Chapter 2

Analysis of Brain Epigenome: A Guide to Epigenetic Methods

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Abstract

The brain is the most complex tissue in the body. The development and diversity of different brain regions and cell types, as well as neuronal plasticity is controlled by the epigenetic mechanisms. This chapter describes the key epigenetic events at gene promoters, gene bodies, and 3′-untranslated regions that are critical for control of gene expression especially in the brain. Sections 2–4 of the chapter overview different methods for the analysis of DNA methylation (Sect. 2), histone modifications (Sect. 3) and noncoding RNAs (Sect. 4). Each section briefly introduces the main steps and output, advantages, considerations, and limitations of the methods, as well as some alternative approaches further developed from the original method.

Key words Neuron, Nucleosome, DNA methylation, 5-methylcytosine, 5-hydroxymethylcytosine, Histone modification, Exon and intron, lncRNA, microRNA, Bisulfite analysis, ChIP, MeDIP, In situ hybridization

1 Introduction

Epigenetic processes are the main driving mechanism for development of all types of tissues in the body from a single zygote cell, and for genomic response to environmental stimuli throughout life. Originally, epigenetics was defined as a field of science that studies inherited changes in gene expression without changes in DNA sequence. Nowadays, epigenetics focused on epigenomic modifications that are not only transmitted from mother to daughter cell but also alter gene expression in non-dividing cells such as neurons. The epigenetic changes define the genomic plasticity that largely contributes to neural plasticity.

The epigenome within a given cell at a given moment depends on genotype, genetic elements (transposons, noncoding RNAs, or ncRNAs) and chromatin modifications (DNA methylation and histone modifications). The coordinated action of these players is controlled by the DNA- and RNA-binding proteins and the

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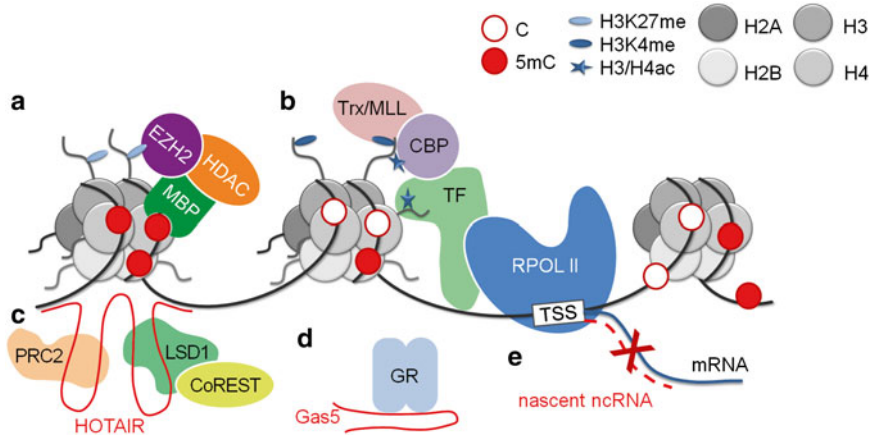

Fig. 1 The main mechanisms of the epigenetic control of gene transcription. (**a**) High DNA methylation level at the gene promoter correlates with repressive chromatin state: recruitment of methylated DNA-binding proteins and the Polycomb chromatin repression complex (PRC) including histone methyltransferases (HMT, e.g., H3K27-specific HMT EZH2) and histone deacetylases (HDAC), resulting in silenced gene transcription. (**b**) Decreased DNA methylation level correlates with increased gene transcription through recruitment of activating Tritorax (Trx) complex including H3K4-specific HMT MLL, histone acetyltransferases (HAT, e.g., CREB-binding protein (CBP)), transcription factors (TF) and RNA polymerase II (RPOL II). (**c**) The scaffold lncRNA, such as HOTAIR, nucleates at specific genomic sites, to which it guides the repressive PRC2 complex and H3K4-specific histone demethylase LSD1/REST repressor CoREST complex which is required for the repression of neuron-specific genes. This mechanism is closely associated with (**a**). (**d**) The decoy ncRNA growth arrest-specific 5 (Gas5) competes with endogenous glucocorticoid response element (GRE) for binding to the glucocorticoid receptor (GR). As a result, GR-mediated activation of GRE-containing genes is repressed. (**e**) RNA interference: Different types of nascent or aberrant ncRNAs act in *cis* to abort the transcription of specific genes in a homology-dependent manner. For example, when transcribed from one DNA strand, ncRNAs may repress the transcription from the other strand due to simultaneous occupancy of both strands, or by antisense-mediated chromatin alteration with Polycomb complex. C, unmethylated cytosine; 5mC, methylated cytosine; H2A, H2B, H3 and H4, histones; TSS, transcription start site

chromatin remodeling complexes (Fig. 1). Knowing the sequence of a gene is often not sufficient to understand its physiological function. The chromatin structure around the gene can strongly affect the gene expression levels in different cell types during different developmental stages. This, for instance, may partially explain somewhat frequent failure of gene knockouts "models" of some disorders if the knocked-out gene has been chosen on the basis of its reduced expression only at the specific time point in that disorder. The knowledge of disease epigenetics could also ameliorate at least partially the effectiveness of translational approach in research seeing especially frequent failure of the drug clinical trials that were based on preclinical findings. For example, one of the important factors to consider when administering drugs is the possible circadian variation of gene expression levels in different organisms (see Iuchi et al. Chap. 15), which is controlled by the epigenetic machinery (see below).

The structural features and function of DNA methylation, histone modifications and ncRNAs, as well as the analytical methods to study them, are described in Sects. 2–4 of this chapter and, more in detail, in several chapters of this volume. The current Sect. 1 highlights the most critical epigenetic processes in the developing and mature nervous system in normal and disease states (*see* Sect. 1.1) and the questions to address when starting the epigenetic analysis in your lab (*see* Sect. 1.2).

1.1 Epigenetics in Health and Disease

The most drastic changes in epigenome take place during development—the process of generating a functional multi-tissue organism from a single zygote. In mammalian development after oocyte fertilization, the transcription-repressive DNA methylation marker 5-methylcytosine (5mC) is globally erased from maternal and paternal DNA molecules [1, 2]. This gives rise to the expression of pluripotency-associated genes until the blastocyst stage, while cell- and tissue-specific genes are silenced by histone modifications, particularly by repressive histone marker H3K27 methylation. During the differentiation, DNA methylation marks are established de novo; pluripotency-associated genes are silenced by increased DNA methylation, which has a life-long protective effect against cancer for example. At this time, the repressive H3K27 methylation mark is being increasingly replaced by the activating H3K4 methylation mark, and cell- and tissue-specific genes begin to be expressed. Cell type-specific epigenetic markers are established at gene regions and can either be transmitted to the daughter cell, or remain stable throughout cell life (e.g., neuron), or be flexible in response to environmental signals. The imprinted genes and repetitive elements escape from the global DNA demethylation after fertilization, and show the similar cycle (DNA demethylation—expression of pluripotency-associated genes—de novo DNA methylation—expression of cell type-specific genes) during the formation of embryonic germ cells, which corresponds to the mouse embryonic age E8-E13 [1]. Any interference with the correct epigenetic programming during in utero development might influence phenotype or lead to neurodevelopmental and other disorders in the growing generation, while incorrect reprogramming in embryonic germ cells may affect even the next generation.

What are the mechanisms of epigenetic regulation of gene expression?

1.1.1 Promoter/5′ UTR Region

The 5mC mark is recognized by methyl-DNA binding proteins (MBPs) that mediate the effect of DNA methylation on gene transcription. MBPs recruit the Polycomb repressive complex (PRC) containing histone deacetylases (HDACs) that remove activating mark histone acetylation resulting in more tight binding of DNA to nucleosome histone core; in this state, the DNA is less accessible to transcription factors [3] (Fig. 1). The mutation in methyl-DNA

binding domain of the MBP MeCP2 is associated with severe neurological disorder Rett syndrome [4]; treatment with HDAC inhibitors increases the expression of many genes including brain-derived neurotrophic factor (BDNF) [5]. In addition to HDACs, PRC member EZH2, a H3K27 histone methyltransferase (HMT), introduces a repressive mark H3K27me3 that inhibits the deposition of chromatin activating marks [6] (Fig. 1a). The guiding of repressive complexes, which lacks DNA-binding domain but has RNA-binding motifs, to specific genomic loci can be facilitated by locus-specific long noncoding RNAs (lncRNAs). LncRNAs are especially enriched in the brain and play a critical role in control of gene expression, including scavenging transcriptional factors by mimicking their DNA responsive elements, such as a pair lncRNA Gas5/glucocorticoid receptor GR (Fig. 1d) [7]. A lncRNA HOTAIR recognizes the HOXD locus and binds PRC2 complex, which promotes H3K27 methylation, and lysine demethylase LSD1 complex, which removes activating histone mark H3K4me [8]. LSD1-mediated H3K4 demethylation is essentially supported by the neuron-restrictive silencer complex REST/CoREST [9, 10] that regulates neuronal/glia lineage maturation and neural plasticity [11] (Fig. 1c).

The active promoter state is associated with decreased 5mC levels and increased binding of the Tritorax group member MLL, an H3K4-specific HMT, and histone acetyltransferases (HATs), such as CREB-binding protein CBP [6] (Fig. 1b). The mutations in MLL gene are associated with tumorigenesis [6] and in CBP gene—with a wide spectrum disorder Rubinstein-Taybi syndrome and neurodegenerative Huntington's disease [12]. One of the most known HATs is the circadian master-pacemaker CLOCK that heterodimerizes with BMAL1 (or ARNTL) and activates transcription from the E-box containing promoters, whereas a HDAC protein SIRT1 cointeracts with the HAT-activity of CLOCK:BMAL1 complex in a circadian manner [13]. Up to 10 % of genes, including critical for brain function BDNF [14] and GR [13], are expressed in a circadian manner.

The insertion of transposable elements that comprise about 20 % of mammalian genomes could significantly affect gene transcription (see Griffiths and Hunter Chap. 11). In humans, increased retrotransposition of LINE-1 element could contribute to pathophysiology of brain diseases, e.g., schizophrenia [15]. LINE-1 inserted upstream of a gene can become methylated and induce formation of heterochromatin that can spread into nearby promoter regions and silence transcription. On the other hand, an active LINE-1 might facilitate binding of transcription factors and ectopically activate a nearby gene [16]. The neuronal retrotransposition of LINE-1 is at least partially controlled by MeCP2, which implicates the de-regulation of transposable elements in the etiology of neurodevelopmental disorder Rett syndrome [17].

1.1.2 Gene Body Region

Although the epigenetic marks in gene bodies are much less studied that in promoters, DNA methylation and ncRNAs were implicated in maturation of pre-mRNAs, or splicing. The brain is the organ the most enriched in alternatively spliced transcripts coding for different isoforms of proteins. A number of excellent reviews about the alternative splicing-mediated pathogenesis of neurodegenerative and psychiatric diseases are available [18, 19]. The increased DNA methylation at the exons compared to introns in some genes, or the decreased methylation at the exon–intron boundaries in other genes help the spliceosome to recognize between the exon and intron already during transcription [20], suggesting that the altered methylation pattern could modulate alternative splicing (Fig. 2). The spliceosome itself is the complex of proteins and non-coding small nuclear RNAs (snRNP complex). The key protein component involved in assembly of splicing snRNPs is a survival motor neuron (SMN) protein, deficiency of which causes the devastating neurological disorder spinal muscular atrophy (SMA) [21, 22]. The spliceosome could as well recognize the functional splice sites

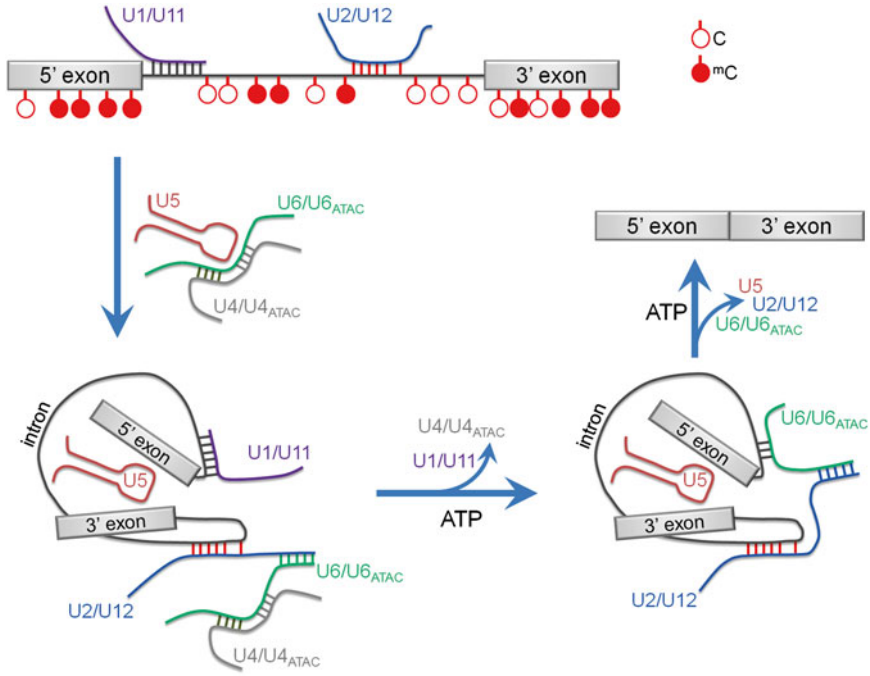

Fig. 2 The mechanisms of RNA splicing. The decreased DNA CpG methylation in introns or at the exon–intron boundaries aims at facilitating splicing of specific introns already during transcription. After transcription, the two types of introns, U2 and U12 types, are recognized and spliced by the major and minor spliceosome complexes, respectively, in an ATP-dependent manner. The majority of introns are of U2-type: flanked by GU/AG dinucleotides (GT/AG in DNA sequence) and spliced by the snRNP complex with U1, U2, U4, U5, and U6 snRNAs. The minority of introns, including those in genes affected in SMA disease, are of U12-type: flanked by AU/AC dinucleotides (AT/AC in DNA sequence) and spliced by the snRNP complex with U11, U12, $U4_{ATAC}$, U5, and $U6_{ATAC}$ snRNAs

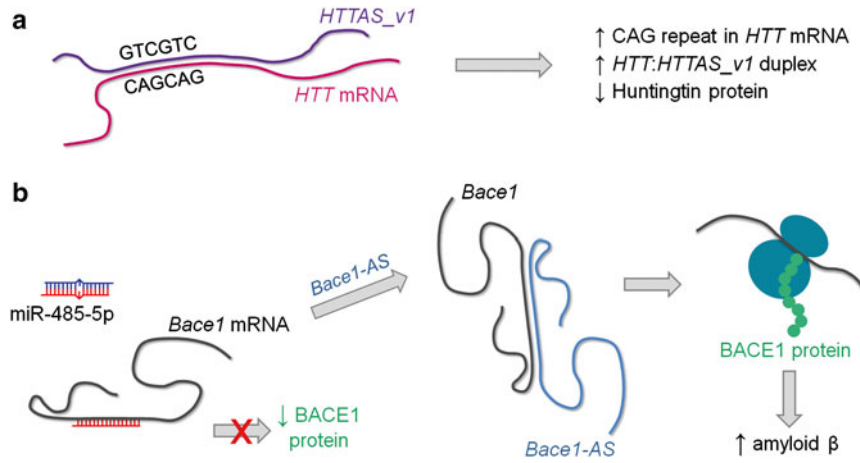

Fig. 3 Some basic mechanisms of posttranscriptional control of mRNA levels by antisense lncRNA. (**a**) The lncRNA *HTTAS_v1* binds *huntingtin* mRNA with more affinity for transcripts with extended CAG repeats, and leads to degradation of the duplex RNA and depletion of HTT protein. (**b**) The *BACE1* mRNA could be targeted by microRNA *miR-485-5p* and subsequently degraded, whereas the lncRNA *BACE1-AS* competes with microRNA for binding and stabilizes *BACE1* transcript, leading to the increased expression of BACE1 protein

within mammalian LINE-1 element; thus, when LINE-1 is inserted into introns, it can induce aberrant splicing resulting in alternative mRNA transcripts [16].

Recently, another important mechanism of regulation of gene translation has been discovered: the binding of protein-coding mRNA by antisense lncRNA (AS-lncRNA). Depending on the target transcript, both overexpression and reduction in AS-lncRNA can promote the development of severe neurological disorders (reviewed in [23]). For example, upregulated lncRNA *HTTAS_v1* binds *Huntingtin* transcript, depletes the expression of HTT protein, and potentially involved in the pathogenesis of Huntington's disease (Fig. 3a) [24]; decreased lncRNA *SCAANT1* leads to increased expression of ataxin 7 and the development of spinocerebellar ataxia type 7 [25]; upregulated lncRNA *BACE1-AS* binds the *β-secretase-1* transcript and stabilizes it, leading to increased production of BACE1 protein and amyloid-β, thus potentially contributing to the development of Alzheimer's disease (Fig. 3b) [26].

1.1.3 3′ UTR

To date, the most investigated mechanism of 3′-UTR-regulated gene expression is controlled by miRNAs. MiRNAs are small RNAs, which mediate epigenetic silencing of multiple target RNAs by RNA interference. MiRNAs are especially enriched in the brain. Majority of miRNAs families increase in their expression during postnatal development and repress their plasticity-related target genes in adulthood, suggesting the key molecular mechanisms for impaired neuronal plasticity [27]. The miRNAs are described more in detail in Sect. 4.

1.2 Getting Started: Things to Consider

1. Scope of the research project: global level, genome-wide or locus-specific epigenetic changes (see "*Output*" for each method in Sects. 2–4). The main applications of the methods that are very useful but currently very rarely applied in neuroscience research are indicated in Sects. 2–4.
2. Expected perspective studies. For example, if the study is focused on analyzing the efficacy of different treatment strategies, such as systemic or local administration of epigenetic drugs (see Sales et al. Chap. 10), a preliminary brain region-specific analysis of epigenetic markers should be performed. Moreover, although a rapid effect of drug administration on gene expression or behavioral phenotype is possible, it is unlikely to be mediated by true epigenetic mechanisms which require a time to have an effect. The experiments with a drug incubation time for at least few hours are recommended.
3. Setting time for behavioral and/or physiological analysis and tissue collection. Because epigenetic changes are correlated with circadian clock at least for some genes critical for brain function [13, 14, 28], planning the analyses, drug administration [29] or brain dissection is recommended at approximately the same time of the day. If the project includes a lot of animals, the analyses/dissection should be done during up to 4 h/day in several days (see for example [30]) and the animals from different groups must be randomized. When analyzing postmortem human samples, it might be useful to record the information about the time of death if available, and then examine the significance of the time factor in the statistical analysis of obtained data.

In addition, the choice of the epigenetic marker to analyze (e.g., DNA methylation, miRNA, or both) also depends on:

4. Price. High-throughput or mass-spectrometry analyses are very costly at the stage of purchasing the equipment.
5. Amount of tissue material. At least few milligrams of tissue are required for some types of analyses, e.g., immunoprecipitation approaches, such as MeDIP (*see* Sect. 2.3.1) or ChIP (*see* Sect. 3). For the parallel correlative analyses of DNA, RNA, or protein levels more material is needed. At least few sections per animal brain or human postmortem tissue block are required for immunohistochemical (*see* Sect. 2.3.2) or in situ analyses (*see* Sect. 4.2).
6. Fresh/frozen/fixed tissue material and its storage conditions. The storage of the tissue sample in the RNA protective solution RNAlater leads to inefficient bisulfite conversion (*see* Sect. 2.1). The formalin fixation is not recommended for MSRE analysis (*see* Sect. 2.2) and damages the nucleic acids that results in reduced amount of good quality DNA or chromatin,

although not significantly interfere with the analyses themselves. Some optimizations for the immunoprecipitation-related approaches, such as ChIP, are recommended when working with small FFPE specimen or laser-microdissected material from postmortem human samples [31, 32].

2 Methods for DNA Methylation Analysis

DNA methylation in mammals mostly occurs in 5′-C-phosphate-G-3′ (CpG) dinucleotides by the transfer of a methyl group from the donor *S*-adenosylmethionine (SAM) to the C5 position of the cytosine (5mC). The process is catalyzed by DNA methyltransferases (DNMTs). Increased DNA methylation at the promoter/first exon regions is often associated with the transcriptional repression of the gene [33]. Normally, both DNA strands are symmetrically methylated at the CpG site. Following DNA replication, a new symmetric CpG is synthesized on the opposite DNA strand and is transiently unmethylated, generating a hemimethylated CpG site. This hemimethylated CpG is recognized by the *maintaining* DNA methyltransferase DNMT1 that introduces the symmetric methylation mark into newly synthesized DNA strand, thus transmitting the methylation pattern into daughter cell [34]. In contrast, the de novo DNA methylation does not need a hemimethylated CpG and is processed by the methyltransferases DNMT3a and DNMT3b independent to DNA replication. The methylated CpG is recognized by the methylated DNA-binding proteins (MBPs) that link DNA methylation with the transcriptional activity of the gene via recruitment of the chromatin remodeling complex [34]. Recent studies on active DNA demethylation process identified several deaminated and oxidized derivatives of 5mC, among which a 5-hydroxymethylcytosine (5hmC) is currently under the most intensive investigation [35].

This section outlines the main approaches used in DNA methylation analysis (Fig. 4).

2.1 Bisulfite Analysis

Methylated DNA bisulfite analysis is based on a chemical reaction of sodium bisulfite with DNA that converts unmethylated cytosine to uracil (Fig. 5a), while methylated cytosine remains not converted (Fig. 5b) [36, 37]. Next, the bisulfite-treated DNA is amplified by polymerase chain reaction (PCR): during the first cycle, in the synthesized antisense strand adenine is incorporated complementary to uracil which is then amplified as thymine in the sense strand (Fig. 5c). The methylated cytosine is amplified as cytosine (for simplicity, the nucleoside names are used instead of nucleotides which are actually incorporated by PCR). Thus, the main output of bisulfite analysis is a C:T ratio at each CpG site unless otherwise stated (*see* Sects. 2.1.1–2.1.7). In published papers, the

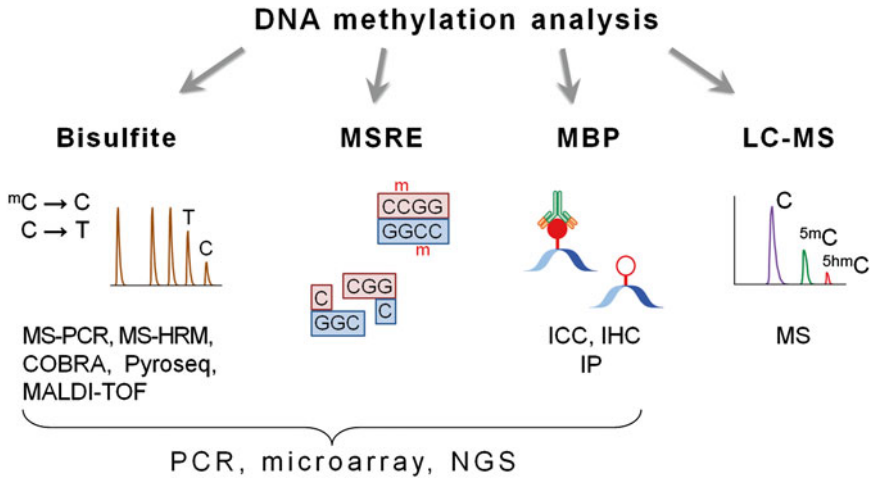

Fig. 4 The types of DNA methylation analysis. PCR, microarray and next generation sequencing (NGS) are the common procedures for bisulfite, MSRE and MBP (particularly, immunoprecipitation, or IP, with MBP) analyses. *MSRE* methylation-sensitive restriction enzyme, *MBP* methylated DNA binding protein, *LC-MS* liquid chromatography-mass spectrometry, *MS-PCR* methylation-specific PCR, *MS-HRM* methylation-sensitive high-resolution melting, *COBRA* combined bisulfite restriction analysis, *MALDI-TOF* matrix-assisted laser desorption ionization time-of-flight, *ICC* immunocytochemistry, *IHC* immunohistochemistry, *MS* mass spectrometry

results of the analysis are often presented as the percentage of methylation: $\%^{m}C = [C/(C+T)] \times 100$.

Main advantages:

1. Wide range of DNA methylation changes can be analyzed: from single CpG-site modification to global methylation content.
2. Requires only a small amount of DNA, which make bisulfite analysis compatible with the following complications in neuroscience research:
   (a) Small human biopsies available after brain surgery; small tissue punches from animal brain sections.
   (b) Laser capture microdissection (LCM) for DNA methylation analysis in isolated specific cells or cell layers [38, 39]. This analysis is critical for a spatial resolution of methylation patterns when studying a complex tissue, such as the brain.
   (c) Formalin-fixed paraffin-embedded (FFPE) postmortem human tissues in which the DNA may be degraded [40]. Can be combined with fluorescent immunostaining method and subsequent LCM for the analysis of specific cell type.
   (d) Neuron-specific methylation pattern can be evaluated using DNA purified from neuronal and non-neuronal cell fractions obtained by the Fluorescence-Assisted Cell Sorting (FACS) [41] (see Bundo et al. Chap. 7).

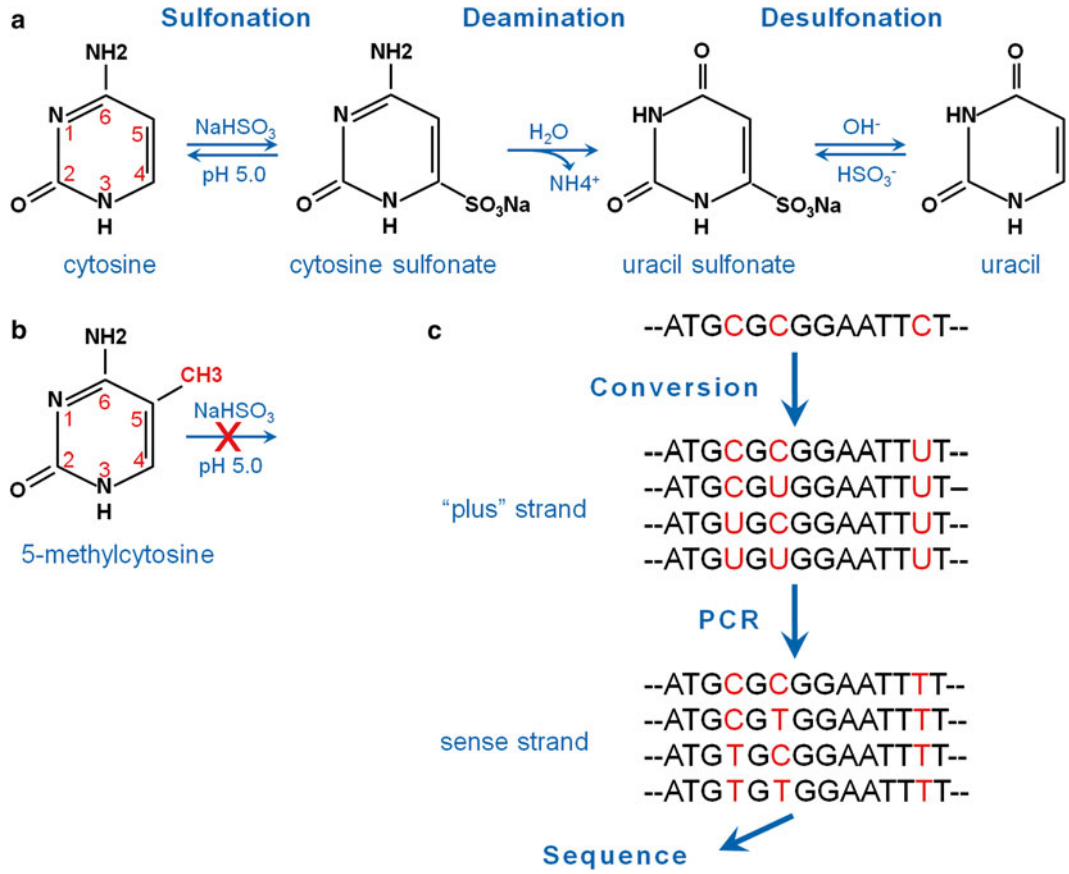

Fig. 5 The principle of the bisulfite analysis of DNA methylation. (**a**) A chemical reaction converts unmethylated cytosine to uracil. The DNA is denatured, then treated with freshly made solution that contains highly concentrated sodium bisulfite and added antioxidant, pH adjusted to 5.0, at elevated temperature for extended time. Next, intermediate sulfonated DNA must be desulfonated by high-pH NaOH for complete conversion. (**b**) Methylated cytosine is resistant to bisulfite conversion in the described reaction conditions. The positions 1–6 of a cytosine ring are indicated. (**c**) Following conversion, the reaction mix consists of the pool of different ssDNA molecules; here only possible converted "+"strands are shown. During PCR, uracils are replaced by thymines, thus generating a mix of different dsDNA fragments (here only sense strands are shown) which are then analyzed by sequencing. An example of a mouse/rat *Bdnf* promoter 4 with the CpG sites -111 and -109 is used for presentation

General considerations/limitations:

1. Complete conversion of DNA (Fig. 5a) is essential to avoid false-positive results. It has been reported that the storage of tissue samples in RNA-preserving reagent, such as an RNAlater, for the parallel analyses of DNA and RNA, significantly reduces the efficiency of bisulfite conversion either by self-made reagents or by commercially available kits (Dr. Pavel Uvarov, University of Helsinki, personal communication; RNAlater did not affect the MeDIP performance) [14]. Bisulfite sequencing analysis may provide an "internal reference standard" for

completeness of bisulfite conversion by analyzing the presence of cytosines in non-CpG context.

2. Bisulfite conversion does not discriminate between 5mC and 5hmC.
3. The DNA after bisulfite-conversion has long mononucleotide stretches that may cause problems during PCR or sequencing reactions (nonspecific primer annealing, or poor amplification due to polymerase slippage), the conditions of which should be optimized. Alternatively, the commercially available kits designed for PCR or sequence of bisulfite-converted DNA should be used.
4. The high-fidelity polymerases may not amplify U-containing template in the first rounds of PCR.
5. The "+" and "−" DNA strands after bisulfite conversion are no more complementary to each other, which has two main consequences: (a) converted DNA is less stable than non-converted DNA and should be stored at −20 °C; (b) for a PCR step, both forward and reverse primers should be designed for only one strand, either "+" or "−".
6. Bisulfite-converted DNA has reduced complexity (mainly composed of three bases) thus challenging the design of unique gene-specific primers. Primer design software, such as a Methyl Primer Express® free online primer design tool, may be used (see also *Umemori and Karpova* Chap. 4).
7. For approaches involving PCR: Highly methylated DNA regions of several hundred base length with increased CG-content may be less efficiently amplified than unmethylated resulting in wrong estimation of C:T ratio. Optimized analysis should be used, for example, when studying methylation in the sequences with expanded CG-containing repeat [42], such as CGG in the FMR1 gene and CCG in the FMR2 gene (these genes are linked to different types of fragile X syndrome-related diseases, a group of triplet repeat expansion disorders) [43, 44].

The bisulfite-related analytical approaches, most used currently or important for development of modern technologies, are listed below in the following order: analysis of a single CpG; analysis of multiple linked CpGs at the locus; analysis of multiple CpGs at the locus with a single-nucleotide resolution; and analysis of global methylation content.

2.1.1 Methylation-Sensitive Single Nucleotide Primer Extension (MS-SNuPE)

1. Denature and bisulfite-convert DNA.
2. Design PCR and SNuPE primers for converted DNA: (a) PCR primers specific for CpG-free areas flanking each region of interest; (b) one SNuPE primer/each CpG designed to anneal immediately upstream of the analyzed CpG site.

3. Perform PCR to amplify a unique DNA fragment and separate a PCR product on two fractions, each for extension reaction with an appropriate radiolabeled nucleotide: (a) complementary to methylated template, and (b) complementary to unmethylated template.
4. Following extension step, determine C:T ratio by comparing the radioactive signals from two fractions.

Output: single CpG site-specific.

Advantages: quantitative detection of minor changes in methylation levels, as well as analysis of low-methylated targets.

Considerations:

1. Simple for detection of methylation changes at one particular CpG site, but very labor-intensive if many sites should be analyzed.
2. Requires individual primer for each CpG, optionally the primer should be from CpG-free region and can be designed either for "+" or "−" DNA strand [45, 46]. The method is not recommended for CpG-dense regions.
3. Fluorescence-labeled nucleotides could be used instead of radioactive but the cost of the procedure will increase.

Further development: (a) By attaching a unique sequence tag to each primer, the multiplex extension reactions can be performed and analyzed using a microarray approach [47]. (b) A single base-extension principle is used by pyrosequencing and next-generation sequencing technologies (*see* Sect. 2.1.5).

2.1.2 Combined Bisulfite Restriction Analysis (COBRA)

1. Denature and bisulfite-convert DNA.
2. Design PCR primers for converted DNA specific for CpG-free areas flanking the region with an appropriate restriction site (containing CG in the recognition sequence, e.g., TaqI, BstUI, and HpyCH4IV). Following bisulfite conversion and PCR, unmethylated C is converted to T, and the restriction site is lost.
3. Perform PCR to amplify a unique DNA product and digest it by an appropriate enzyme.
4. Determine a C:T ratio at the CpG site by analyzing the amount of fragments, non-digested vs. digested with the restriction enzyme [48] (see Hayakawa et al. Chap. 3).

Output: sensitive and quantitative analysis of single or few CpG sites (depends on the number of restriction sites present in the amplified DNA fragment).

Advantages: simplicity and relative rapidity; low-cost.

Considerations:

1. Limited to the CG-containing sequences recognized by restriction enzymes.
2. Requires complete digestion by an appropriate enzyme.

Further development: (a) A simultaneous digestion with multiple enzymes increases the number of sites analyzed if the fragments generated by digestion differ in size. (b) The quantitative analysis of COBRA digestion patterns in large sample sets is performed by electrophoresis in microfluidics chips (Bio-COBRA) [49, 50].

2.1.3 Methylation-Specific PCR (MS-PCR)

Main applications: The MS-PCR is a very good option for the analysis of genomic regions with relatively high methylation level, or for the diagnostics of disease states where the pathological and normal methylation patterns are very distinctive (repetitive elements; imprinted genes; analysis of tumors).

1. Denature and bisulfite-convert DNA.
2. Design PCR primers that overlap the region with a CpG site, optionally located at the 3′-end of the primer. Two sets of primers are used for each region of interest: specific for methylated and unmethylated CpG.
3. Perform PCR with each set of primers and compare the amount of products amplified from the ^{m}CpG-template specific primer vs. unmethylated template-specific primer.

Output: qualitative analysis of multiple linked CpG at the locus (if several CpG sites are covered by the primer) or CpG site-specific.

Advantages: simplicity and rapidity; low-cost (if the PCR conditions have been already optimized, see Considerations below).

Considerations:

1. Standard Taq-polymerases and many real-time PCR mixes, although being excellent for routine PCR, will not efficiently amplify bisulfite-converted DNA template (personal observation and see, e.g., Hayakawa et al. Chap. 3). Moreover, real-time PCR master mixes containing uracil DNA glycosylase UNG should not be used with the template containing uracils. Optimization of PCR conditions or specific PCR kit for converted DNA is required.
2. The strong dependence of PCR efficiency on reaction conditions leads to poor reproducibility of results. If the primer covers only one non-3′-end located CpG site, the methylated- and unmethylated-template-specific primers differ by only one nucleotide and can anneal to nonspecific template. To increase the assay sensitivity and reproducibility, it is recommended using the primers that cover several CpG sites.

Further development: The MethyLight [51] and more sensitive digital MethyLight [52] assays are reproducible and accurate fluorescence real-time PCR (TaqMan®)-based techniques, for which the commercially available kits are developed; it is compatible with the allele-specific discrimination which is important in the analysis of imprinted genes.

2.1.4 Methylation-Sensitive High-Resolution Melting (MS-HRM)

1. Denature and bisulfite-convert DNA.
2. Design PCR primers specific for the CpG-free areas flanking the region of interest.
3. Perform real-time PCR followed by a high resolution melting (HRM) step. The end product analysis is similar to a single nucleotide polymorphism (SNP) analysis (e.g., by Roche LC480 Gene Scanning software).

Output: total methylation level at the locus.

Advantages: high-throughput; sensitive and reproducible (see Considerations); cost-effective.

Considerations:

1. Requires real-time PCR mix designed for HRM analysis (commercial mixes are available).
2. For sensitive and reproducible results, requires multiple replication of each sample (at least, triplicate) and control samples for standard curve analysis to be included in each rt-PCR plate [53, 54].

2.1.5 Bisulfite Sequencing (BS)

1. Denature and bisulfite-convert DNA.
2. Design PCR and sequencing primers for converted DNA specific for non-CpG regions flanking the region of interest.
3. Perform PCR to amplify a unique DNA fragment.
4. Determine a C:T ratio for each CpG site in the amplified DNA fragment using one of the sequencing technologies:
   (a) TA-cloning with subsequent sequencing of fragment-containing plasmids purified from at least ten bacterial colonies/DNA sample [55–57]
   (b) Pyrosequencing: one of the PCR primers is biotinylated allowing a PCR product to be immobilized, then sequenced by a base-pair extension ("sequencing-by-synthesis") technique [58, 59]
   (c) Illumina next-generation multiplex sequencing using a "sequencing-by-synthesis" technology: the PCR products from many samples are sequenced simultaneously in one reaction if one of the PCR primers has a sample-specific 5′ extension (a DNA barcode tag recognized by a next-generation sequencing platform). Requires individual barcode, thus, individual primer, for each sample.

Output: Locus-specific C:T ratio.

Advantages: quantitative analysis of methylation status with a single-nucleotide resolution; reproducibility; detection of the low-methylated targets (TA-cloning and next-generation sequencing).

Considerations:

1. Labor-intensive (especially TA-cloning) and expensive. The equipment required for pyrosequencing and next-generation sequencing is not commonly available.
2. Pyrosequencing: requires optimization of the assay conditions to obtain a unique amplified DNA fragment without forming dimers between PCR and sequencing primers. The accuracy of the assay for a low-methylated (less than 10 %) CpG site may be not sufficient.
3. There is a large number of gene-specific validated pyrosequencing assays (available for example from Qiagen or pyrosequencing service companies). However, although they could be specific for the gene of interest, they are not necessarily designed for the region of interest. For example, in our experience, none of multiple commercially available Bdnf gene-assays were specific for Bdnf promoters, which are the regions of interest in many labs studying this critical for brain function gene. Custom assays should be designed.
4. Multiplex sequencing: requires individual barcode, thus, individual primer, for the PCR with each sample. Alternatively, the samples can be amplified with the same PCR primer set; then individual barcodes could be attached to the PCR fragments from each sample during additional post-PCR step.
5. It is optional to validate next-generation sequencing data in a few samples by another method (e.g., TA-cloning).
6. Relatively short read length (usually less than 150 bases) by pyrosequencing and next generation-sequencing technologies: only a few CpG sites could be analyzed in the low-dense CpG regions.

2.1.6 Matrix-Assisted Laser Desorption Ionization Time-of-Flight Mass Spectrometry (MALDI-TOF MS)

Main applications: This method is sensitive but currently more often used in the analysis of the genes that show highly diverse patterns during developmental stages (e.g., imprinted genes in neurodevelopment [60]) or in pathological conditions (e.g., cancer). Recently, this method facilitated testing the FMR1 methylation levels in blood samples of individuals with fragile X syndrome (FXS) [61, 62].

1. Denature and bisulfite-convert DNA.
2. Design PCR primers located in the CpG-free areas outside of the region of interest with one primer tagged with a T7 promoter sequence for RNA transcription.

3. Perform PCR to amplify a DNA fragment that will be used for transcription.
4. For base-specific cleavage, transcribe a reverse strand from T7 promoter and digest with RNAse A that performs C-/U--nucleotide specific cleavage of RNA. By adding into transcription mix cleavage-resistant nucleotide dCTP instead of CTP, only U-specific cleavage is achieved.
5. Perform a MALDI-TOF MS analysis of the cleavage products to obtain a characteristic mass signal pattern, the excellent explanation of technological approach can be found at http://www.methylogix.com/genetics/maldi.shtml.htm. The sequence changes from G (complementary to unconverted C) to A (complementary to cytosine converted to T) yield 16-Da mass shift per each ^{m}CpG site.

Output: semiquantitative analysis of the C:T ratio at single or multiple CpG sites at the locus.

Advantages: high-throughput due to using a universe T7 promoter; sensitive for low-methylated template; single-nucleotide resolution; cost-effective if the equipment is available (see Considerations).

Considerations:

1. The required equipment is expensive and not commonly available.
2. In high-throughput setting, multiple cleavage products of the same mass could be produced, thus complicating their realignment to reference template sequences. By adding into separate transcription mixes different cleavage-resistant nucleotides, either dTTP (instead of UTP) for C-specific cleavage or dCTP (instead of CTP) for U-specific cleavage, the assay is highly sensitive [63].
3. The analysis of both "+" and "−" DNA strands reduces errors produced by RNA-polymerases but increases the cost of the procedure.

2.1.7 Whole Genome Bisulfite Sequencing (BS-seq)

1. Denature and bisulfite-convert DNA.
2. Perform a high-throughput genome-wide analysis using a DNA methylation array or sequencing platforms [64]. Multiplex sequencing of differently tagged samples is optional.

Output: genome-wide C:T ratio.

Advantages: quantitative analysis of methylation status with a single-nucleotide resolution; detection of the low-methylated targets.

Considerations:

1. Time-consuming (analysis of the next-generation sequencing results by realignment to the reference genome) and expensive.
2. Due to reduced complexity of bisulfite-converted DNA and relatively short sequence reads, the methylation at some CpG sites cannot be analyzed because the surrounding sequence context cannot be uniquely mapped in the genome. This issue is especially problematic in the analysis of repetitive elements.
3. It is optional to validate the next-generation sequencing data by a low-throughput methylation analysis of the region of interest.

2.2 Methylation Sensitive Restriction Enzymes (MSREs)

MSREs were isolated from bacteria, where DNA methylation serves as a primitive immune system: the host DNA is methylated at some adenines and cytosines and protected from MSRE-mediated cleavage, whereas the foreign DNA, such as of the virus that infected bacterial cell, is cleaved. Restriction enzymes used in DNA methylation analysis of eukaryotes cut DNA at specific nucleotide recognition sequences, or restriction sites, that contain CG dinucleotide. MSREs do not cleave DNA if the cytosine residue within their restriction sites is methylated, leaving methylated DNA intact and, thus, amplifiable by PCR. By definition, in the course of MSRE-analysis it is possible to detect only ^{m}CpGs within certain sequences, such as TCGA (by HpyF30I enzyme), CCGG (by HpaII enzyme), CCGC (by SsiI enzyme), or GCGC (by Hin6I enzyme).

The MSRE-based methods of DNA methylation analysis use native DNA which is more stable than bisulfite-converted. Due to PCR step, the MSRE analysis is successfully used when only a small amount of DNA is available [65]. MSREs work on DNA isolated from human frozen and RCL2-fixed samples (RCL2 is formalin-free fixative for human specimen) [66] but was shown to be ineffective with FFPE-fixed samples [67].

2.2.1 MSRE-PCR

1. In the region of interest, select a fragment up to 200 bp with 1–6 sites of methylation-sensitive restriction enzyme, such as SsiI (AciI), Hin6I (HinP1I), and HpaII. Design PCR primers specific for restriction site-free areas flanking the fragment of interest.
2. Digest genomic DNA with an appropriate enzyme and perform real-time PCR with the designed primers.
3. Determine methylation level in the fragment: if at least one CpG within restriction sites is unmethylated, the fragment is digested by the enzyme and not amplified by PCR, while fully methylated sequence is amplified by PCR.

Output: relative methylation level at the locus, or at the single CpG (if there is one restriction site per analyzed fragment).

Advantages: simplicity and rapidity; low cost; relatively high-throughput.

Considerations:

1. Limited to CpGs within recognition sites of methylation-sensitive restriction enzymes.
2. Requires complete digestion of DNA. Partial undigestion produces false-positive data (increases actual methylation level). By adding an equal amount of internal control unmethylated DNA into each sample of genomic DNA, the quality of digestion can be monitored [68].

Further development: (a) An excellent microarray-based approach for genome-scale assessment of allelic DNA methylation patterns: MSRE-digested DNA mix is amplified and hybridized to single nucleotide polymorphism (SNP) mapping arrays (available for example from Affymetrix) [69]. (b) Methylation-sensitive and insensitive isoschizomers (recognize the same sequence) of restriction enzymes are used in MSRE-enriched microarrays. (c) 5hmC can be distinguished from 5mC using a following approach: T4 β-glucosyltransferase (T4-BGT) transfers the glucose moiety of uridine diphosphoglucose (UDP-Glc) to the 5hmC residues in double-stranded DNA, making beta-glucosyl-5-hydroxymethylcytosine [70]. This modified residue is resistant to cleavage by a restriction enzyme CviQ1, which cleaves unmethylated C, 5mC, and 5hmC (commercial kits are available form Millipore), and by a restriction enzyme MspI, which is methylation-insensitive isoschizomer of HpaII (commercial kits are available, e.g., from NEB New England Biolabs; genome-wide 5hmC analysis can be performed by Reduced Representation Hydroxymethylation Profiling RRHP™ available from ZymoResearch).

2.2.2 MSRE-Enriched Microarray

1. In separate reactions, digest genomic DNA with methylation-sensitive/insensitive isoschizomers. The following pairs of enzymes are appropriate: HpaII/MspI (used in HpaII tiny fragment Enrichment by Ligation-mediated PCR, or HELP) [71], HpyF30I/TaqI. The digestion with methylation-insensitive enzymes (MspI or TaqI) is used as a control to ensure that the target CpG is not deleted or mutated.
2. Subject both digested fractions to adapter linking by ligation-mediated PCR followed by dual-label microarray (commercially available) [65, 71].

Output: genome-scale relative methylation level.

Advantages: high-throughput; sensitive for low-methylated targets; compatible with the analysis of copy-number variation (due to digestion with methylation-insensitive enzyme); cost-effective.

Considerations:

1. Genome coverage by microarrays depends on frequency and distribution of restriction sites.
2. Requires complete digestion of DNA.
3. Validation of the array data by low-throughput method is optional.

Further development: For complete genome coverage, digested DNA fractions can be analyzed by massively parallel sequencing platform [65].

2.3 Methylated DNA Binding Proteins (MBP)

The structural characteristics of methylated cytosines enabled researchers to develop an important tool for DNA methylation analysis by affinity-based enrichment with methyl-DNA binding proteins, or MBPs. Two main types of proteins are used for the approach that can be generally called methylated DNA immunoprecipitation, or MeDIP: the MBD-containing MBPs (see, e.g., methylated island recovery assay, or MIRA [72, 73]), and the antibodies against cytosine modifications [74]. The 5mC or 5hmC antibodies are not true MBPs because they may potentially recognize modified cytosine in RNA molecules. The preferential specificity of MBD-MBPs and antibodies for a single CpG site or multiple closely located CpGs (see *Karpova and Umemori* Chap. 6) should be considered when choosing a protocol for MeDIP analysis.

The antibodies against cytosine modifications are successfully used in the spatial analysis of DNA methylation, or immunohistochemistry IHC [75] (see Abakir et al. Chap. 8), and in the global methylation analysis by enzyme-linked immunosorbent assay, or ELISA [76]. These analyses discovered, for instance, that, in contrast to 5mC, the levels of 5hmC modification are tissue-specific during development [75] and especially high in the adult brain [76].

2.3.1 Methylated DNA Immunoprecipitation (MeDIP)

1. Shear DNA to obtain fragments of 200–1000 bp size, optionally by sonication for unbiased fragmentation; alternatively by restriction enzyme MseI which is used for MIRA.
2. Perform immunoprecipitation (IP) of fragmented DNA with an appropriate MBP: anti-5mC or anti-5hmC antibody for a standard MeDIP, or MBD (MBD2b/MBD3L1 protein complex) for a standard MIRA. Several commercial IP kits are available.
3. Using both IP and input (non-precipitated control) fractions, determine methylation level at the region of interest by one of the following approach:
   (a) MeDIP-PCR: perform real-time PCR with the primers specific for any region of interest [14] (see Karpova and Umemori Chap. 6).

(b) MeDIP-chip: perform dual-label microarray analysis; commercial DNA microarrays, including specific for promoter regions, are available from several manufacturers [41, 77, 78].

(c) MeDIP-seq: perform DNA methylation profiling by next-generation sequencing platforms [79–81].

Output: relative methylation level at locus-specific (MeDIP-PCR) and genome-wide (MeDIP-chip, MeDIP-seq) scale.

Advantages: high-throughput and/or genome-wide analysis; discriminate 5mC and 5hmC modifications; compatible with FFPE tissue samples.

Considerations:

1. MeDIP with anti-5mC- or 5hmC-antibodies requires denatured ssDNA.
2. Requires relatively high amount of DNA (micrograms).
3. "CpG-density factor": not recommended for low-methylated targets because the DNA-affinity of MBP and antibodies is biased towards highly methylated fragments.
4. Nonspecific immunoprecipitation: DNA fragments can bind magnetic or sepharose beads used during the procedure resulting in low signal-to-noise ratio. Optimization of beads blocking/washing steps may be required. Alternatively, a combination of MIRA with MSRE approach (COMPARE-MS) was shown to increase sensitivity and specificity of the assay [82].
5. MeDIP-chip/MeDIP-seq: general limitations specific for genome-scale methods; the results should be validated by low-throughput approach.

Further development: In combination with MS-SNuPE approach, MIRA and MeDIP are recommended for quantitative assessment of allele-specific DNA methylation [83].

2.3.2 Immunohistochemistry and Immunocytochemistry (IHC and ICH)

1. Fix the tissue or cell culture samples; remove paraffin and dehydrate FFPE tissue sections.
2. Perform immunostaining with appropriate antibodies. A DNA denaturation step is required when using anti-5mC or anti-5hmC antibodies [75, 84].
3. Acquire images and analyze the intensity and spatial distribution of immunopositive signal.

Output: total methylation level.

Advantages: spatial resolution; discriminate 5mC and its oxidized modifications 5hmC/5fC/5caC; compatible with formalin-fixed tissue samples; low-cost.

Considerations:

1. A control immunostaining omitting primary antibody should be performed.
2. For semiquantitative analysis, a detection protocol should be optimized (see for details [85]).
3. High sensitivity if the tyramide signal amplification (TSA) is used. However, the conditions of TSA should be optimized to ensure a linear correlation between the signal intensity and incubation time (see Abakir et al. Chap. 8).

Further development: Simultaneous immunostaining with different antibodies can be performed [75] (see Abakir et al. Chap. 8), e.g., cell-type specific methylation levels are accessed by multiple immunostaining with cell-specific markers. In this case, the cell-type specific staining should be tested for efficacy under DNA denaturation conditions, and applied either before or after denaturation step.

2.4 High Performance Liquid Chromatography (HPLC)

Liquid chromatography is an analytical chemistry approach which is based on the physical separation of the molecules and can be combined with the mass analysis by mass spectrometry (MS). Because each nucleoside, including modified cytosines, is of a particular mass, the HPLC-MS analysis provides the most accurate information about the global levels of DNA methylation. This approach requires an average amount of DNA around 1 μg, but can be successfully adapted to lower amount, such as 50 ng (see Ross and Kaas Chap. 5), which makes it possible to use with small tissue specimen or brain-region specific punches from brain slices.

1. Hydrolyze genomic DNA into individual nucleosides, either by formic acid [86] or by enzymatic digestion (kits are available from ZymoResearch, Epigentek etc.) [84, 87].
2. Perform column separation and detection of nucleosides by mass spectrometry [86, 87].
3. Measure the percentage of the nucleoside of interest relative to other nucleosides.

Output: global methylation level.

Advantages: quantitative; the most sensitive (at global level); discriminates simultaneously different modifications due to mass difference.

Considerations:

1. Requires including internal standards into genomic DNA sample for nucleoside-specific calibration curve.
2. For low-frequent modifications, the initial amount of DNA should be increased up to several micrograms (see Ross and Kaas Chap. 5).

3 Analysis of Histone Modifications by Chromatin Immunoprecipitation (ChIP)

Histone is the main protein component of a nucleosome, a first level of chromatin compactization. A nucleosome is composed of 146 bp of DNA in 1¾ helical turns are around of an octamer of core histones (2x H2A, H2B, H3, H4) and a linker histone H1 (Fig. 6). Hydrophilic histone tails comprise 25–30 % of mass of histones and enriched in lysine (K) methylation, acetylation and ubiquitination, serine (S) phosphorylation, arginine (R) methylation, etc. Specific histones modifications inhibit or enhance association of the histone tail with DNA and chromatin remodeling complex. ATP-dependent chromatin remodeling complex influences DNA accessibility for transcriptional factors via histones modifications. For example, lysine acetylation catalyzed by the histone acetyl transferases, HATs, and their cofactor acetyl-coenzyme A neutralizes the basic charge of DNA–histone interaction and

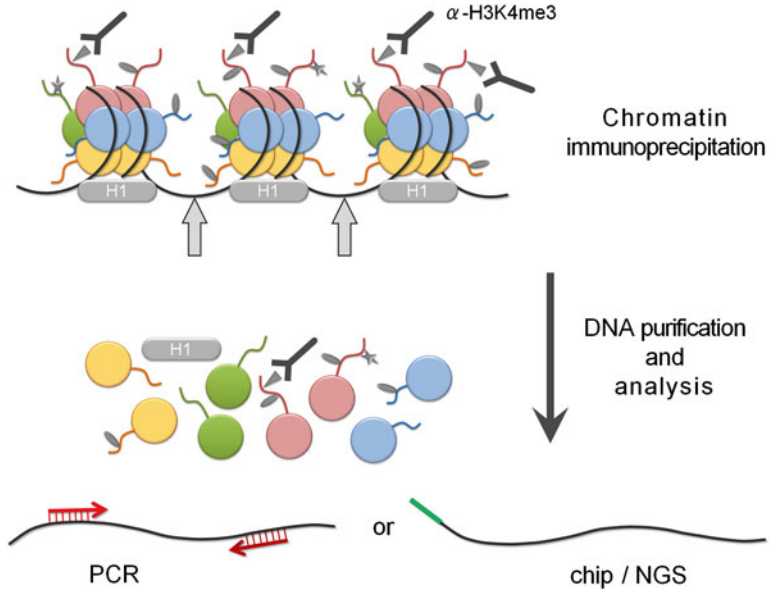

Fig. 6 The principle of chromatin immunoprecipitation (ChIP) analysis. A nucleosome is composed of DNA and the octamer of core histones (2x H2A, H2B, H3, H4) linked by the histone H1. The between-nucleosome sites (*light-gray arrows*) are more sensitive to enzymatic chromatin shearing, which is leading to the ladder-like view of digested chromatin on the agarose gel (see *Hayakawa et al.* Chap. 2). The amino-acids in the histone tails are posttranslationally modified and can be recognized by modification-specific antibodies, such as anti-H3K4me3 (three-methylated lysine 4 of histone H3, the marker of active chromatin). The immunoprecipitated DNA fragments purified after ChIP are analyzed either by PCR with gene-specific primers (*light* and *dark red arrows*) or, after adaptor linking (*green line*), by high-throughput microarray on-chip or next-generation sequencing (NGS)

weaken histone–DNA contacts, whereas removal of the acetylated mark by the histone deacetylases, HDACs, stabilizes internucleosomal contact and leads to the formation of DNA less accessible to transcription factors (Fig. 1) [3, 88]. The acetylated histones are normally associated with "permissive" chromatin state and active transcription, whereas histone methylation is linked to "repressive" chromatin, with the exception of histone H3 lysine 4 methylation (or H3K4me). The combination of histone modifications around a gene specifies the time- and cell type-dependent pattern of gene expression and can be analyzed by chromatin immunoprecipitation method [3, 89].

ChIP analysis allows detecting DNA fragments associated with specific transcription factor or modified histone. There are two main types of ChIP: X-ChIP that uses formaldehyde-cross-linking of chromatin contacts [89], and N-ChIP that uses native chromatin [90]. In both types of ChIPs, the DNA-component of chromatin should be sheared to fragments suitable for subsequent detection by PCR or microarray, which is achieved by sonication or enzymatic digestion (see Hayakawa et al. Chap. 9). The ChIP is then performed with the antibodies against histone modification or transcription factors, such as methylated DNA binding protein MeCP2. For histone-ChIP, wide range of original material can be used, from low-cell number samples [91] to dozens ng-tissue pieces [46, 92]; moreover, the human FFPE specimen are compatible with the ChIP analysis after the paraffin-removal and rehydration step. For analysis of transcription factor-binding sites, larger samples may be needed.

Here, an overview of the histone-ChIP procedure is present:

1. Fix cells or tissue with formaldehyde (for cross-linking the chromatin DNA/RNA/protein complex in X-ChIP) and lyse the sample (in X- and N-ChIP).
2. Prepare chromatin and shear it to 1–3-nucleosome size, or 150–500 bp, by one of the following methods:
   (a) Sonication for X-ChIP
   (b) Enzymatic shearing (e.g., using a kit from Active Motif) for X-ChIP (see Hayakawa et al. Chap. 9)
   (c) Micrococcal Nuclease for N-ChIP
3. Perform immunoprecipitation IP with an appropriate ChIP-grade antibody followed by re-cross-linking (for X-ChIP) and DNA purification.
4. Using the DNA derived from both IP and input (non-precipitated control) fractions, determine histone modification levels at the region of interest by PCR (ChIP-PCR), microarray (ChIP-on-chip) or next generation sequencing (ChIP-seq).

Output: locus-specific (ChIP-PCR); genome-wide (ChIP-on-chip; ChIP-seq).

Advantages: high range of amount of original material; growing number of ChIP-grade antibodies; sensitive; cost-effective.

Considerations:

1. In vivo, one histone modification may cancel or promote another modification [3]. To investigate the relationship between gene transcription and chromatin state at its promoter, the analysis of several modifications may be required.
2. For chromatin preparation from tissue samples, it is important to lyse and homogenize the sample to obtain the suspension of individual nuclei.
3. N-ChIP: because the chromatin is not cross-linked, the nucleosomes might be displaced from their original position.
4. The antibodies should be validated experimentally with a number of control samples or ChIP-grade (commercially available from several companies, such as Upstate, Abcam, Active Motif etc.).
5. Requires including control IP reactions without primary antibodies or with immunoglobulins obtained from the same species as the primary antibodies.
6. Signal-to-noise ratio can be regulated by stringency of washing steps following IP reaction.
7. For consistent results with "difficult" samples, such as the samples of small size or FFPE specimen, it is recommended using commercially available ChIP kits offered by multiple suppliers (see above).
8. For validation of genome-wide data, locus-specific PCRs should be performed.

Further development: For very small or rare samples, the linear DNA amplification (LinDA) technique can be used to increase the amount of DNA available after the ChIP procedure [93].

4 Analysis of Noncoding RNAs: Focus on miRNAs

Noncoding RNAs regulate many biological processes including the process of transcription itself. NcRNAs affect the expression of thousands of genes in response to developmental or environmental signals. The function and the list of ncRNAs that regulate RNA expression are being rapidly discovered, suggesting that new methods for analysis of different ncRNAs will continue to be developed. In the brain, lncRNAs and miRNAs are particularly enriched. The analysis of their expression, especially lncRNAs, is similar to classical mRNA analytical approaches and includes gene-specific quantitative PCR or transcriptome-wide chip and NGS assays, fluorescent in situ hybridization (FISH) and RNA immunoprecipitation (Fig. 7).

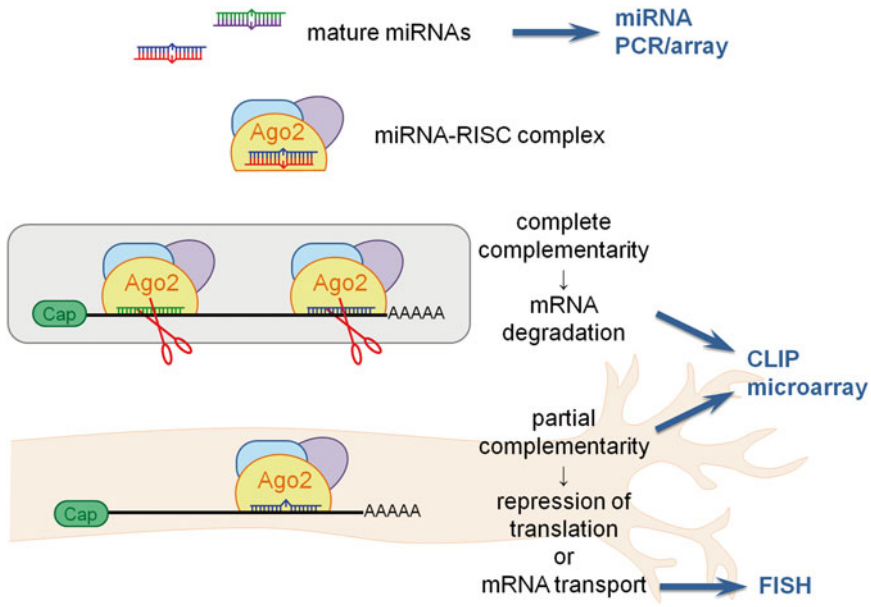

Fig. 7 The diversity of miRNA analysis is based on miRNA biogenesis and cellular function. The purified mature miRNAs are analyzed by the quantitative approaches, miRNA-specific PCR or high-throughput array or microarray. The RNA-induced silencing complex (RISC, miRNA+ protein Ago2) with target RNA is analyzed by the cross-linking immunoprecipitation (CLIP). The fluorescent in situ hybridization (FISH) method aims at investigating the cell-type or cellular compartment-specific localization of miRNA and/or target transcript

This section is focused on the peculiarities of miRNA analysis. In contrast to DNA and histone modifications, as well as nascent or some lncRNAs (Fig. 1), miRNAs are especially involved in post-transcriptional repression of gene expression as well as targeting the transcripts to distal cellular compartments, the process which is critical for neuronal plasticity and activity-dependent local translation in neurons [27, 94] (see also *Orefice and Xu* Chap. 12).

Original long transcript (pri-miRNA) with a hairpin structure is released by RNase III Drosha to double-strand pre-miRNA inside the nucleus. In the cytoplasm, RNase III Dicer removes the hairpin loop and cleaves the pre-miRNA into 21–23 nucleotides duplexes of mature miRNAs. Duplexes bind to the Argonaute, AGO2 protein complex RISC [95]. Within the RNA-induced silencing complex, or RISC, miRNAs bind the target messenger RNA, mRNA, and repress its translation [27] (Fig. 7). Each miRNA may recognize several target mRNAs and the strength of miRNA–mRNA duplex, and consequently the translation repression, depends on sequence similarity between the miRNA and mRNA: if completely complemental, the targeted mRNAs are cleaved [96]; if partially complemental, the translation of the targeted mRNAs is repressed, or the targeted RNA within the AGO2 complex is included in transporting neuronal granules [97].

Current miRNA-analytical approaches are mainly focused on the analysis of mature miRNAs (see this section and the Chaps. 13 and 14 by Silahtaroglu et al. and Hollins et al. for detailed protocols); however, these approaches should be followed by the miRNA-target prediction and identification of potential molecular pathways affected in your study. The sequence similarity and the chemical properties of the secondary structure of miRNA–mRNA duplex are used by many programs to predict miRNA targets. To identify potential target genes regulated by miRNAs, the bioinformatics analysis should be performed using numerous computational tools, such as PicTar, TargetScan, DIANA-microT, miRanda, rna22, and PITA. freely available at umm.uni-heidelberg.de, mirbase.org, microrna.org, ncrna.org, targetscan.org etc. Although these tools may predict potential target, they do not provide with the information about actual existence of miRNA–mRNA complex. To resolve this problem, recent development of miRNA target-analyzing methods involve RNA co-immunoprecipitation with the antibodies against AGO proteins [98], such as the methods based on UV-mediated cross-linking and immunoprecipitation (CLIP): HITS-CLIP [99], iCLIP [100] and PAR-CLIP [101]. The efficient tools for analyzing CLIP data are being developed [102]. Hopefully, the miRNA–mRNA databases created by these tools could be freely available for research community.

4.1 Quantitative Analysis of miRNA Expression

1. Purify total RNA by any method preserving small RNA molecules, such as using Trizol (Invitrogen), Qiazol (Qiagen) etc. with subsequent precipitation with glycogen. Alternatively, commercial kits for miRNA-enriched RNA purification are available.
2. Reverse transcribe for cDNA synthesis. A standard approach for miRNA-derived cDNA synthesis includes 3′ polyadenylation step followed by reverse transcription using a primer oligo-dT coupled with universal adapter (the universal adapter priming can be used in the next quantitative **step 3**).
3. Using cDNA template, quantitate miRNA expression by one of the following method:
   (a) miRNA-specific qPCR: perform real-time PCR with a miRNA-specific primer and universal primer. Individual miRNA-assays are commercially available from multiple suppliers; it is recommended using the reverse transcription kit and miRNA assay from the same supplier.
   (b) miRNA qPCR-array: add template to ready-to-use PCR array (predefined set of primers designed for MiRNome- or pathway-specific analysis), and perform real-time PCR. The arrays are available from suppliers, such as Qiagen, Exiqon, Life Technologies etc.

(c) miRNA-chip: following labeling step, hybridize samples to microarrays [103] (see Hollins et al. Chap. 14). The diverse microarrays are available from Agilent, Affymetrix, Exiqon.

(d) miRNA-seq: following linker-attachment step, perform high-throughput sequencing, optionally by next-generation sequencing platform [104, 105].

Output: locus or genome-wide quantitative miRNA expression levels.

Advantages: low original amount of RNA; works with FFPE and laser-microdissected specimen; specific (discriminates different members of miRNA-families); sensitive (especially Exiqon microarrays, which use LNA™-based capture probes, and high-throughput sequencing); high-throughput; discovery of new miRNAs (miRNA-seq).

Considerations:

1. Requires working in RNase-free environment until cDNA synthesis step is completed.
2. LNA™-modified primers or probes (Exiqon) strongly increase the specificity and sensitivity of the analysis.
3. High-throughput data (miRNA-array, miRNA-chip, and miRNA-seq) require validation using miRNA-specific qPCR assay.

Further development: (a) Simultaneous profiling of miRNAs and their predicted mRNA targets [106] provides strong background for functional miRNA analysis using miRNA-inhibitors and mimics. (b) The target mRNA prediction is more specific using the data derived from an RNA-binding AGO protein immunoprecipitation microarray (RIP-Chip) analysis [102, 107, 108].

4.2 Fluorescent In Situ Hybridization (FISH) Analysis of miRNA Expression

1. Fix tissue samples/sections, or remove paraffin and dehydrate FFPE sections.
2. Perform in situ hybridization with LNA™-probes, which are available for almost all miRNAs from Exiqon [109, 110] (see Silahtaroglu et al. Chap. 13).
3. Acquire images and analyze the intensity and spatial distribution of immunopositive signal.

Output: spatial and semiquantitative miRNA expression levels.

Advantages: can be optimized for semiquantitative analysis; sensitive for low-expressed targets if LNA™-probes are used; compatible with immunohistochemical analysis for co-localization studies; compatible with FFPE samples if they were prepared in RNase-free conditions; relatively low-cost.

Considerations:

1. Requires working in RNase-free environment until hybridization step is completed.
2. Control reactions, such as omitting probe/antibody or using scrambled-miRNA control probe, are necessary.
3. Requires optimization of the Proteinase K-treatment step.
4. To prevent low-abundant miRNA loss (due to their small size) during the procedure, an optimized fixation of FFPE specimen with 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) has been suggested [111].

Further development: (a) The probes with 2′-O-methyl RNAs (2OMe) and LNA modifications provide superior sensitivity for low-abundant miRNA in the brain [112]. (b) The tyramide signal amplification contributes to highly sensitive miRNA FISH detection (see Silahtaroglu et al. Chap. 13).

5 Conclusions

The complexity of the brain tissue underlines structural (different brain regions, cell types and subcellular compartments) and temporal (developmental, circadian) variations of gene expression in normal and disease state. Chromatin accessibility and spatial regulation modulate the functional status of the genes in each cell in response to developmental and environmental stimuli, such as stress factors or drug administration. The complex nature of processes in neurons and glia necessitates integrative approaches in epigenetic research, in particular, the combination of analyses of DNA methylation, histone modifications and ncRNAs. Moreover, in the field of neuroscience, the high sensitivity of applied methods is essential because, for example, the subtle changes in DNA methylation pattern at gene promoters can lead to long-lasting significant changes in behavior [46, 113, 114]. At the same time, these behavioral alterations could be mediated by normal expression levels but impaired transport of important mRNAs to dendrites, as it was suggested for *Bdnf* transcripts in Huntington's disease [97]. Even if the investigation of the gene promoter regions does not reveal any epigenetic changes associated with the corresponding protein levels or phenotype, a complementary sensitive miRNA analysis may discover a novel posttranscriptional mechanism of disease. The efficient and faithful techniques using low cell-number samples, such as specific neuronal subtype or very small chirurgical brain material, should help in epigenome profiling of disease in parallel with transcriptome and proteome profiling. The data generated by the differential profiling should be effectively integrated using bioinformatics tools and globally distributed.

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