are well established and can complement the genomic and epigenetic profiling from the same cells outlined below.

In summary, changes in transcription and DNA methylation with aging have been shown to occur in rodent hippocampus [40], yet mouse and rat models of aging *cannot* be used for integrated single-cell RNA-seq/methylome profiling because the minute amount of DNA (1–2 pg/cell) requires amplification for sequencing, which in turn erases epigenetic and methylation signatures. At present, this type of single-cell experiment is only possible using *Aplysia*, and the protocols for single-cell RNA-seq and DNA methylation profiling are presented in the following sections.

3 Materials

Reagents

- QIAamp[®] DNA Micro genomic DNA isolation kit (Cat # 56304, Qiagen).
- RNAqueous-Micro[™] (Cat # 1931, Ambion/Life Technologies).
- ArrayControl[™] RNA Spike-in control RNAs (Cat #1780, Ambion/Life Technologies).
- High-Capacity cDNA Archive Kit (Cat # 4322171, Applied Biosystems/Life Technologies).
- TaqMan[®] Universal PCR Master Mix (Cat # 4304437, Applied Biosystems/Life Technologies).
- SYBR Green PCR Master Mix (Cat # 4309155, Applied Bio-systems/Life Technologies).
- MEGAscript[®] T7 Kit (Cat # 1333, Ambion/Life technologies).
- Quick Ligation[™] Kit (Cat# M2200S, New England BioLabs).
 - 10× Quick Ligation Reaction Buffer:
 - 132 mM Tris–HCl.
 - 20 mM MgCl₂.
 - 2 mM dithiothreitol.
 - 2 mM ATP.
 - 15 % polyethylene glycol (PEG 6000) pH 7.6 at 25 °C.

- AMPure XP Reagent (Cat # A63880, Agencourt, Beckman Coulter, Inc.).
- LA Taq™ (Cat # RR002M, Clontech) all buffers included.
- MethylMiner™ (Cat # ME10025, Invitrogen/Life Technologies).
 - Components of the kit include all buffers plus.
Dynabeads® M-280 Streptavidin.
MBD-Biotin Protein.
- End-It™ DNA End-Repair Kit (Cat# ER0720, Epicentre).
- E-Gel® SizeSelect™ 2 % Agarose (Cat # G6610-02, Invitrogen/Life Technologies).
- Agilent Bioanalyzer™ High Sensitivity DNA Kit (Cat #5067-4626, Agilent Technologies).
- Agilent Bioanalyzer™ RNA 6000 Pico Kit (Cat #5067-1513, Agilent Technologies).
- Primers: 0.2 µM scale HPLC purified, IDT, Integrated DNA Technologies, Inc. (*see* Table 1 for sequences).

Equipment

- Agilent Bioanalyzer™ 2100 (Cat # G2947CA, Agilent Technologies).
- DynaMag™-2 magnet (microcentrifuge tube magnet) (Cat # 123-21D, Invitrogen, Life Technologies).
- Covaris S220 Focused-ultrasonicator.
- ABI 7700 Sequence Detection System (Applied Biosystems/ Life Technologies).
- Qubit® 2.0 Fluorometer (Cat # Q32866, Invitrogen/Life Technologies).
- MJ Research Thermo Cycler (Cat # PTC-100, MJ Research).
- E-Gel® iBase™ and E-Gel® Safe Imager™ Combo Kit (Cat # G6465, Invitrogen/ Life Technologies).

4 Methods

4.1 Single-Neuron Identification and Isolation

Aplysia californica has the largest neurons in the animal kingdom with the cholinergic motoneuron R2 measuring up to 1.1 mm in diameter (*see* refs. 24, 41 and Fig 2b). Many of the neurons in the central nervous system of *Aplysia* are identified on the basis of distinct function and phenotype [22, 23] and can be easily mechanically isolated. In many cases, the same neuron can be visually identified from animal to animal. If visual identification is not possible, as in the L7 neuron, electrophysiological testing was performed for identification [34, 41]. The illustrated examples of the isolation of two identified neurons are shown in Figs. 2b and 3a using a glass micropipette [41]. In both cases, the ganglia can be pretreated

with protease treatment to soften and remove the connective tissues and expose neurons of interest. If visual identification is possible, the easiest and most efficient way to isolate neurons is to place the ganglia in cold (4–10 °C) ethanol for a few minutes. This treatment quickly fixes the ganglia and prevents neuronal injury during the isolation without affecting RNA quality and integrity. Next, a pair of glass microelectrodes (like those used for conventional microelectrode recordings) can be used to mechanically remove individual neurons (Fig. 2b). The procedure takes from 1 to 10–15 min to isolate a neuron of interest (it is a matter of practice and skill).

An alternative and complementary method is to isolate living neurons using the same protocols as for cell culture [41] (see detailed description in Lovell and Moroz, 2006); this procedure is illustrated in Fig. 3a. The later procedure can be applied to neurons that require electrophysiological identification and characterization. We have obtained comparable yields of RNA using either of these procedures.

Importantly, as we indicated earlier, it is possible to isolate individual neuronal processes, axons, and even growth cones for transcriptome profiling of extrasomatic RNA. To facilitate the visualization, neuronal processes can be electroporated using fluorescent dyes as described elsewhere [42].

4.2 Single-Neuron RNA Extraction

Once single neurons and/or cell compartments are isolated, total RNA is extracted (*see* Fig. 3a, b). We choose the RNA isolation kit or method that has been experimentally proven best based on the quality and quantity of RNA for a specific animal, tissue, or even single cell [43] (see below for more details and notes). For single neurons, the RNeasy-Micro™ Kit produces excellent and reproducible quality RNA. RNA quality is analyzed using an Agilent 2100 Bioanalyzer™ on a 6000 Nano LabChip. For very small quantities the 6000 Pico LabChip can be used. From the cell body of a single L7 neuron, we extracted up to 100–200 ng of RNA (*see* Fig. 3), and from the processes of sensory neurons in cell culture, we extracted 20–40 pg of total RNA that was used to generate sequencing libraries for the Roche/454 platform.

We show that the total RNA quantity from each neuron is linear in relation to its cell volume ($R^2 = 0.9052$, *see* also Fig. 3). Therefore, the concentration of RNA molecules is constant regardless of cell size in these neurons. From the single R2 neuron shown in Fig. 2b, 1.9 µg of total RNA was extracted (*see* Note 1). Up to 250 ng of genomic DNA was extracted from the same identified neuron, R2, which is the largest known neuron in the animal kingdom. However, the procedure was successfully tested for the entire spectrum of neuronal cell diameters from 30 to 300 µm in *Aplysia*, squid (*Loligo*) axoplasm, and for clusters of 20–100 hippocampal neurons from CA1 and CA3 regions of rat brain. Thus, the presented method is not limited to neurons isolated from *Aplysia*, and our protocol can also be easily adapted to samples with larger amounts of material.

¹With *Aplysia*'s large neurons, it is possible to isolate both RNA and DNA from the same cell with the AllPrep DNA/RNA/ Protein Mini Kit from Qiagen. However, when using an all-in-one kit, the yield is usually sacrificed.

4.3 Single-Cell RNA-Seq Library for 454 Pyrosequencing

All commercially available RNA-seq library kits from Illumina, SOLiD, and the new Ion Torrent PGM sequencers require large amounts of starting material (>1.0 µg) and are not feasible for single cells. The general strategy for all these kits starts with rRNA depleted or total mRNA isolated; the resultant RNA is fragmented either chemically or by heat and then ligated with RNA primers specific to a chosen sequencing technology, followed by reverse transcription to generate cDNA by either template switch reverse transcriptase or the traditional first- and second-strand cocktail of enzymes. The resultant dsDNA is amplified, purified, and sequenced.

Recently, two single-cell approaches to RNA-Seq were published—Tang and colleagues [44, 45] and Linnarsson and colleagues [46]. Both protocols demonstrate single-cell sequencing capabilities. Both protocols also start with capture of single cells and direct processing to reverse transcription with no subsequent RNA isolation, and we have used this method with success. However, after this point the two protocols deviate radically, with the Tang protocol becoming a 100-step process over 6 days time that has only been applied to 30 atypical large early embryonic cells (10–100 times larger than most somatic cells) with no internal controls. The Linnarsson protocol uses a template switch method of generating cDNA and validates their method with spiked-in controls.

Here, we used an unbiased method of library construction for transcriptional profiling that is both quantitative and qualitative, preserves the direction of transcripts, and is applicable for single neurons (*see* Fig. 4a). All materials and methods for this type of RNA-seq library construction have been described [43]. The only current modification to the protocol is the primers and adaptors that are specific for 454 sequencing technology (*see* Table 1). All together for these single-cell 454 specific libraries, we used one cholinergic R2 neuron, one L7 motor neuron, three serotonergic MCC neurons, and a cluster of approximately 200 sensory neurons to validate our method's applicability.

Briefly, library construction starts with total RNA reverse transcribed to cDNA with an oligo-dT primer, and then a second strand is generated (*see* Fig. 4a). The double-stranded cDNA is fragmented with a restriction enzyme, and then 454-specific adaptors are sequentially ligated onto the double-stranded cDNA fragments, which are finally amplified. Fragmented DNA with ligated adaptors is processed through an emulsion-based clonal amplification (emPCR) then captured onto beads as required for subsequent sequencing steps. DNA-captured beads are placed on a PicoTiter-Plate device for pyrosequencing using the 454 GS-FLX platform. We validated both the quantitative aspect with absolute RT-PCR with spiked-in controls and directionality of sequencing (*see* Subheading 4.1, 4.2, 4.4 and Fig. 4b–d).

4.4 Validation of Single-Cell RNA-Seq

RNA-seq, also called “Whole Transcriptome Shotgun Sequencing,” can, by sheer brute force of high sampling, detect RNAs from very low abundance classes (or a rare subpopulation of cells contributing to the sample) and do it unambiguously. Fundamental to all RNA-seq experiments is a validation of quality, quantification, and directionality of the

transcription output for a given sample/ single cell to substantiate the protocols. Validation of our single-cell RNA-seq has been performed with two types of controls: external (six *spiked-in* RNAs of known concentration) and internal (absolute copy number of four cloned and characterized transcripts). We also performed a correlation of sequenced digital output across different cell types with the results of quantitative RT-PCR. Then we used in situ hybridization to confirm cell-specific expression profiling, relative abundance, and expression of predicted antisense transcripts.

4.4.1 Spike-in RNA Controls (External Control)—For the external controls, sequencing was performed with six non-animal oligonucleotides (ArrayControl™ RNA Spike-in control RNAs) spiked-in at various concentrations to the RNA of a single L7 motoneuron at the beginning of the library construction. As a result, we demonstrated a linear correlation between the quantities of each spiked-in sequence added at the beginning of the protocol and the number of reads generated from RNA-seq data (*see* Table 2). Frequency (or “expression level”) is the number of reads divided by the total number of reads in a specific library.

4.4.2 Protocol for Absolute Real-Time PCR (Copy Number Determination)—An internal copy number for four specific transcripts (*see* Table 3) was generated using absolute quantification with the TaqMan® custom gene expression assay from three different individual R2 neurons. The specific transcripts were chosen based on their abundances in a single R2 neuron. The intracellular copy numbers for four transcripts of interest also showed a linear correlation to their abundance (expression) in the sequence data set (*see* Fig. 4b).

Protocol for Absolute RT-PCR is the following (*see* Note 3):

1. Single cells R2, L7, MCC, and sensory cluster had their size measured and were then isolated.
2. Total RNA was extracted from these single cells using RNAqueous-Micro™ as described in Subheading 4.2.
3. cDNA synthesis was produced with random hexamers using the High-Capacity cDNA Archive Kit.
4. Primers and probes were tested for 100 % efficiency by ABI and came premixed in a 20× solution (*see* Table 3 for sequences).
5. Two-Step RT-PCR was performed where the RT and PCR were done separately. The PCR was performed using TaqMan® Universal PCR Master Mix on an ABI 7700 Sequence Detection System.
6. The linear dynamic range of the input RNA (cDNA) from 1 ng to 10 pg was determined. All samples and standards were performed in quadruples.
7. The PCR conditions were the manufacture’s recommendations which were:

One cycle at 95 °C for 10 min.

³The RT-PCR protocol followed manufactures’ recommendations and is applicable to most RT-PCR machines.

Followed by 40 cycles.

95°C for 15 s.

60°C for 1 min.

8. The negative control was a cell proven by in situ hybridization to not contain the tested sequence.
9. The standard curve was generated in the following way:
 - a. A plasmid that contained the sequence of interest was transcribed with MEGAscript® T7 Kit to generate cRNA.
 - b. The concentration of the cRNA was measured with a 2100 Bioanalyzer (Agilent Technologies).
 - c. A known amount of cRNA was added for cDNA synthesis using the same protocol as the samples.
 - d. Once the cDNA was produced, dilutions were made to generate 30–300,000 copy numbers based on the starting cRNA concentration.
 - e. Sample copy number was calculated from the standard curve and standard deviation generated.
 - f. Expression is the number of reads for the gene of interest divided by the total number of reads for that specific sequencing dataset.
 - g. R^2 were generated for three individual R2 neurons Fig. 4b.

4.4.3 Localization by In Situ Hybridization for RNA-Seq Validation—In situ hybridization (ISH) is a well-established method that uses a labeled complementary RNA (cRNA) strand (i.e., probe) to localize a specific RNA sequence at single-neuron resolution. Thus, we used in situ hybridization to further confirm neuron-specific expression profiling of more than 50 selected genes selected from corresponding digital RNA-seq profiles (*see* Note 2). Finally, observed directionality of the specific genes of interest is shown to be neuron specific and differential (confirmed by in situ hybridization, see examples of the multicolor in situ hybridization protocols used in [42]). Numerous expression patterns of selected mRNA transcripts have been mapped to individual cells in the central nervous system (CNS) of *Aplysia* as we reported elsewhere [34, 42]. The relative abundance of select transcripts perfectly correlates with their digital expression profiles (*see* Fig. 4b–d).

4.4.4 Protocol for Quantitative RT-PCR for Identified Neurons—For genes of interest, a correlation between their calculated digital expression profiles in the identified cells with their expression levels using quantitative RT-PCR (QRT-PCR) must be performed to confirm the efficiency of single-cell RNA-seq. For example, expression of the transcript encoding FMRFamide, an R2-specific neuropeptide marker, was confirmed by absolute RT-PCR (Fig. 4d), in situ hybridization (Fig. 4c), and number of sequence reads.

²Digital profiling or RNA-seq refers to quantification of sequencing reads. Frequency or expression is calculated by dividing the number of reads of a specific transcript by the total number of reads in a specific library.

The key steps for the QRT-PCR follow (*see* Note 3 as well as [47]):

1. Single-neuron isolation and RNA extraction are described in Subheadings 3.1 and 3.2
2. cDNA is generated as described in the Subheading 4.3.
3. Sequences of the transcript of interest can be loaded into Primer Express® software (Applied Biosystems) to design specific primers (amplicon lengths are between 75 and 125 bp).
4. Two-step RT-PCR does the RT and PCR separately with the SYBR Green PCR Master Mix on an ABI 7700 Sequence Detection System (following the same PCR setup as in Subheading 4.4.2).
5. The linear dynamic range of the input RNA (cDNA) is 10 pg–1 ng.
6. All primer sets are tested for optimal dissociation curves with amplification efficiencies between 85 and 100 % (all primer sets not meeting the standards should be redesigned).
7. We usually normalized all runs to *Aplysia* 18s RNA as an endogenous control.
8. The relative standard curve method was employed for analysis and an expression ratio calculated for each sample pair.
9. Ideally all data should be performed in technical triplicate and from at least two biological replicates.

4.4.5 Sense and Antisense Digital Profiling—The library construction protocol presented here preserves directionality and allows us to measure sense and antisense by digital profiling, while the external spiked-in controls allow us to monitor the quality of the library. We also confirmed the directionality with the spike-ins and only 0.7 % were sequenced in the antisense direction.

In contrast, when 317 known *Aplysia* full-length cDNAs (*see* Note 4) were mapped to all the single-neuron transcriptomes, 94.12 % were in the sense orientation and 5.88 % were antisense. These antisense reads were not randomly distributed and were only present for approximately 44 % of the full-length *Aplysia* cDNAs examined. Antisense reads aligned to specific genome regions on the cDNA in one neuron were absent from others. While antisense reads matching specific cDNAs appeared independently in samples derived from different neuronal types and aligned to the same sections along the cDNAs, the percent of antisense was also variable between cells. For example, Fig. 5 demonstrates differential expression and complex patterns of abundance and directionality of specific transcripts between two neurons: the peptidergic motoneuron L7 vs. serotonergic interneuron MCC (the red-colored reads are antisense and the blue-colored reads are sense).

⁴*Aplysia californica* full-length cDNAs are all NCBI reported clones and sequences as well as an additional set of unreleased cDNAs cloned in our lab.

4.5 Single-Cell Enriched Methylated Genomic DNA Library (MethylMiner™)

4.5.1 Introduction to DNA Methylation Protocols—Determining the epigenetic signature of a single cell is an enormous challenge. It would be reasonable to assume that every neuron, because of its unique properties and wiring, might have its own unique transcriptome as well as methylome. *Aplysia*'s large neurons are fully accessible for direct DNA methylation profiling at the entire genomic scale (single-neuron methylome, *see* Note 5). In general, it is possible to use the same set of neurons as described for RNA-seq experiments. Specifically, we outline here an enrichment method of 5-methylcytosine (5-mC). It is a more practical first step for methylation profiling, in contrast to bisulfite sequencing, because of the limited amount of genomic DNA even from large *Aplysia* neurons (5–250 ng). The bisulfite sequencing technique relies on the conversion of every unmethylated cytosine residue to uracil, which is then sequenced and recognized as a thymine. If conversion is incomplete the result is false positive calls for methylation sites. A major challenge in bisulfite sequencing is the degradation of DNA that takes place at the same time as the conversion. The conditions necessary for complete conversion, such as long incubation times, elevated temperature, and high bisulfite concentration, can lead to the degradation of ~90 % of the sample DNA. Since the starting amount of genomic DNA in single cells is limited, the extensive degradation associated with bisulfite sequencing can be problematic. A final concern with bisulfite sequencing is that the treatment greatly reduces the level of complexity in the sample to mostly three bases making molecular manipulations more difficult.

Here we present an enrichment capture technique for methylated (5-mC) DNA using the MethylMiner™ approach followed by high-throughput sequencing which is adapted to single neurons (*see* Fig. 6 and Note 6).

The most important advantage to the MethylMiner™ enrichment is that, unlike bisulfite treatment, there are no harsh denaturing conditions causing severe degradation and loss of DNA. Genomic DNA is isolated from single cells then fragmented to 150 bp. Methylated DNA is enriched from fragmented genomic DNA via binding to the methyl-CpG binding domain of human MBD2 protein, which is coupled to paramagnetic Dynabeads® M-280

⁵The large *Aplysia* neurons can go through up to 16 rounds of duplication generating 50–250 ng of gDNA and over 1 µg of RNA. The polyploidy might be viewed like a “photocopy” or “nature PCR machine” multiplying the entire genome complement and thus generating substantial amounts of genomic DNA from a single neuron. The functional significance of neuronal polyploidy is not clear in *Aplysia*. It was determined by histochemical measures that the amount of gDNA in large neurons of *Aplysia* correlates to the number of genomic duplication and can reach 100,000 genome copies per cell [57]. Although the physiological changes that accompany a polyploidy state are poorly understood, there are several potential advantages to polyploidy. Increase in cell size due to polyploidy might provide a metabolic benefit to support extensive branching of axodendritic processes innervating very large areas. A gene copy redundancy might, in principle, protect polyploid cells from deleterious mutations. However, it is important to know whether all genomic copies in the same *Aplysia* neuron are equally methylated.

To address these questions we looked at the epigenetic mark of 5-mC DNA methylation. Will all copies of the same genome carry the same epigenetic mark of 5-mC DNA methylation? We investigated selected genes involved in synaptic function. An example of the methylation status of DNMT in polyploid neurons is shown in Fig. 8. Here bisulfite sequencing of a band from a PCR product was performed. *The starred cytosines in the sample chromatogram show no background from other nucleotides, thus indicating all the copies of the genome in polyploid neurons are equally methylated.* If some of the cytosines were not methylated, there would be multiple peaks in the chromatogram indicating a mixed population, but this is not the case here because there are no background peaks in any of the cytosines marked. Therefore, we conclude that in *Aplysia* all the copies of the genome in polyploid neurons have the same methylation status.

⁶We also think that novel commercial kits exploring similar approaches (e.g., EpiXplore from Clontech) can be adapted for integrated RNA-seq/methylome profiling. However, their applicability for small amounts of starting material needs to be validated separately.

Streptavidin via a biotin linker from the MethylMiner™ kit. It should be noted that *Aplysia* has the MBD protein (Acc# ADM34183.1) which shares 98 % identity with the binding domain of the human MBD2 used in this kit (*see* Fig. 6). The enriched gDNA is eluted with one high-salt elution step. Resultant gDNA is ligated with the appropriate P1 and P2 SOLiD adaptors and amplified. The products were sequenced directly using SOLiD technology (*see* Note 7).

4.5.2 Library Construction

1. Genomic DNA was isolated from single cells using a QIAamp® DNA Micro genomic DNA isolation kit (*see* Note 8).
2. Isolated genomic DNA was fragmented to 150 bp with a Covaris S220 Focused-ultrasonicator (*see* Note 9).
3. End repair of fragmented gDNA.

34.0 µL	DNA to end repair
5.0 µL	10× end-repair buffer
5.0 µL	dNTP mix
5.0 µL	ATP
1.0 µL	End-repair enzyme mix
50.0 µL	Total volume

4. Mixture is incubated 20 min at room temperature.
5. The mixture is purified with 1.8 volumes of AMPure XP Reagent (90.0 µL) as described above and eluted with 35 µL of nuclease-free water. This fragmented DNA is the template for the methylation enrichment.
6. Methylated DNA was enriched following manufacturer's directions (MethylMiner™).
 - (a) Initial bead wash of the Dynabeads® M-280 Streptavidin.
 - i. 10.0 µL of beads were added to the DNA for a final volume of 100.0 µL
 - ii. Tubes were placed on a magnetic rack for 1 min to concentrate the beads, and then the liquid was discarded.

⁷A potential limitation with a simple MethylMiner™ enrichment is the lack of single-nucleotide resolution. In principle, bisulfite sequencing can be conducted after MethylMiner™ enrichment. Nevertheless bisulfite sequencing may require more material (*see* Fig. 6) and additional control experiments need to be performed regarding compatibility between MethylMiner enrichment and bisulfite treatment. For example, it is true that bisulfite conversion can degrade DNA, but magnetic bead capture of methylated DNA might also lead to some aspecific binding. Thus, a combination of protocols with amplification after capture of methylated DNA and following bisulfite treatment can be tested for further single-nucleotide resolution applications. Here, a complementary methyl-sensitive PCR validation for selected genome regions would also be useful.

⁸We like the QIAamp® DNA Micro genomic DNA isolation kit because there is a very low void volume for the columns, thus allowing for low volumes (15–20 µL) to elute samples compared to 100–200 µL for most other kits.

⁹Other sonicators besides a Covaris such as a Bioruptor® can be used to fragment gDNA.

- iii. An equal volume of 100.0 μ L of 1 $\times$ bind/wash buffer was added to the beads, the beads were resuspended by pipetting, and then the above step was repeated.
- (b) The Dynabeads[®] M-280 Streptavidin capture with the MBD-Biotin Protein.
 - i. Add 7.0 μ L of MBD-Biotin Protein to 100.0 μ L of 1 $\times$ bind/wash buffer.
 - ii. Transfer the diluted MBD-Biotin Protein to the tube of resuspended beads and mix on a rotating mixer at room temperature for 1 h.
 - iii. Wash the coupled MBD-beads as in steps (b) (ii)
- (c) Methylated DNA capture on MBD-beads.
 - i. Transfer the DNA/buffer mixture to the tube containing the MBD-beads and mix overnight at 4 °C.
- (d) Removal of non-captured DNA from the Beads.
 - i. Place the tube on the magnetic rack for 1 min then remove the supernatant.
 - ii. Wash the beads with 200.0 μ L of 1 $\times$ bind/wash buffer (*see* Note 10).
 - iii. Mix the beads on a rotating mixer for 3 min. (iv) Place the tube on the magnetic rack for 1 min then remove the supernatant (*see* Note 11).
- (e) Elution of the captured methylated DNA.
 - i. The enriched methylated DNA is eluted in 200.0 μ L of the high-salt elution buffer (2,000 mM NaCl) provided in the kit (*see* Note 12).
 - ii. Beads are incubated on a rotating mixer for 3 min.
 - iii. Place the tube on the magnetic rack for 1 min then remove the supernatant.
 - iv. **Steps 6** (e) (i–iii) are repeated and supernatant combined with first elution.
 - v. The enriched methylated DNA from the two high-salt elutions is ethanol precipitated.

7. Sequencing adaptor ligation.

¹⁰This step removes residual non-captured DNA.

¹¹This saved liquid is a non-captured DNA wash fraction.

¹²We chose to do one high-salt elution to capture all methylated DNA in one fraction. Another option is a multi-fraction elution with a stepwise NaCl gradient that separates distinct populations based on the number of methylation sites per molecule.

- (f) Resultant gDNA is ligated with P1 and P2 SOLiD adaptors and amplified.

10.0 µL	10× Quick Ligation Reaction Buffer
5.0 µL	Quick T4 DNA Ligase
10.0 µL	Adaptors P1/P2 (mix 10 µM each) (<i>see</i> Table 1)
75.0 µL	dsDNA
100.0 µL	Total volume

- (g) This mixture is incubated for 10 min at room temperature.
- (h) The ligation mixture is purified with 1.8 volumes of AMPure XP Reagent (38 µL) as described above and eluted with 35.0 µL of nuclease-free water.

8. PCR amplification is performed on the adaptor-ligated purified dsDNA with LA Taq™.

5.0 µL	10× LA PCR Buffer II (Mg ²⁺ plus)
8.0 µL	dNTP Mix (2.5 mM each)
2.0 µL	Primers mix A and B (10 µM each)
35.0 µL	Ligated dsDNA
0.5 µL	Takara LA Taq™ DNA Polymerase (5 U/µl)
50.0 µL	Total volume

9. The above mixture was amplified with the following conditions for eight cycles:

95°C for 30 s.

8 cycles:

95°C for 30 s.

58°C for 30 s.

72°C for 1 min.

10°C hold.

10. The amplified PCR product is visualized on a 2 % agarose gel to check for adequate concentration.
11. The amplified PCR product is purified with 1.8 volumes of AMPure XP Reagent (90.0 µL) as described above and eluted with 20.0 µL of TE.
12. The appropriate size fractionation is performed on an E-Gel® SizeSelect™ 2 % Agarose.
13. Samples were sequenced by SOLiD technology.

5 Data Analysis

As for *Aplysia* neurons, initial annotation of the presented data can be viewed using the *Aplysia* genome browser which outlines ongoing collaborative efforts in the analysis of this newly sequenced genome. The *Aplysia* genome consortium was formed in 2004 (<http://www.genome.gov/Pages/Research/Sequencing/SeqProposals/AplysiaSeq.pdf>), and the *Aplysia* genome sequencing was performed as a collaborative effort between the Broad Institute, Columbia University (E.R. Kandel and J. Ju's laboratories), and our lab at the University of Florida. The initial *Aplysia* draft (Sanger) genome is located on the UCSC browser <http://genome.ucsc.edu/>. We created a mirror of the UCSC browser with an updated assembly (<http://128.227.123.35:8889/cgi-bin/hgGateway>), and ongoing sequence data is added as tracks to the browser. For example, one can now view the methylation and expression status at the same time for a given gene of interest (Fig. 7). Globally, quantification of methylation reads can be handled in a manner similar to RNA-seq.

Even a single test with these reported protocols will generate a massive data set and require substantial bioinformatics efforts for efficient integration and interpretation of both RNA-seq and methylome results (which certainly should be a subject for separate publications under overall concepts and scope of the ongoing ENCODE projects <http://www.nature.com/encode/?gclid=CMmLjtWlq7MCFQvznAodKEEAiQ#/thread>). Although space restriction prevents a detailed presentation and computational data analysis, careful selection of cells and design of time-course series is required for integration of transcriptional data, chromatin states, and reconstruction of gene regulatory circuits (*see* Note 13).

6 Future Directions

To the best of our knowledge, nobody has yet been able to achieve integrated transcriptome/methylome profiling from any single cell or identified neuron. Nobody was able to follow up the entire dynamics of aging at the level of any functionally characterized neuron. Technically, it is very difficult using mammalian neurons [44, 46, 48], but now it is entirely possible using selected invertebrate models such *Aplysia* [24, 34, 49]. Although the presented protocols have been successfully validated, one of the major bottlenecks has been

¹³It is important to remember that separate examination of gene expression arrays from DNA methylation profiles can lead to erroneous and misleading conclusions. The interpretation of the data can also be biased depending on whether you approach this integration from the gene expression perspective or you do the methylation first and then search for its relevance to gene expression. The most typical questions are: Does a specific change in methylation have an impact on gene expression? Likewise can a change in gene expression be due to alterations in DNA methylation? To address these questions, a carefully selected series of experiments with multiple time points should be designed to follow the time course of dynamic genomic organization. Ideally, these should be complemented by capture of nascent RNAs at given time intervals. Similarly, tools of reverse genetics (e.g., RNAi or injections of relevant molecular constructs) should also be applied. Currently, none of these integrative studies have been performed at the level of single identified neurons. However, the implementation of presented and relevant single-cell protocols on relatively simpler models with functionally characterized neurons would be critical for progress in the field.

Finally, epigenetic modifications in neurons can be both stable and dynamic as a result of programmable changes during development, hormonal or transmitter stimulation, and activity-dependent processes that highly transient in nature [58]. To specifically identify an age-related event in senescent neurons, all of these factors have to be considered together with some environmental challenges or manipulations to show that the epigenetic landscape can change over time given gene-environment interactions. It is achievable using accessible *Aplysia* neurons but, because of enormous heterogeneity and lack of identified neurons, similar tests are extremely challenging using neural circuits in the mammalian brain. Combined, these remarks emphasize the critical importance of *Aplysia* and related species as novel and powerful models in the biology of aging and fundamental neuroscience. We would like to stress this vision and contrast it to growing concerns about the translational value of *Aplysia* that favors short-term applied research over long-term basic research about the integrative genomic biology of aging, circuit organization, and memory in particular.

the prohibitive cost of sequencing required for multiple time points and biological replications. However, advances in next generation sequencing make these types of experiments achievable even within the logistics of individual laboratories. Indeed, in addition to the more conventional *Illumina* approach with HiSeq2500, it is now possible to employ novel state-of-the-art sequencing platforms such as a cost-efficient semiconductor sequencer (*Ion Torrent Proton*) that can be fully adapted to single-neuron RNA-seq applications and DNA methylation profiling. This innovative approach allows one to perform multiple single-neuron RNA-seq experiments at the lowest possible cost today (< \$100 cell) with just under 2–3 days turnaround time, from cell sampling to sequencing and initial annotation. Ideally, experimental design can also target both transcriptome and methylome profiling from the same single neuron—which is currently technically possible using *Aplysia* preparations.

It also should be noted that emerging single-molecule real-time sequencing (*Pacific Biosciences*) will allow one to map distribution of 5mC and 5hmC at single-nucleotide resolution. Currently, the platform is relatively expensive for the entire genome-scale coverage. However, it can be a complementary approach to target DNA methylation of specific genes and their regulatory regions.

7 Conclusion

It is recognized that at least several thousand genes dynamically and persistently change their expression in virtually any neuron in memory-forming circuits to maintain long-term plasticity or compensatory changes in aging. However, the enormous heterogeneity of neurons and unprecedented complexity of their connectivity currently prevent reliable identification of individual neurons in mammalian circuits. Due to the very small sizes of mammalian neurons (10–40 μm in diameter), the equally considerable challenge is an ability to perform efficient single-cell transcriptome or epigenomic profiling. Although such an analysis is technically difficult, if not impossible, in vertebrate preparations, it can be successfully completed today using the simpler nervous systems of invertebrates such as *Aplysia*, where most of the neurons and synaptic connections in the circuits mediating major forms of learning have been identified. Just as important as the technical advantages offered by the neuroanatomy of *Aplysia* is the extensive background information available on the behaviors mediated by its well-studied neurons. Thus, we are confident that *Aplysia* offers a powerful experimental model to study cell and epigenomic biology of aging at a level that is difficult to achieve elsewhere.

However, the presented approach is not constrained by low input RNA abundance and can be applied to other cells and smaller cell populations such as 20–100 hippocampal neurons, axoplasm from *Loligo*, and embryonic cells, as proven in our experiments. Thus, since the protocol we presented is not limited in its applications to *Aplysia* neurons, we further advocate this model as an efficient preparation to couple a truly integrative analysis of genomic functions with real-time physiological measurements from the very same neurons as they learn and remember, age, or respond to induced injury.

Here, we also would like to comment on the constantly growing concerns about the so-called translational value of *Aplysia* research or research on related invertebrate species. This is also a common critical point from major funding agencies who tend to disfavor most invertebrate models (save *Drosophila* and *C. elegans*) vs. mammalian work. The major complaint to the comparative/ invertebrate models research community is that their translational value to vertebrates and in particular to humans might not be justified. This view is detrimental for basic research and overlooks the fact that separation between deuterostome (including vertebrates) and protostome (including molluscs) lineages occurred within a relatively short evolutionary interval, possibly less than 50–100 million years between each other in the later Ediacaran period (it is ended 542 million years ago), while fishes (vertebrates) and human lineages are divided by at least 450–500 million years of independent evolution started during (or after) the “Cambrian explosion” [50, 51]. As a result, the major molecular and genomic blocks underlying bilaterian organization and epigenetic regulation are remarkably similar and highly conservative despite the large evolutionary distances. In fact, even prebilaterian animals, such as the sea starlet anemone *Nematostella* or the placozoan *Trichoplax*, have greater chromosome synteny with humans than flies and nematodes with humans [52, 53]. Ironically, *Drosophila* and *C. elegans* lost a significant part of DNA methylation machinery due to the rapid evolution in these lineages and short life cycles, whereas *Aplysia* generally preserved this machinery from a common animal ancestor [1, 34, 43, 54]. Moreover, *Aplysia* shares more common genes with humans (including those involved in neurological disorders) than both flies and nematodes [34].

In summary, the current volume is dedicated to methods in biology of aging that are not restricted to vertebrates and humans. Thus, *Aplysia* has been specifically chosen to address questions in neurobiology of aging that currently are technically difficult, if not impossible, to address in humans. For example, it is impossible to work on identified neurons in humans, and there are no reliable protocols for isolation of both DNA and RNA from the very same neuron from intact vertebrate/human brains immediately after physiological tests. Yet, the same procedures are very straightforward in *Aplysia*. Considering that the basic DNA composition and the presence of 5-mC and 5-hmC in *Aplysia* are comparable to what we know in vertebrates [55], the same basic chemistry should work on mammalian models as well. In fact, previous and existing molecular information [19, 56] indicate significant conservation of both signaling and epigenetic mechanisms between *Aplysia* and vertebrates.

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Fig 1.

Aplysia is an emerging model for cell biology and epigenomics of aging. The life expectancy of *Aplysia* is approximately a year, and the animal can grow very rapidly from a small microscopic swimming larva, less than 0.5 mm in diameter to up to a 1–3 kg mature adult comparable in its size with a rabbit. Yet, the *Aplysia* central nervous system consists of only 10,000 neurons of different coloration. Some of these neurons are the largest in the animal kingdom [57] and are visible to the naked eye. As a result of their surface location and unique morphological and functional properties, these neurons can be reliably identified and mechanically isolated for microchemical and genomic analysis. The isolated cell on a penny is one of the large cholinergic motoneurons (modified from [24, 59, 60])

Fig 2.

Different neurons age differently. Comparison of age-related changes in gene expression between two identified cholinergic neurons LPI1 and R2 (modified from [1]). (a) The schematic representation of the reference design microarray experiments to compare two different cell types R2 and LPI1 during the aging process in *Aplysia*. In these microarray tests, individual neurons were compared to the same reference CNS sample. The individual circles represent single neurons (LPI1—blue tones; R2—orange tones) from young or old animals. (b) Photograph of the freshly dissected right abdominal semi-ganglion with the position of R2 and R14 neurons marked (connective tissues from the ganglionic surface were removed and the natural coloration of cell somata were preserved). This R2 cell is the largest neuron ever photographed reaching 1.1 mm in diameter. When this cell was isolated (insert) and fixed in 100 % ethanol it lost its pigmentation. 1.9 µg of total RNA was obtained from this neuron. (c) We found that only 58 neuronal transcripts (~0.1 %) are differentially expressed when LPI1 and R2 neurons are compared from young animals. In contrast, when the same cells were directly compared from old animals, we identified 2,508 differentially expressed transcripts (~4.5 %). This suggests that identified cholinergic motoneurons are more similar to each other in younger animals than the same neuronal types in older animals. (d) The landscape diagram is modified from Waddington, C. H., 1956 (Principles of Embryology, op. cit., p. 412; [61]). Following Waddington's visual schematics, the ball represents a neuronal fate. The valleys are the different fates a given neuron might roll into. At the beginning of its journey, development is plastic, and a cell can have many fates. However, as development and aging or memory proceeds, certain molecular events occur randomly and this can lead to different underlying molecular phenotypes and decisions that cannot be reversed

Fig 3.

Isolation of single identified neurons and their RNA/DNA content. (a) Panel of a series of photographs showing the isolation of the L7 neuron with a glass suction pipette (modified from [41]). Below is a schematic depiction of a single neuron and its neurites; the diagram shows the amounts of RNA isolated from neuronal somata and axodendritic processes. (b) The amount of RNA isolated from a single *Aplysia* neuron is directly proportional to the volume of that cell. The gigantic R2 neuron yielded 1.9 μg of total RNA and 250 ng of gDNA

Fig 4.

Single-cell RNA-seq and its validation. **(a)** The diagram presents the workflow of the RNA-seq protocol outlined in the text. Single *Aplysia* neurons were isolated, RNA extracted, and 454 sequencing libraries constructed. **(b)** Absolute RT-PCR was used to generate the intracellular copy numbers for four transcripts of interest. This copy number showed a linear correlation to transcript abundance (expression) in the sequence data set. **(c)** In situ hybridization [42] was performed on one of the neuron-specific and quite abundant transcripts (the neuropeptide FMRFamide) in the R2 neuron. The photo had been captured in 100 % ethanol. Note, the white nuclei (blue asterisks) can be seen in many neurons. **(d)** Quantitative RT-PCR of transcripts of interest (FMRFamide) displayed a correlation between the digital profile in the different single cells and their corresponding quantitative RT-PCR expression. Expression profiles for both the frequency of sequencing reads and the QRT-PCR displayed similar patterns. However, caution should be taken for interpretation of the RNA data, since abundant transcripts known to be selectively transported to synaptic terminals and located on neuronal somata can also be captured by RNA-seq (e.g., in contrast to R2, L7 does not express FMRFamide). Thus, to test cell specificity of expression, a complementary in situ hybridization should be performed **(c)**

Fig 5.

Neuron-specific expression of sense and antisense transcripts in identified neurons. The library construction protocol reported here preserves directionality and allows quantification of sense (*red*) and antisense (*blue*) as revealed by RNA-seq for two identified neurons: the gill motoneuron L7 and feeding interneuron MCC. Differential expression is demonstrated by the abundance of reads for L7-specific secretory peptide (confirmed by in situ hybridization for the same transcript in L7, see *insert*) compared to the interneuron MCC which does not express this gene. Remarkable transcriptional complexity is evident even from a small region of the genome shown here

Fig 6.

Single-cell Enriched Methylated Genomic DNA Library constructed using the MethylMiner™ approach. The diagram presents the workflow of the reported MethylMiner™ enriched sequencing library protocol. Genomic DNA is first isolated from single neurons, then fragmented to 150 bp. Methylated DNA is enriched from fragmented genomic DNA via binding to the methyl-CpG binding domain of human MBD2 protein, which is coupled to paramagnetic Dynabeads® M-280 Streptavidin via a biotin linker from the MethylMiner™ kit. The enriched gDNA is eluted with one high-salt elution step. Resultant gDNA is ligated with the appropriate P1 and P2 SOLiD adaptors and amplified. At this point this product can be directly sequenced for regional mapping, bisulfite sequenced for single-nucleotide resolution or used for absolute RT-PCR for copy number determination of genes of interest

Fig 7.

Data analysis of both expression and methylation profiling. RNA-seq and methylation sequence data are loaded as tracks on the UCSC browser <http://genome.ucsc.edu/>. For a specific gene, the correlation between expression and methylation can be viewed

Fig 8.

Methylation in polyploidal neurons occurs in all DNA copies. In this illustrated example, after bisulfite sequencing, bands from the amplified PCR reactions were sequenced for DNMT1 gene. The starred cytosines in the sample chromatogram show no background from other nucleotides, thus indicating all the genome copies in *Aplysia* polyploidal neurons are equally methylated. If some of the cytosines were not methylated, there would be multiple peaks in the chromatogram indicating a mixed population. However, this is not the case here because there are no background peaks in any of the cytosines marked

Table 1

Adaptors and primers for 454 and SOLiD libraries

Primer name	Primer sequence
Trsa	5'-CGCAGTCGGTAC (T) ₁₃ -3'
454 libraries	
A adaptor	5'-GCCTCCCTCGCGCCATCAG-3'and 5'-CCTGATGGCGCGAGGG-3'
B adaptor	5'-GCCTTGCCAGCCCCGCTCAG-3'and 5'-CTGAGCGGGCTGGCA-3'
A PCR	5'-GCCTCCCTCGCGCCATCAG-3'
B PCR	5'-GCCTTGCCAGCCCCGCTCAG-3'
SOLiD libraries	
P1 adaptor	5'-CCACTACGCCCTCCGCTTTCCTCTCTATGGCAGTCGGTGAT-3' and 5'-ATCACCGACTGCCCATAGAGGAAAGCGAGGCGTAGTGGTT-3'
P2 adaptor	5'-AGAGAAATGAGGAACCCGGGGCAGTT-3'and 5'-CTGCCCGGGTTCCTCATTCTCT-3'
P1 PCR	5'-CCACTACGCCCTCCGCTTTCCTCTCTATG-3'
P2 PCR	5'-CTGCCCGGGTTCCTCATTCT-3'

Table 2External controls: *Spike-in* controls starting copy numbers and resulting read frequencies

Spike number	Starting copy number	Reads	Frequency (468,723 total)
Spike_1	2.50E ⁺⁰⁸	5,268	1.13E ⁻⁰²
Spike_2	2.50E ⁺⁰⁷	3,830	8.12E ⁻⁰³
Spike_3	1.87E ⁺⁰⁶	218	4.65E ⁻⁰⁴
Spike_4	1.67E ⁺⁰⁶	109	2.33E ⁻⁰⁴
Spike_5	1.83E ⁺⁰⁴	15	3.20E ⁻⁰⁵
Spike_6	1.27E ⁺⁰²	1	2.13E ⁻⁰⁶

Table 3

Primer and probe sequences for absolute and quantitative real-time PCR

Transcript	Accession number	Forward primer	Reverse primer	Probe
Custom TaqMan				
<i>FMR1</i> amide 0045477209 Custom TaqMan	P08020	5'-GATGACGATGTGCAGGATCTGA-3'	5'-CCCAAAACCTCATGAACCGTTTATTAC-3'	5'-FAM-ACCATCACCACCAATATATCC-3'
<i>b-Tubulin</i> 0045477646 Custom TaqMan	AAP13560	5'-TGGATGTCGTCAGGAAAGAGTCT-3'	5'-CCACCCAAGGAGTGTGTCAATT-3'	5'-FAM-CCTGCAGACAAATCACA-3'
<i>MIP</i> 185429001 Custom TaqMan	AF454399.1	5'-GCTATGGCTCCGAAAGTTTTTCG-3'	5'-CTTGTGTCCAGAGCCAATTGTTC-3'	5'-FAM-TCCCACGAGTAATTCT-3'
<i>Shab</i> 0016112793 Custom TaqMan	S68356	5'-GGGCTCGCTCATCAGCAT-3'	5'-CCCTTGGTGGTGTGATGGT-3'	5'-FAM-CCTGTGTCGATCATCCCC-3'