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The detection, function, and therapeutic potential of RNA 2'-O-methylation

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Abstract

RNA modifications play crucial roles in shaping RNA structure, function, and metabolism. Their dysregulation has been associated with many diseases, including cancer, developmental disorders, cardiovascular diseases, as well as neurological and immune-related conditions. A particular type of RNA modification, 2'-O-methylation (Nm) stands out due to its widespread occurrence on all four types of nucleotides (A, U, G, C) and in most RNA categories, e.g., mRNA, rRNA, tRNA, miRNA, snRNA, snoRNA, and viral RNA. Nm is the addition of a methyl group to the 2' hydroxyl of the ribose moiety of a nucleoside. Given its great biological significance and reported association with many diseases, we first reviewed the occurrences and functional

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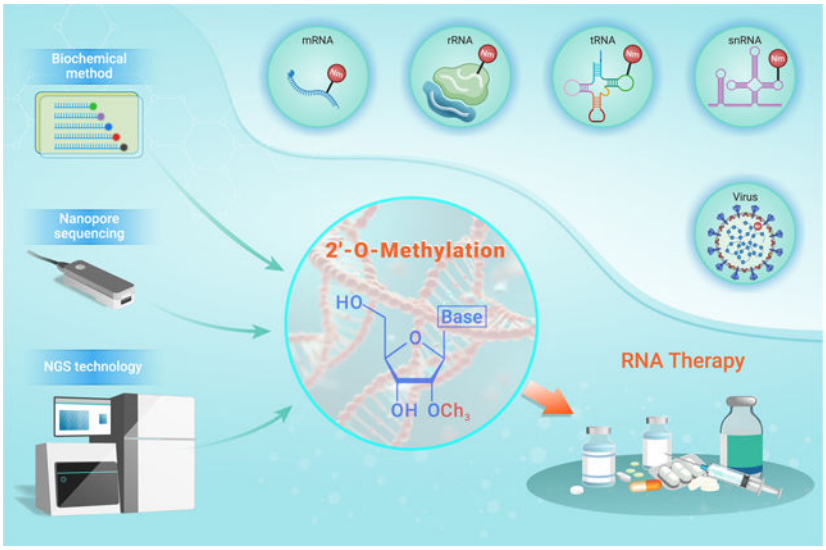
implications of Nm in various RNA species. We then summarized the reported Nm detection methods, ranging from biochemical techniques in the 70's and 80's to recent methods based on Illumina RNA sequencing, artificial intelligence (AI) models for computational prediction, and the latest nanopore sequencing methods currently under active development. Moreover, we discussed the applications of Nm in the realm of RNA medicine, highlighting its therapeutic potential. At last, we present perspectives on potential research directions, aiming to offer insights for future investigations on Nm modification.

GRAPHICAL ABSTRACT



INTRODUCTION

RNA modifications play diverse and critical roles in regulating RNA functions, gene expression, and cellular processes.[1] Dysregulation of RNA modifications are often associated with developmental disorders and diseases, including cancer,[2,3] viral infections,[3] and autoimmune diseases.[3] Understanding the functions of RNA modifications in disease processes can therefore provide insights into disease mechanisms and potential therapeutic targets. More than 170 types of RNA modifications have been identified in cells, highlighting the complex nature of post-transcriptional RNA processing.[4,5] Several RNA modifications that can be mapped with base-resolution, including N6-methyladenosine (m^6A), 5-methylcytosine (m^5C), pseudouridine (Ψ), A to I editing, 2'-O-methylation (Nm or 2'-O-Me), 5-hydroxymethylcytosine (hm^5C), and N1-methyladenosine (m^1A), are known to play functional roles in mRNA stability, splicing, export, translational regulation, RNA-protein interactions, etc.[6–10] However, such mappable RNA modifications account for only a small proportion of the many reported RNA modification types.

Among the many types of RNA modification, Nm stands out due to its widespread occurrence in the transcriptome on all of the four types of nucleotides (A, U, G, C).[11] Nm consists of a methyl group being added to the oxygen atom that is attached to the second carbon in the ribose sugar of a nucleotide (Figure 1A).[12] Nm is recognized as a critical

regulator of the structure, splicing, and metabolism in almost all major RNA categories, such as in messenger RNA (mRNA) (Figure 1B),[13,14] small nucleolar RNA (snoRNA) (Figure 1C),[15] ribosome RNA (rRNA) (Figure 1D),[16] transfer RNA (tRNA) (Figure 1E),[17] viral RNAs (Figures 1F–G), small nuclear RNA (snRNA),[18] and microRNA (miRNA).[19] Nm is first reported in rRNA and tRNA, and recently being detected in a diverse range of mRNA and viral RNAs.[1] These findings highlight the previously underappreciated roles for Nm in regulating RNA molecules beyond the traditional understanding of its function in non-coding RNAs. The enzymes responsible for the addition of the methyl group are known as RNA methyltransferases. More than a dozen proteins, including methyltransferases and their co-factors, have been reported to play roles in the installation or modulation of Nm in diverse RNA molecules (Table 1). Despite the wide involvement of these proteins in complex diseases such as cancer, Alzheimer's, autoimmune diseases, and many others,[3] the exact role of Nm in these pathological conditions remains to be explored. Understanding the mechanisms and implications of Nm will provide valuable insights into the molecular basis of these diseases and may contribute to the development of novel therapeutic strategies.

Although less extensively studied compared to some other modifications such as the m^6A and m^5C, significant research progress has been made in Nm in the past decade. This significant progress benefits largely from advancements in detection technology based on the next-generation sequencing (NGS) methods and nanopore sequencing technologies.

In this review, we first introduce the occurrences and functions of Nm in different RNA categories. We then provide a comprehensive overview of the methods employed to detect Nm in RNAs. Finally, we introduce the applications and discuss the future perspectives of Nm investigations.

OCCURRENCES AND FUNCTIONS OF 2'-O-METHYLATION

Nm in rRNA

The addition of Nm to rRNA is carried out by the protein complex known as box C/D small nucleolar ribonucleoproteins (Box C/D snoRNPs), which consist of the proteins fibrillarin (FBL), SNU13, NOP56, and NOP58.[56,57] Each complex contains a snoRNA belonging to the C/D box family that are predominantly localized in the nucleolus,[58] which is a subnuclear compartment dedicated to rRNA processing. Different snoRNP complexs contain different snoRNAs. The snoRNAs act as guides, utilizing their complementary base-pairing interactions with the rRNA molecule to precisely direct the site-specific Nm modification[56,59] (Figure 1D).[12] This intricate mechanism ensures the accurate placement of Nm modifications at specific nucleotides within the rRNA, thereby contributing to the structural and functional integrity of the ribosome and its role in protein synthesis.[57,60]

The enzyme FBL is a vital component of the box C/D snoRNP complex and catalyzes the 2'-O-Me.[25] FBL-mediated Nm in rRNA influences biological processes such as protein translation and ribosomal biogenesis.[16,61,62] FBL, as well as other proteins in the snoRNP complex, is associated with several types of cancer[25] and autoimmune disease.[21] For example, high FBL protein level is often linked to modifications in rRNA methylation, leading to tumori-genesis and higher mortality rate among breast cancer patients.[25] In the

breast cancer cell model, the increase in expression of FBL, which leads to a higher level of rRNA Nm and abnormal translation of IRES (internal ribosome entry site) oncogenes,[25] is caused by the mutation of tumor suppressor gene TP53.[63] In addition, identified by the scleroderma antiserum, FBL was found solely in the fibrillar area of the nucleolus.[21]

Besides FBL, there are several other factors that influence 2'-O-methylation (2'-O-Me) of rRNA through writer-associated mechanisms. For example, NPM1 protein actively governs the Nm sites on rRNA through direct interactions with snoRNAs, and its inactivation is found to be associated with disruptions of hematopoiesis in mice,[22] immune cell function in adults,[22] as well as with the pathology of dyskeratosis congenita in humans.[26] Mechanistically, NPM1 is associated with macrophage effector functions, which are partly regulated by its ribosome regulation. Since macrophages play a significant role in the immune system's reaction to various pathological conditions, loss of NPM1 and the associated ribosome would most likely imply a compromised immune system.[22] Recently, we revealed that EZH2 (enhancer of zeste homologue 2), a well-defined lysine methyltransferase for H3K27me3, could interact with FBL directly to regulate the rRNA Nm in an methylation-independent manner.[27,64] Mechanistically, EZH2 plays a role in enhancing the interaction between FBL and NOP56, facilitating the assembly of box C/D small nucleolar ribonucleoprotein. Notably, EZH2 deficiency decreases the rRNA Nm, disrupting the translation process as well as the initiation of translation through internal ribosome entry site (IRES) in cancer cells.[27] These findings shed light on the previously unknown function of EZH2 in translational regulation associated with cancer.[27] Furthermore, dysregulations or mutations affecting box C/D snoRNPs are associated with cancer,[15] highlighting their potential role in oncogenesis. Additionally, defects in box C/D snoRNPs have been implicated in Prader-Willi syndrome (PWS), a genetic disorder characterized by developmental abnormalities and metabolic dysfunction.[47] More recently, Liu. *et. al* revealed that the lncRNA INHEG, interacting with NOP58, promotes rRNA 2'-O-methylating complex assembly and plays an important role in the glioma stem cell maintenance and tumorigenicity through regulating rRNA 2'-O-methylation.[28]

Nm in mRNA

The recent discovery of Nm at internal sites of mRNA molecule has revealed its essential role in the regulation of gene expression,[46] and has opened an new avenue of research into how gene expression might be subtly controlled at the post-transcriptional level. Before then, the most prevalent site for Nm in mRNA is reported at the mRNA 5' cap,[13,14] (Figure 1B[12]) which is a protective structure found at the 5' end of eukaryotic mRNA and plays a critical role in pre-processing and stabilizing mRNA.[65]

The Nm modification on the first nucleotide in the mRNA cap, known as N1 Nm (Cap1), is specifically catalyzed by the enzyme cap methyltransferase 1 (CMTR1) that adds a methyl group onto the 2'-O-ribose of the first transcribed nucleotide.[38] Meanwhile, CMTR2 is responsible for ribose Nm at the second transcribed nucleotide (Cap2).[45] With synthesized tetranucleotide cap analogues used for RNA capping during in vitro transcription, Karolina *et al* revealed an impact of Cap2 Nm on protein production levels and cell type-specific nature of the functions exerted by this Nm modification in the process of protein synthesis.[44]

The Nm modification also protects mRNA from being decapped and degraded by the protein DXO, thus contributing to the maintenance of stability and integrity of the mRNA molecule.[13,14] Additionally, cap Nm plays a crucial role in masking mRNA from the innate immune surveillance mechanisms, preventing the misrecognition of mRNA as non-self or viral RNA.[44] Hence, deregulation in CMTR1 expression affects immune system-related pathways, inducing diseases such as asthma[39] and the Alzheimer's disease (AD).[40] Based on data from an independent microarray study, CMTR1 mRNA showed an increased expression in nasal lavage samples from asthma patients.[39] In the case of AD, CMTR1 is identified as one of 20 upregulated proteins in AD mice model's brain (5xFAD mice), which strengthens the expression of genes associated with amyloid plaque formation and AD development.[40] Moreover, Cap2 modification can be found on all mRNAs and is formed gradually as mRNAs age in the cytosol, converting from Cap1 to Cap2 and leading to an enrichment of Cap2 on long-lived mRNAs.[41] A significant increase in Cap1 abundance activates RIG-I, especially when the expression of RIG-I is heightened; however, the convertion of Cap1 to Cap2 substantially reduces the ability of RNAs to bind to and activate RIG-I.[41] This finding indicates that 2'-O-methylated Cap1 plays an immunostimulatory role, whereas Cap2 functions to decrease the activation of the innate immune response.[41]

Besides its functions in the cap structure, Nm can also occur at internal positions in mRNA molecules, influencing the downstream process of translation. Notably, the presence of Nm within the coding regions of mRNA has been demonstrated to interfere with pivotal stages of tRNA decoding, resulting in a reduction in translational efficiency and affecting protein production, which is a crucial aspect of gene expression and cellular function.[17] In recent years, it has been discovered that m6A was installed in a co-transcriptional manner in mRNA.[66–68] Nm was described as a post-transcriptional modification on the Cap structures of mRNA and rRNA, which is catalyzed by CMTRs[69] and FBL guided by snoRNAs, respectively. The presence of Nm in mRNA of *Pxdn* gene, which is mediated by snoRNA U32, suggests that Nm in mRNA might be added post-transcriptionally.[46] However, kinetic labeling experiments[70] and Ribometh-seq[71] both suggested that Nm is predominantly co-transcriptional in 18S rRNA and co- and post-transcriptional in 25S rRNA from yeast. Therefore, whether the transcriptome-wide Nm is post-transcriptional or co-transcriptional is still underinvestigated.

Regarding Nm distribution along an mRNA sequence, previous studies using Nm-seq indicated that Nm displayed a global distribution, as the modifications were also present inside mRNAs apart from the 5' cap.[72,73] In addition, NJU-seq revealed that the CDS and 3' UTR have more Nm in the Nm-modified transcripts.[74] Nm-mut-seq[75] and our Nanopore-based method NanoNm[43] have revealed Nm could happen in the whole transcript but with an enrichment in the region around the stop codon. Functionally, Nm in mRNA increases the mRNA expression levels but inhibit translation levels of Peroxidasin (Pxdn) in vitro and in vivo.[46] Furthermore, the internal Nm also contributes to the stability of the secondary structures of mRNAs.[76] Interestingly, the internal Nm modification sites have been linked to viral infections. Nm depletion has been shown to trigger innate immune response when FBL is down-regulated leading to the decrease in Nm levels.[42] This discovery suggests a novel role for Nm in the cellular response to viral invasion, thereby highlighting the importance of Nm in a broader context of cellular defense mechanisms. Recently, our work has revealed

thousands of Nm sites from prostate cancer cells, where we show that the widespread Nm at mRNA internal sites promotes mRNA stability and the 2'-O-Methylated mRNAs were linked to shortened 3'-UTR, which contributes to its longer mRNA half-life.[43]

These findings suggest that Nm represents an intricate layer of post-transcriptional gene regulation, adding a dimension of complexity to our understanding of gene expression regulation and immune response.

Nm in tRNA

Nm also plays important roles in the structure and function in tRNA.[77,78] In tRNA, Nm modifications occur at various positions and are crucial for the proper functioning of the molecule (Figure 1E).[12] Typically, RNA modifications can stabilize the L-shaped tertiary structure of tRNAs, increasing its thermal stability, and aiding in its correct folding.[79] These modifications can also impact the codon recognition process by the tRNA anticodon during translation.[80] tRNA Nm dysregulation occurs when there is an imbalance in the cellular mechanisms responsible for tRNA modifications, potentially due to genetic mutations, environmental factors, or disruptions in associated enzymatic pathways. Dysregulation of tRNA Nm modifications can affect protein synthesis and has been implicated in diseases, such as cancer and neurological disorders.[81,82] In human tRNA, FTSJ1 acts as the tRNA 2'-O-methyltransferase, and a study from tRNA Nm in bacteria has revealed roles of FTSJ1 in regulating stress and innate immune responses.[32] Meanwhile, snoRNAs are also closely connected to tRNAs since C/D box snoRNA SNORD113–6 protects tRNALeu (TAA) from being degraded by guiding Nm activities.[48] Pathologically, FTSJ1 is associated with mental retardation, as the dysregulation of FTSJ1 gene and altered Nm patterns in tRNAs have been identified as its cause.[33,35,36]

Nm in snRNA

snRNA is involved in the processing of pre-mRNA in the nucleus, particularly in splicing - the removal of introns and joining of exons to create the mature mRNA.[83,84] In snRNA, Nm modifications are less common but important. They often occur near splice sites and may play a role in the splicing mechanism, potentially influencing splice site recognition and/or snRNA-snRNA interactions.[85] Dysregulation of snRNA Nm modifications can potentially disrupt splicing, leading to erroneous mRNA and protein sequences, and potentially contributing to diseases such as cancer and splicing-related genetic disorders.[86] For instance, defects in U2 snRNA can disrupt mRNA alternative splicing and give rise to cerebellar ataxia and neurodegeneration.[49] Thus, Nm modifications in snRNA are vital for their respective functions in mRNA processing, giving rise to the fine-tuning of gene expression.

Nm in piRNA

Nm modification can also be present in PIWI-interacting RNAs (piRNA). piRNA represents a type of small non-coding RNA molecules that stabilize the genetic material through suppression of transposable elements in both reproductive and gonadal non-reproductive cells.[87] In male germ cells, piRNA is often 2'-O-methylated at the 3' termini end, and this modification plays a role in biogenesis.[88] Moreover, the study from Lim et al. demonstrated that the protein HENMT1 is an essential modulator for the Nm of mammalian piRNAs.[50,51]

Specifically, HENMT1 is an enzyme involved in the methylation of the 3’ end of piRNAs. The dysregulation of HENMT1 can be associated with instability of piRNAs, leading to male sterility due to germ cell arrest during spermatogenesis in mouse models. Therefore, Nm in piRNA serves as a critical regulatory mechanism that not only influences piRNA stability and functionality, but also exerts a profound impact on male reproductive health.

Nm in human viruses, including HIV and COVID-19

The recent surge in virology has illuminated novel roles of Nm modification in human viruses, such as COVID-19 (Figure 1F)[12] and HIV (Figure 1G).[12] For instance, Ringeard et al. showed that the addition of Nm by FTSJ3 is associated with HIV’s avoidance of immune sensing,[52] as acknowledged by El Kazzi et al. (2023).[53] Their studies revealed that the FTSJ3 enzyme, which adds the Nm modification to HIV RNA, aids in disguising the virus from the host’s immune surveillance.[52] Nm essentially “camouflages” the viral RNA, making it less recognizable to the host’s immune system. By preventing the recognition of the viral RNA as foreign, the Nm modification assists HIV in avoiding immune responses, thus enhancing its ability to persist within the host.[52,53] Moreover, Nm is also found to be added to viral mRNA by the nsp16/nsp10 enzyme complex in the SARS-CoV-2 to evade the immune system.[55] These findings offer crucial insights into the molecular strategies employed by SARS-CoV-2 and other viruses to survive and replicate within their hosts. Furthermore, the findings suggest potential therapeutic strategies for targeting these modifications. For example, antiviral drugs can be designed to inhibit the function of the nsp16/nsp10 complex, thus stripping the virus of its “camouflage” and making it more susceptible to the host’s immune response.[89]

Although Nm modifications are abundantly distributed across various RNA species, their methylation levels vary significantly. In rRNA, the methylation ratio could as high as 95% and also be less than 80%, based on data from MS-spectrometry, RiboMeth-seq, and Nanopore sequencing.[43,90,91] For tRNA, the methylation ratio is similarly high, as shown by RiboMeth-seq.[92] In mRNA, for the Nm at cap1, a very early study indicated that in HeLa cells, almost every mRNA molecule has a cap1 structure, and only ~50% of mRNA molecules contain a cap2.[93] For the Nm in cap2, the methylation levels, an recent study revealed significant variability in cap2 abundance across mammalian cell lines, ranging from 25% in mouse embryonic stem cells to 56% in MCF-7 cells, while it was nearly absent in Caenorhabditis elegans and found at low levels in fruitfly and zebrafish mRNA.[41] Additionally, cap2 levels varied considerably among mouse tissues, from 8% in the brain to 30% in the spleen.[41] However, at the internal site of mRNA, Nm-mut-seq,[75] NJU-seq,[74] and our NanoNm[43] data reveal a much lower methylation ratio, ranging from 0.2 to 0.5, 0.01 to 0.3, and averaging around 0.25, respectively. In snRNA, methylation is highly abundant, with stoichiometric methylation at all Nm sites in human tissues exceeding 90%, as confirmed by RiboMeth-seq.[94] Human piRNAs, like plant miRNAs, exhibit complete 2’-O-methylation, whereas mammalian miRNAs, such as those in mice, lack this modification.[95] Recent findings indicate that human miRNA methylation occurs in lung cancer, catalyzed by HENMT1, a homolog of the plant enzyme HEN1.[96] Viral RNA also shows high Nm levels, with RiboMeth-seq data indicating methylation ratios approaching 60% in HIV mRNA.[52]

In SARS-CoV-2 (COVID-19) mRNA, Nm-seq detected 130 Nm sites across the genome, although precise methylation levels could not be determined due to method limitations.[54]

There are several valuable bioinformatics databases related to RNA modifications such as m6A[97] and 2'-O-methylation. For example, MODOMICS offers detailed positional information of Nm in rRNA and tRNA.[98] RMBase v3.0 contains data on 1,074,100 RNA modification sites spanning 73 types of RNA modifications across 62 species, including 5,596 Nm sites in human mRNA and rRNA.[99] Furthermore, RMDisease v2.0[100] and RMVar[101] provide information on genetic variants that affect RNA modifications and their associations with diseases and traits. DirectRMDB, utilizing Nanopore direct RNA-seq, provides Nm site data derived from this sequencing technology.[102] In addition to these, snoRNA databases like snoDB2.0[103] and Snopy[104,105] are valuable resources for identifying snoRNAs that target Nm in rRNA. Sci-ModoM provides a comprehensive quantitative database of transcriptome-wide, high-throughput RNA modification sites, including the stoichiometric information of Nm modifications.[105] RNAcentral also offers a comprehensive repository for snoRNA-related information.[106]

In summary, Nm holds immense biological significance due to its involvement in crucial biological processes, such as stress response,[32] protein synthesis,[16] immunogenic pathway,[107] and cellular aging.[108] Besides its biological importance, the pathological implications of Nm in cancer, virus infection and immune response, have positioned it as a promising candidate for therapeutic applications. Thus, development of innovative approaches for the detection and quantification of Nm will contribute to unraveling its biological significance, pathological implications, and therapeutic potential. Accurate detection approaches will advance our ability to study Nm in a precise and comprehensive manner. However, due to the relatively lower levels of Nm in cells compared to some other RNA modifications, its detection and measurement remains challenging. As a result, the study of Nm in patients lags behind the investigation of other modifications. Recently, new methods and techniques have been developed to overcome these limitations and enhance our understanding of Nm. In the next section, we will provide a comprehensive overview of the latest methodologies employed for the accurate detection of Nm in RNA. These innovative approaches will open new avenues for studying the distribution, dynamics, and functional consequences of Nm modifications, shedding light on their roles in various biological processes.

METHODS TO DETECT THE 2'-O-METHYLATION

In this section, we aim to provide a comprehensive and up-to-date review of the methods for Nm detection – spanning from biochemical methods in the mid-20th century to recent methods based on high-throughput NGS, AI-driven computational prediction, and the latest nanopore sequencing-based approaches. We first give an overview of the initial biochemical methods used for Nm detection. Then we summarize the NGS method to detect Nm. Subsequently, a comprehensive survey will be conducted on the recent AI-driven models for computational prediction of Nm. Lastly, we discuss the latest third-generation sequencing technique – nanopore-based methods – used for Nm detection and contextualize it within the landscape of broader types of RNA modifications, such the m^{6}A and pseudouridine (Ψ).

Conventional techniques for Nm detection

Traditional non-sequencing methodologies have been pivotal in enhancing our understanding of Nm. Prior to the advent of NGS technologies, an array of diverse techniques contributed significantly to the exploration of Nm modification sites. These methodologies include, but are not limited to, biochemically based procedures, chromatographic techniques, mass spectrometry (MS), and primer-extension (PE) based methods. Each method has contributed unique insights, underscoring their indispensable roles in the investigation of Nm (Figure 2 & Table 2).

The earliest biochemical methods for RNA analysis can be traced back to the mid-20th century, marked by different enzymatic probing attempts to sequence RNA nucleotides using ribonuclease to partially digest radioactive rRNA, or targeting RNA molecule labeled at the terminal.[110,111] The latter case is known as direct rRNA sequencing based on end labeling. For the adaptation of this method to detect modified rRNA nucleotides, experiments were conducted on alkali-degraded yeast rRNA utilizing chromatography for identification of methylation sites,[138] as well as on rabbit 18S rRNA where Nm and Ψ sites were identified.[139,140] Later, method for sequencing modified rRNA nucleotides expanded the repertoire of model organisms including *E coli, S carlsbergensis, Xenopus laevis*, and *Homo sapiens*.[112] Enzymatic probing, primarily utilizing *in vivo* labeling with radioisotopes and chromatography, has demonstrated high precision in identifying and quantifying Nm sites.[114]

Chromatography and mass spectrometry also contributed to a deeper understanding of Nm. Chromatography appeared in many forms and settings, including thin-layer chromatography[113] in 1968, gas chromatography[141] in 1972, column chromatography[142] in 1974, and homochromatography[143] in 1980, despite the limited specificity and quantification for Nm modification. On one hand, chromatography separated various components of a complex mixture based on their varying affinities to a stationary phase and a mobile phase. On the other hand, the basic mass spectrometry hinged on the principle of ionization and subsequent separation of ions according to their mass-to-charge ratio, with the earliest methods of Nm detection focusing on the resulting unique sugar fragment.[144] Regarding chromatography, high-performance liquid chromatography (HPLC) and reverse phase (RP)-HPLC signified the transition from semi-quantitation to quantitation of RNA modification with higher resolution and sensitivity. This transition allowed researchers to identify new methylation sites in the 18S rRNA of *S. cerevisiae*[115] and to locate all ribose and base modifications in yeast rRNA to a single nucleotide resolution.[90,116] Similarly, more refined and accurate versions of MS were applied on *E. coli* and *S. pombe* rRNAs.[129,130] qMS was also introduced to detect Nm sites in *S. cerevisiae* and *H. sapiens* 80S rRNAs.[127,128] Moreover, the boundary between MS and chromatography began to blur, begetting a new class of integral techniques such as LC/MS on *E. coli* 16S rRNA and tRNA-derived small RNAs (tmRNA)[131,132] and LC-MS/MS on human tRNA-derived small RNAs (tsRNA).[133] Capable of identifying and quantifying Nm modification, these tools involved enzymatic digestion of RNA into nucleosides, followed by MS analysis to measure the relative abundance of modified nucleosides compared to unmodified ones. Despite the advantages of MS-based techniques, the complexity of sample preparation and the need

for expensive instrumentation can be limiting factors. Additionally, the requirement for the digestion of RNA into individual nucleotides before MS analysis results in the loss of information about the site-specific location of modifications.[127–130]

With reverse transcription primer extension (RT-PE) as an initial example, sequencing of rRNA by reverse transcription was introduced on eukaryotic 28S rRNA in the early 1980s.[117] Thereafter, reverse transcription-based Nm detection were carried out in human and *Xenopus* 18S rRNA in 1995.[118] Nevertheless, RT-PE suffered from several weaknesses, including the lack of specificity, the need to know the approximate position of the Nm site in advance, and poor sensitivity for low-abundance transcripts. A factor that hampered the precision of this method was that RNA modifications beyond Nm could still impede the RT.[122] PE can also be conducted in conjunction with partial alkaline hydrolysis, as demonstrated in 1996 in human U62-U63, yeast U24, and *Xenopus* U25–U31 snoRNAs.[120,121] Moreover, PEs were performed using varying concentrations of dNTPs to identify the Nm locations on rice 25 S rRNA.[119] The initial RT-PE methods exhibit a certain level of sensitivity; however, they also produced false positive and negative signals[118] and did not detect sites equally well as some sites completely escaped detection.[123] To address these limitations, enzymes such as RNase and DNAzymes were utilized for stoichiometric analysis,[135,136] which was based on the resistance of target Nm sites to cleavage by RNase H. Despite the greater technical complexity, enzyme-based approach was still useful, given that the cleavage patterns can then be examined to give stoichiometric assessment of the relative abundance of modified versus unmodified RNA residues.[135,136]

Inspired by the observation that RT-enzymes encounter hindrance when encountering a Nm modification under conditions of low deoxynucleotide triphosphate (dNTP) concentrations, a method called Reverse Transcription Ligation PCR (RTL-P) has been developed to address the challenge of detecting Nm modifications in low-abundance RNAs. RTL-P utilizes a combination of reverse transcription and ligation PCR to enable the targeted detection of Nm modifications. By overcoming the limitations of low abundance, RTL-P provides a valuable tool for studying the presence and functional significance of Nm modifications in RNAs that were previously challenging to analyze. RTL-P contained a semi-quantitative PCR procedure added following RT-PE, and it was tested on human and yeast rRNAs.[134] However, RTL-P could not accurately measure the Nm level.[134] RT-qPCR (Reverse Transcription-quantitative Polymerase Chain Reaction) was developed and improved upon RTL-P with more precise quantitative measurements, more efficient workflow, and enhanced sensitivity.[124] RT-qPCR (real-time quantitative PCR)[125] is more sensitive to Nm,and it can be utilized to measure the Nm at the 3’ terminus of small RNAs and its alterations under different biological statuses.[95] It was employed on miRNAs in A. *thaliana* and mouse, as well as piRNA in *H. sapiens* for Nm analysis.[95] These methods allow for the quantification of specific RNA modifications and provide their relative abundance compared to unmodified residues. Applications of these methods were constrained by limitations in throughput and scalability, highlighting the need for alternative approaches. Nevertheless, these quantitative approaches made possible of the high-throughput paradigm for Nm study.

High throughput NGS methods and their optimized variants

Previous methods have focused on identifying Nm sites and relied on chemical treatment or enzyme digestion followed by reverse transcription and PCR amplification. Recently, the advent of high-throughput NGS techniques has broadened our understanding of the functional implications of RNA modifications to a transcriptome-wide scale. Advancements in sequencing methodologies have facilitated the precise and comprehensive profiling of Nm sites either within the rRNA transcriptome or the entire transcriptome, revealing their widespread dynamics and functional roles. These reported methods include RiboMethSeq, Nm-seq, RibOxi-seq, 2OMe-Seq, Nm-rep-seq, Nm-mut-seq, and several others (Figure 3 & Table 3).

RiboMethSeq.—Relying on conducting high-throughput sequencing on rRNA segments ending near Nm sites, RiboMethSeq includes alkaline fragmentation of rRNA, followed by library preparation and NGS.[71,145] Its underlying principle primarily exploits the resistance of Nm sites to cleavage by alkaline. After sequencing, bioinformatics analysis allows for the quantification of the methylation level at each site, which reflects the ratio of methylated to unmethylated molecules. RiboMethSeq was used on Nm in rRNAs of yeast,[71] Archaea,[146] and human[147,148] as well as tRNAs in *E. coli* and *S. cerevisiae*.[92] RiboMethSeq excels in high-throughput, accurate, and site-specific quantification. However, it requires a substantial amount of input RNA and a great sequencing depth, making it unsuitable for many mRNAs and short RNAs (<50nt), such as sncRNA.[92,149] Thus, Marchand *et al* improved RiboMethSeq to be compatible with minimal RNA input while enabling accurate quantification of Nm levels under moderate sequencing depth.[145] To render it more applicable to sncRNA, Gumienny *et al* facilitate Nm site discovery by mapping snoRNA–targeted interactions through a combined, high-throughput approach of CLIP-seq and RiboMeth-seq.[150] In 2020, Pichot *et al* optimized RiboMethSeq to allow for both a better false discovery rate and an improved Nm quantification.[151] With these improvements, RiboMethSeq has the potential to precisely measure the Nm levels in RNA, because the protection signal from RiboMethSeq exhibits a linear correlation with the methylation level.[152] In most studies, the RiboMeth-Seq was applied to a specific cell line or mouse model. Recently Yuri et.al used RiboMeth-seq to perform the profiling of Nm sites in rRNA from 60 samples originating from various human cell lines, revealing the conversiton and variability of rRNA sites in different cell lines.[153]

***Nm-seq*.—**Nm-seq functions by fragmenting RNA using an oxidation-elimination-dephosphorylation (OED) solution. Multiple rounds of OED cycles are performed to selectively enrich the fraction of RNA molecules with Nm modifications at their 3’ ends. In the last round of OED, the process is stopped after the elimination step, resulting in unligatable 3’ phosphate ends. The modified RNA is converted into a cDNA library and sequenced via NGS, and its Nm modification sites are then pinpointed through bioinformatics analysis.[73,155] The key benefit of Nm-seq is that it demands less sequencing depth. So far, Nm-seq has detected thousands of Nm sites at mammalian mRNA internal sites.[73,155] An improved variant is proposed by Hsu *et al* to detect Nm sites in lower-abundance RNA species as well as to allow the removal of PCR duplicates and RT mispriming products through custom 3’ and 5’ adaptors.[156]

RibOxi-seq.—In RibOxi-seq,[157] RNA is subjected to periodate treatment, oxidizing the Nm in ribose sugars. This process makes the RNA more prone to cleavage by endonuclease Benzonase (while Nm modification sites resistant to it) upon subsequent aniline treatment. The resulting RNA fragments are then transformed into a library for NGS. Although this method demands substantial starting material, it benefits from needing a lower sequencing depth. Later, an optimized version was introduced in 2022, enabling it to provide precise profiling of Nm sites in rRNA with minimal RNA input and demonstrating its robustness in detecting Nm sites within introns and exons of mRNA.[158]

2OMe-Seq.—The primary feature of 2OMe-Seq is its utilization of the unique reverse transcription primer extension process, where dNTPs are kept at low concentrations. Under this condition, as the greater likelihood of reverse transcription termination or pause due to Nm, shortened cDNA products are produced, which can then be sequenced to infer Nm locations from the reverse transcription stopping sites. 2OMe-seq involved the creation of libraries using whole cell RNA, where random-primed reverse transcription was performed at two different dNTP concentrations (1 mM and 0.004 mM). Subsequently, adapter ligation followed, and the libraries were sequenced using the Illumina platform.[159] While 2OMe-Seq exhibits superior specificity compared to some other methods because of the enhanced resistance of various modified residues against partial alkaline hydrolysis treatment, it is limited by the lack of stoichiometric information.[163] Moreover, it provides information about the relative rather than absolute abundance of Nm modifications at specific sites.[159] Thus, the sequencing data often needs to be validated and interpreted carefully.

Other emerging sequencing-based methods to detect Nm in mRNA.—There are several other novel techniques reported very recently for Nm studies, such as MeTH-seq,[160] Nm-REP-seq,[161] NJU-seq,[163] CLAM-Cap-seq,[41] and Nm-mut-seq.[162] MeTH-seq allows transcriptome-wide, site-specific mapping of Nm and it has been tested on yeast mRNAs.[160] In 2023, Nm-REP-seq is devised to enable for the first time the identification of 3’-end Nm on ncRNAs in drosophila, mice, and humans by combining sequencing and the elimination of Nm-modified nucleosides by RNA exoribonuclease and periodate oxidation.[161] More recently, the introduction of NJU-seq and Nm-VAQ succeeded in identifying thousands of new Nm sites in mouse and human mRNAs.[163] While MeTH-seq, Nm-REP-seq, and NJU-seq all detected some mRNA Nm sites, the stoichiometric information cannot be directly measured by their chemical treatment- or enzyme digestion-based methods involved, and thus only sites are detected. To address this limitation and provide stoichiometric information of Nm sites for transcriptome-wide analysis, two recent methods have been introduced. One technique, termed CLAM-Cap-seq, relies on cap-specific enzymatic cleavage and has been tested on mRNAs in mES, HEK293T, and MCF-7 cells. Specifically, it involves the process of first decapping mRNA and ligating the cap tag to the cDNA, and then remove mRNA except for the cap tag before converting it into sequencing library. It has the strength of offering the comprehensive and stoichiometric measure of Cap2 distribution across the mRNA; however, due to the steps of reverse transcription and removal of mRNA during the RNase T2 step, it is prone to bias and loss of information concerning Cap modifications.[41] The other newly developed approach, Nm-Mut-seq, enables the stoichiometric analysis of transcriptome-wide Nm sites in low

abundant RNAs at single-base resolution, including Am, Cm, and Gm. Using rRNA in HeLa cells, the developers first selected the mutagenic reverse transcriptase variant capable of incorporating a mutation at Nm sites. This variant, RT-41B4, was developed through iterative rounds of selection using a fluorescence-based reverse transcriptase selection platform. The RT-41B4 variant, along with optimized NGS conditions, was used to build the Nm-Mut-seq pipeline.[162] Nm-Mut-seq was unable to detect Um.[162]

While numerous methods have been developed to identify Nm sites, pinpointing a definitive gold standard remains challenging. The selection of a method largely hinges on the specific research objectives. For instance, RiboMethSeq[71] is a traditional approach for studying Nm in rRNA. However, for those aiming to investigate Nm in mRNA, newer techniques like Nm-mut-seq,[162] Nm-REP-seq,[161] NJU-seq,[163] and MeTH-seq,[160] present viable options. Notably, the latest approach, the Nanopore-based method (described in more detail in a later section), could be a valuable tool to detect RNA Nm sites de novo, with a single-base resolution across the full length of each single mRNA molecule, and directly measure the stoichiometry of Nm in mRNA with high sensitivity.[43] It's worth noting that current high throughput detection methods often exhibit limited concordance between each other when identifying mRNA sites.[161] It's crucial to validate the results using independent techniques as complementary approaches to ensure the comprehensiveness and accuracy of the findings, for example, to use some low-throughput method such as RTL-P[134] and site-specific RT-qPCR after oxidation–elimination–dephosphorylation.[162] The limited concordance between methods can be due to the unsaturated sequencing depth of current datasets. For instance, although Nm-Mut-seq and Nm-seq detected few common mRNA Nm sites, 69 (60%) of 115 mRNA Nm sites detected by Nm-Mut-seq could be successfully verified by a targeted version of the Nm-seq method. Therefore, a big overlap between these methods would be achievable with deeper sequencing. We conducted a down-sampling analysis (unpublished) and verified that the sequencing depth in current Nm-Mut-seq data (300 million reads) did not yet reach saturation. Meanwhile, Nm sites revealed by our recent Nanopore-based method showed a significant (40-fold) overlap with the sites revealed by Nm-mut-seq, reaching at 300 to 400 overlapped sites in each analyzed cell type. These observations suggest that Nm-mut-seq might be a favorable NGS-based method to detect Nm in mRNA at its current sequencing depth.[43]

Machine learning models to predict 2'-O-Methylations

Complementary to biochemical and NGS-based methods, computational models based on machine learning (ML) have been developed to predict Nm in different RNA species. In this section, we summarize the support vector machine (SVM), random forest (RF), and neural network (NN) methods for the prediction of Nm (Figure 4 & Table 4).

Support vector machine (SVM).—To predict Nm sites, Chen *et al* devised an SVM-based method that characterized RNA sequences using nucleotide chemical attributes and compositions and achieved an accuracy rate of 95.58% in recapitulating Nm sites within the human genome.[164] Yang *et al* developed the SVM-based model iRNA-2OM on *H. sapiens* dataset for Nm site predictions, which achieved a higher accuracy of 97.95%.[165] Moreover, in 2023, a further boost in model performance on human dataset is observed by integrating

SVM with XGBoost, producing an overall accuracy of 84.3 % on the independent test set.[166]

Random forest (RF).—An RF-based method called NmSEER V2.0, which relies on random forest algorithm and multi-encoding scheme, achieves an AUROC of 0.862 and AUPRC of 0.254 in the independent test.[167] In combination of random forest and light gradient boosting for hybrid feature set construction, the model NmRF obtained the accuracies of 89.069 and 93.885% and AUC of 0.9498 and 0.9832.[168] More recently, Pichot *et al* created a RF-based model trained by RiboMethSeq datasets, and it has been evaluated on other eukaryotic rRNAs such as *S. cerevisiae* and *A. thaliana*.[169]

Neural network (NN).—In 2018, Mostavi *et al* proposed the Deep-Nm model that incorporated convolutional neural networks (CNN) to refine features.[170] Based on the training on the Nm-seq data, Deep-Nm obtained AUC and auPRC scores of 90%.[170] Subsequently, iRNA-PseKNC (2methyl) obtained an accuracy of 98.27% and sensitivity of 96.29% on the *H. sapiens* dataset.[171] Besides that, the DeepOMe model also emerged, which combined CNN with Bidirectional Long Short-term Memory (BLSTM).[172] Furthermore, there came the transformer-based model Bert2Ome, which reached the prediction accuracy and AUROC score of 99.15% and 0.99, respectively, on human data based on a 5-fold cross-validation.[173]

The third generation long-read sequencing of native RNA

While NGS methods are widely utilized, several technical limitations remain to be addressed. These limitations include biases introduced during PCR enrichment, difficulties to obtain highly specific antibodies, and complicated treatment with chemicals.[174] However, the advancement in third-generation sequencing technology, particularly nanopore-based sequencing, presents a promising solution to address these challenges. This advanced sequencing approach allows for transcriptome-wide analysis of RNA modification at a single-nucleotide resolution across the whole body of each single RNA molecule, enabling direct measurement of Nm stoichiometry.

Nanopore sequencing represents a distinctive third-generation approach to sequencing, enabling direct sequencing of native RNA molecules without amplification. In short, nanopore sequencer requires three major components: the polymer membrane, the nanoscale protein pore embedded in the membrane, and the motor protein on the pore.[175,176] Nanopore sequencing operates by directing individual strands of RNA molecules through a protein pore at a nanoscale level. During their passage through the pore, variations in ionic current are recorded and utilized to decipher the sequence. The pore incorporates a motor protein that guides the molecule through, facilitating the real-time sequencing of nucleic acids.[177] Notably, nanopore sequencing offers several advantages over NGS methods, such as the ability to sequence the full length of RNA molecule and the capacity to analyze native RNA without the need for reverse transcription and PCR amplification. Consequently, it provides direct, real-time sequencing data at the single-molecule level. However, one significant drawback of this method is its biased error profile when compared to NGS, which can present challenges in accurately identifying modified nucleotides.[178,179]

Nanopore sequencing, as a revolutionary method for direct RNA sequencing, relies on a series of computational steps for data analysis (Figure 5A). The computational pipeline begins by converting the raw signal data into nucleotide sequences through a process known as “base calling,” typically employing software like Albacore or Guppy.[180,181] Subsequently, the resulting base-called reads are aligned to a reference sequence using minimap2,[182] enabling the determination of their precise genomic location. Finally, feature extraction is performed, wherein specific patterns within the aligned reads are identified to detect the presence of Nm modification. For this purpose, the tool Nanopolish[183] or customed scripts are employed, leveraging signal-level information to accurately identify Nm modification events. These computational steps collectively establish the foundation for analyzing nanopore-sequenced data.

Nanopore direct RNA sequencing involves sequencing RNA molecules directly without reverse transcription to generate cDNA.[184] It provides information about the RNA sequence as well as potential RNA modifications present in the sample. Nanopore direct RNA sequencing, while a promising emerging strategy, currently cannot distinguish between different types of RNA modifications unless modification type-specific computational methods are developed. As a result, this approach has been limited to the analysis of only a few specific RNA modification types such as the m^6A,[185–196] pseudo-U,[197,198] m^5C,[199] and A-to-I editing.[200] Recently, there are several methods aiming to detect multiple RNA modifications with one software. For example, NanoSPA[201] could detect pseudo-U and m6A simultaneously, while CHEUI[202] could detect m6A and m5C at once. Addtionally, TandemMod[203] could detect multiple RNA modifications, including m^1A, m^6A, m^5C, and m^7G, and it remains applicable across species and conditions.

To date, only a limited number of studies have employed nanopore sequencing to detect Nm (Figure 5B). Two recent studies have observed a shift of current signals at a few known Nm sites in rRNAs but cannot analyze new Nm sites *de novo* in any type of RNA.[207] In 2021, Begik *et al* predicted the stoichiometry of Nm-modified sites using a two-category (Nm-modified or unmodified) K-nearest neighbors (KNN) classification algorithm in yeast mitochondrial rRNA.[198] In 2022, DeepNm and HybridNm were proposed, with the former employing a multi-scale framework to accurately capture subtype-specific sequence features of 2’-O-Me.[208] Nm-nano unifies RF and XGBoost with k-mer embedding models to pinpoint Nm sites within nanopore direct RNA sequencing reads from human Hela and HEK293 cell lines. It has recaptured Nm sites in mRNA as “yes” or “no” events but cannot analyze stoichiometry.[205] In this light, developing a computational method that effectively utilizes nanopore sequencing signals for *de novo* stoichiometric analysis of Nm with the single-base resolution and at the transcriptome scale was still an unsolved challenge.[209] To fill this gap, we developed a machine-learning method NanoNm (https://github.com/kaifuchenlab/NanoNm) to perform the *de novo* stoichiometric analysis of Nm with the single-base resolution from Nanopore direct RNA-seq data.[43] In addition to validating the Nm sites in human, yeast, and fly rRNA, with stoichiometry exceeding 90%, we identified thousands of Nm sites on mRNAs at single-base resolution, with a median stoichiometry of 0.25 per site.[43] Our investigation unveiled a favorable impact of FBL-mediated Nm modification on both mRNA stability and expression levels. Notably, over-activation of FBL in cancer cells coincides with increased levels of 2′-O-methylated mRNAs involved in

cancer pathways, suggesting a pivotal role for FBL in post-transcriptional regulations.[43] In addition, various software tools —such as xPore,[194] Nanocompore,[189] Yanocomp[193]—are designed specifically for comparing RNA modifications between two samples (Table 5). These tools exhibit the potential to detect Nm modifications by comparing between a control sample and a sample with the depletion of Nm methytransferases, which might be able to offer valuable insights into the comparative analysis of RNA modifications.

Taken together, nanopore-based direct RNA sequencing presents a promising solution to address the technical limitations of NGS methods, enabling transcriptome-wide stoichiometric analysis of RNA modifications at a single-nucleotide resolution.

APPLICATIONS OF 2’-O-METHYLATION IN RNA THERAPY

Nm can be utilized to enhance the therapeutic properties of RNA-based components. Burmeister *et al* demonstrated that Nm-based aptamer ARC245 can act as a potential anti-angiogenesis therapeutic to vascular endotheliasl growth factor (VEGF) by inhibiting its activity.[210] Mechanistically, ARC245 is a 23-nucleotide antisense small RNA that binds to VEGF with Kd values of 2 nM, as determined from nitrocellulose filter binding assay.[210] An RNA bearing Nm modification also appears antagonistic to TLR7 that mediates immune stimulation in inflammatory and autoimmune diseases, as revealed by Robbins *et al* on human peripheral blood mononuclear cells (human PBMCs) and murine Flt3L dendritic cells (Flt3L DCs).[211] Specifically, a small-molecule, TLR7 agonist loxoribine, which preferentially activates human and mouse TLR7, has been utilized to investigate the inhibitory effect of Nm RNA on cytokine production.[211]

Additional therapeutic application of Nm involves enhancing the efficiency and precision of the CRISPR system. For instance, Ha *et al* indicate that ribosyl-Nm U-rich crRNA boosts dsDNA digestibility and CRISPR-Cas12a’s specificity in mouse zygotes.[212] Similarly, Ke *et al* also focus on CRISPR-Cas12a and discover that Nm-modified guide RNA (gRNA) can increase Cas12a’s specificity by a minimum of two folds while decreasing its binding affinity to off-target DNA.[213] Furthermore, some other RNA modifications besides Nm also possess the capacity to chemically modify CRISPR RNA, including 2’-fluoro (2’F)-pyrimidines,[214] 2’-deoxy[215] and phosphorothioates.[215] In summary, the chemical modifications of CRISPR RNAs, such as Nm, have the potential to enhance gene-editing activity and improve specificity in RNA therapy. These RNA modifications exemplify the power of manipulating CRISPR technology to achieve more precise and efficient genetic editing.

CONCLUDING REMARKS AND FUTURE PERSPECTIVES

In summary, the detection technologies, and our understanding of the biological significance of Nm, have undergone significant advancements.[216] Nm has been established as a crucial post-transcriptional modification that plays vital roles in RNA stability, structure, and function, therefore influencing diverse biological processes. Classical biochemical techniques laid the foundation for our investigation of Nm, and the field has witnessed a paradigm shift with the advent of NGS techniques. Deep-sequencing methods, such as

RiboMethSeq, Nm-seq, RibOxi-seq, and 2OMe-seq – along with their refined versions – have revolutionized the mapping of Nm across the transcriptome. These techniques have provided unprecedented sensitivity and resolution in detecting Nm sites. Machine learning models have shown promising potential, facilitating the prediction of Nm sites and improving our understanding of the epitranscriptomic landscape.

Furthermore, the unprecedented development of nanopore sequencing, a third-generation sequencing technique, offers real-time, single-molecule sequencing capabilities that addresses the limitations of previous methods. This advancement holds immense potential for achieving absolute quantitation of Nm levels. In the future, single-cell nanopore RNA sequencing might hold promise for uncovering the heterogeneity and dynamics of Nm modifications within individual cells. It can potentially provide insights into the role of Nm in cellular processes, such as development, differentiation, and disease progression. Additionally, it may enable the identification of cell type-specific Nm sites, providing a deeper understanding of their functional significance.

Despite the significant advancements in Nm detection, several challenges persist in the field. Issues related to potential sequencing bias, the requirement for extensive computational resources, and the relatively high error rate in nanopore sequencing methods need to be addressed. One key mystery is the lack of significant overlap between the mRNA internal Nm sites detected by different methods. This might be that different methods are inherently biased towards detecting different subsets of modification sites. Given the extensive distribution of the Nm, the individual existing methods may have only identified a minor subset, which is too small to provide enough statistical power for examining overlaps thoroughly. Interestingly, when it comes to conserved Nm sites, the majority are typically found on rRNA. Most next-generation sequencing (NGS) methods can reliably detect Nm sites in rRNA, setting a gold standard. However, there is a twist when it comes to mRNA. The Nm-REP-seq[161] study, for example, showed that Nm sites in mRNA were not conserved, but many genes (50%) were overlapped when compared to Nm-seq. Such inconsistency could be attributed to three main factors. First, Nm in mRNA tends to exist with ultra-low stoichiometries and varies greatly between different cell types; Second, technical limitations and background noise can negatively impact the accuracy of Nm site detection; Third, insufficient sequencing depth can reduce the chances of detecting the full spectrum of RNA modification sites in mRNA.[217,218] Our recent study revealed ~400 overlapped Nm sites revealed by the NanoNm technique and the Nm-mut-seq technique in mRNA.[43] A similar case existed in the detection of RNA modification of ac4C. The ac4C was proven detectable using LC-MS in yeast mRNA[219] and Cryo-EM with high sequencing depth in mammalian 80S rRNA.[220] However, when the low depth of sequencing (less than 20 million reads per sample) was employed, as conducted by Sas-Chen *et al*, the ac4C sites were not detected in human and yeast mRNAs.[221] In another study, Daniel et.al used a high-depth (about 100 million reads per sample) sequencing library that could capture more ac4C sites in mRNA.[222] Two back-to-back manuscripts[217,218] from these two groups also discussed this inconsistency in more detail. Recently, more studies using ac4C-seq further revealed mRNA ac4C modifications in heart disease[223] and self-renewal of stem cell.[224] Nevertheless, studying modifications on mRNA remains instructive and sheds light on potential novel biological functions.[5]

In this light, future research should focus on improving existing methodologies, developing novel computational tools, and integrating multi-omics data to gain a more comprehensive understanding of the biological and pathological implications of Nm. Notably, the emerging role of Nm in cancer research provides exciting opportunities to explore the disease's biology and identify potential therapeutic targets. As new studies continually expand and enable scientists to more precisely detect and quantify Nm, it is anticipated that new avenues for therapeutic interventions will be unveiled.

In conclusion, the remarkable progress made in our understanding of Nm and its detection methodologies has opened vast possibilities for future research and therapeutic applications. Continuing efforts in improving techniques and integrating various omics data hold the key to unlocking the full potential of Nm in advancing our understanding of biological processes and developing targeted interventions.

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PUBLIC SUMMARY

- 2’-O-methylation (Nm) in non-coding RNA and mRNA is vital in human biological and pathological processes.
- Various Nm detection methods include biochemical assays, RNA sequencing, and AI-based prediction.
- Nanopore sequencing offers quantitative, single-molecule, and context-aware Nm detection.
- Nm improves RNA efficacy, stability, and durability, holding promise to advance RNA therapies.

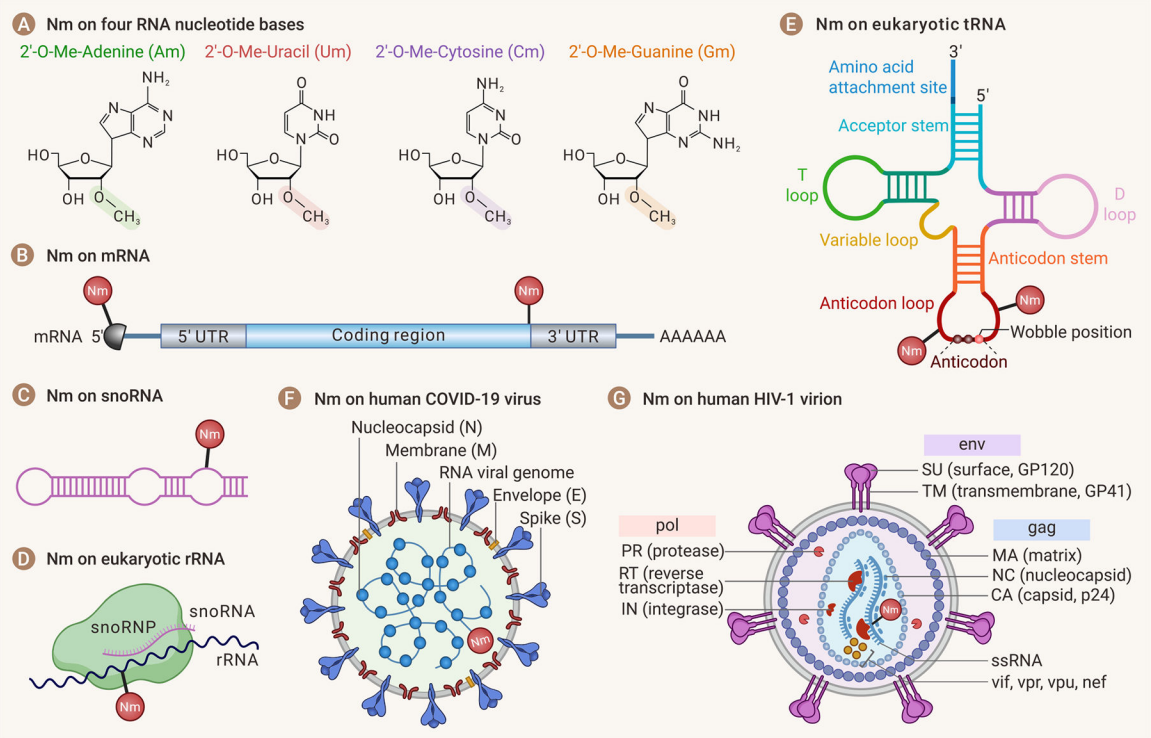

Figure 1. Nm on various RNA and viruses

(A) Illustrations of Nm on the four RNA nucleotides; (B) Nm on mRNA; (C) Nm on snoRNA; (D) Nm on eukaryotic rRNA; (E) Nm on eukaryotic tRNA; (F) Nm on human COVID-19 virus; (G) Nm on human HIV-1 virion.

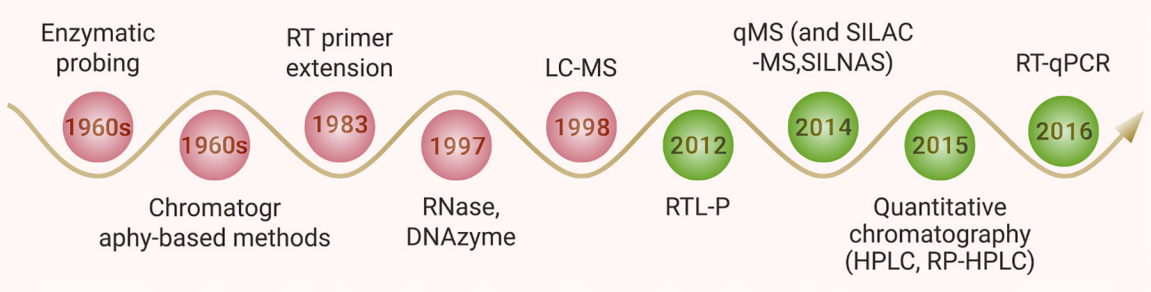

Figure 2.
Timeline of the development of biochemical methods for Nm detection.

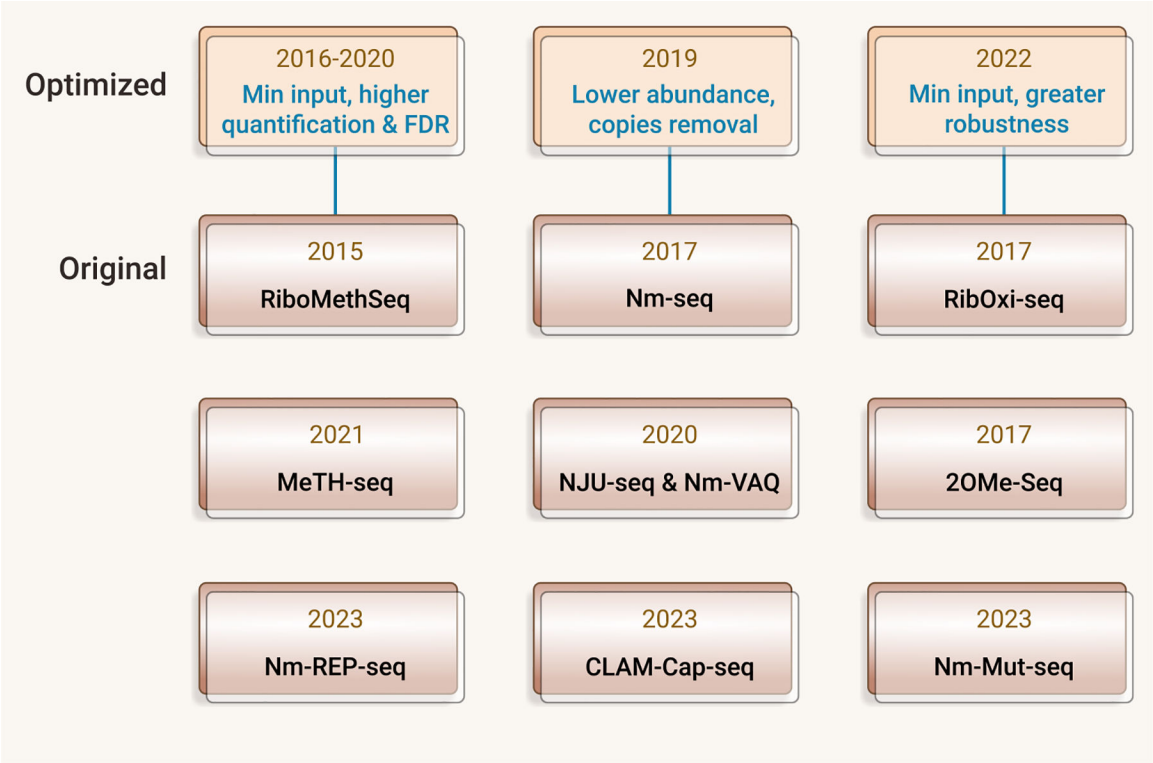

Figure 3.
Chronology of next-generation sequencing (NGS)-based techniques for Nm detection.

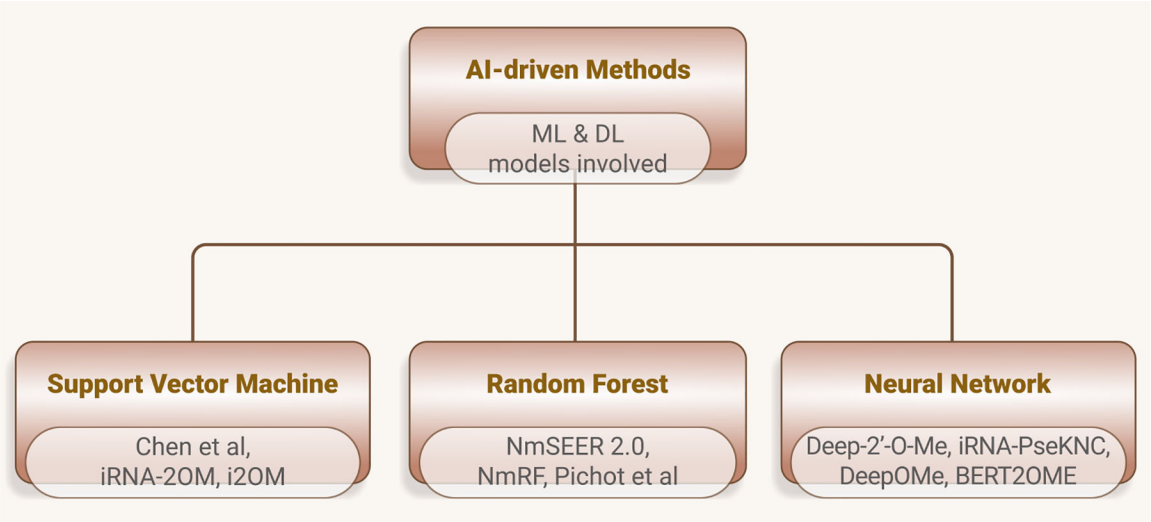

Figure 4.
Artificial intelligent methods for Nm detection.

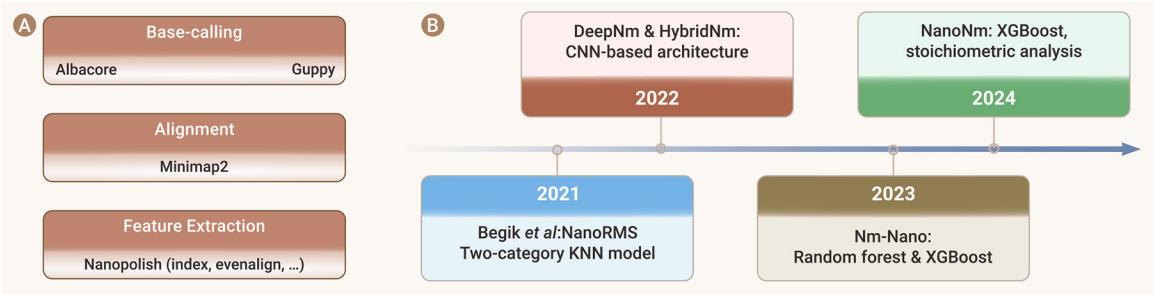

Figure 5.
Computational pipelines and AI-driven methods for nanopore sequencing data processing in recent years.

Table 1.

Nm in different RNA categories

	Nm regulatory factor	Example role/property	Related pathological condition
Nm in rRNA[16]	FTSJ2[20]	Involved in cell proliferation[20]	Autoimmune disease,[21] hematopoiesis,[22] Cancer,[23–25] Dyskeratosis congenita,[26] Translational impairment[27] Glioma stem cell maintenance and tumorigenicity[28]
	FBL[29]	Participate in pre-rRNA processing,[29] regulate rRNA transcription[30,31]	
	NPM1[26]	Regulate C/D box snoRNAs translation and Nm through direct binding[26]	
	LncRNA INHEG	Interact with NOP58, promotes rRNA 2'-O-methylating complex assembly[28]	
	EZH2[27]	Facilitate the assembly of box C/D small nucleolar ribonucleoprotein[27]	
Nm in tRNA[17]	FTSJ1[32–34]	Suppress growth defects of yeast TRM7Δ mutants[34]	Stress & immune responses,[32] intellectual disability[33–36]
	FBL[37]	Involved in snoRNA-tRNA Nm	Regulate stem cell differentiation[37]
Nm in mRNA[13,14]	CMTR1[38]	2'-O-methylate the N1[38]	Asthma,[39] Alzheimer's,[40] mRNA ageing,[41] viral infection,[42] prostate cancer[43]
	CMTR2[44]	2'-O-methylate the N2[45]	
	FBL	Regulate mRNA expression,[46] stability, and linked to alternative polyadenylation[43]	
	DXO[13,14]	Possess decapping & exoribonuclease activities toward incompletely capped RNAs[13,14]	
Nm in snoRNA[15]	C/D box[15,47]	Guide sequence-specific Nm and facilitate RNA folding & cleavages[15,47]	Cancer,[15] PWS[47]
	SNORD113–6[48]	Protect against site-specific fragmentation of tRNALeu (TAA)[48]	
Nm in snRNA[18]	U2 snRNA[49]	Lead to distortion of pre-mRNA splicing if dysfunctional[49]	Cerebellar ataxia & neurodegeneration[49]
Nm in piRNA[50,51]	HENMT1[50,51]	Ensure piRNA stability and its bulk & length[50,51]	Male sterility[50,51]
Nm in human virus[52–55]	FTSJ3[52,53]	Methylate viral HIV-1 RNA and help evade innate immune recognition[52,53]	HIV[52,53] COVID-1 9[54,55]
	nsp16/nsp10 enzyme complex[55]	Facilitate Nm of the first nucleotide and help the virus evade innate immune recognition[55]	

Abbreviations: FBL: fibrillarin; PWS: Prader-Willi syndrome.

Table 2.

Comparison across classic biochemical methods for Nm detection. Only the most relevant ones are displayed here for the sake of space and clarity

	Principle	RNA(s) used	Resolution	Key advantage	Major disadvantage
Enzymatic probing[109–112]	Differential reactivity of Nm to ribonuclease[109–111]	rRNA in yeast, vertebrate, *H sapien*[112]	Depending on the exact protocols	Precise, site-specific stoichiometric estimation[109–111]	Incomplete transcript coverage, require radioisotopes[109–111]
TLC[113,114]	Separation by different migration rates on stationary phase[113,114]	Radiolabeled RNA[113,114]	Semi-quantitative	Quick, cheap, versatile[113,114]	Limited resolution, require radiolabel[113,114]
HPLC (RP-HPLC)[90,115,116]	Separation by different interactions with stationary and mobile phases[90,115,116]	rRNA in S. *cerevisiae*[115] and yeast[90,116]	Depending on the exact protocols	High resolution and sensitivity[90,115,116]	Limited for uncharacterized retention time[90,116]
RT-PE[117]	RT followed by primer extension[117]	18S rRNA,[118] in yeast and rice,[119] snoRNAs[120,121]	Nucleotide-level	High sensitivity, site-specific analysis[117]	Limited throughput and resolution, prone to bias and false positives[122,123]
RT-qPCR[124]	PCR with an engineered DNA polymerase[124]	miRNAs in *A. thaliana,* piRNA in *H. sapiens*[95]	Nucleotide-level	High sensitivity, directly quantitative[124,125]	Prone to false positives[126]
qMS[127–129]	Mass spectra shift from isotope-labeling[127–129]	rRNA in *E. coli*[129] 80S rRNA in S. *cerevisiae,* and *H. sapien*[127,128]	Quantitative	High accuracy and sensitivity[127–130]	Complex and time-consuming preparation[127–130]
LC-MS[131,132] (and LC-MS/MS[133])	Nm-purified RNA fragments with enzyme hydrolysis[131–133]	*E.coli* tmRNA & 16S rRNA,[131,132] human tsRNA[133]	Depending on the exact protocols	High sensitivity and throughput[131–133]	Time consuming, unsuitable for low abundance RNA[131–133]
RTL-P[134]	Nm-induced RT terminates in low [dNTP][134]	rRNA in human and yeast[134]	Not site specific	High sensitivity and specificity[134]	Prone to false positives[134]
RNase/DNAzymes[135,136]	Resistance of Nm to cleavage[135,136]	18S & 28S rRNA from *Xenopus*[135] and S. *cerevisiat*[136]	Not site specific	Specific, stoichiometric analysis[135,136]	Require highly efficient enzymes[137]
Nm-VAQ[74]	Nm inhibited the cleavage of RNase H	rRNA and mRNA of human cells	Site specific	Precise quantification of Nm ratio[74]	Require RNase from Mycoplasma genitalium[74]

Abbreviations: TLC: thin-layer chromatography; (RP)-HPLC: (reverse-phase)-high-performance liquid chromatography; RT-PE: reverse transcription primer extension; PCR: polymerase chain reaction; RT-qPCR: reverse transcription-quantitative polymerase chain reaction; qMS: quantitative mass spectrometry; LC-MS: liquid chromatography–mass spectrometry; RTL-P: Reverse Transcription Ligation PCR; Nm-VAQ: an RNase H-based Nm validation and absoluste quantification tool.

Table 3.

Comparison across major NGS methods for Nm detection

	Mechanism	Detection method	RNA used	Resolution	Advantage	Limitation
RiboMethSeq[71]	Resistance to alkaline fragmentation[71]	High-throughput sequencing	rRNA in yeast,[71] *Archaea,*[146] and human[147,148]	Nucleotide-level	High throughput, accurate, quantitative[154]	High RNA input and read depth[154]
Nm-seq[73,155]	Resistance to periodate oxidation[73,155]	High-throughput sequencing	Mammalian mRNA[73,155]	Nucleotide-level	Transcriptome-wide profiling[73,155]	Laborious, high RNA input needed[156]
RibOxiseq[157]	Resistance to periodate oxidation[157]	Oxidation-specific assay and sequencing	rRNA, mRNA in human[157]	Nucleotide-level	High sensitivity, lower sequencing depth[157]	High RNA input, not quantitative[158]
2OMe-Seq[159]	Stop/pause of RT at Nm site[159]	RT and sequencing	18S and 28S rRNAs[159]	Nucleotide-level	High sensitivity, site-specific mapping[159]	Unable for absolute quantitation of Nm[159]
NJU-seq[74]	RNA hydrolysis by MgR at the Nm+1 sites[74]	High-throughput sequencing	Huma, mouse mRNA	Nucleotide-level	Accurate, high throughput Nm site screening	Lack of stoichiometric information
MeTH-seq[160]	Oxidative bisulfite conversion[160]	Bisulfite conversion and sequencing	yeast mRNA[160]	Nucleotide-level	Transcriptome-wide, quantitative[160]	False negatives due to sequence capture bias[160]
Nm-REP-seq[161]	Elimination of Nm by RNA exoribonuclease and periodate oxidation[161]	High-throughput sequencing	ncRNA, mRNA in humans, mice, and drosophila[161]	Nucleotide-level	Transcriptome-wide, site specific[161]	Hard to identify Nm in ncRNAs and mRNAs[161]
CLAM-Cap-seq[41]	RT, ligation, conversion to sequencing library after removing mRNA[41]	High-throughput sequencing	mRNA in mES, HEK293T, and MCF-7 cells[41]	Nucleotide-level	Transcriptome-wide, stoichiometric measure of Cap2 in mRNA[41]	Potential bias and loss of information[41]
Nm-Mut-seq[162]	RT that accounts mutations at Am, Cm, and Gm sites[162]	High-throughput sequencing	mRNA/rRNA in HeLa and HepG2 cells[162]	Nucleotide-level	Transcriptome-wide, stoichiometrically quantify Nm sites[162]	Unable to quantify Um sites[162]

Abbreviation: RT: reverse transcription.

Table 4.

Tabular comparison across the most recent ML approaches for Nm prediction.

	Model	RNA used	Algorithm(s)	Features for site prediction
Support vector machine (SVM)	Chen et al's[164]	*H. sapiens*	SVM	Physical-chemical based binary encoding
	iRNA-2OM[165]	*H. sapiens*	SVM	Physical-chemical based binary encoding
	i2OM[166]	*H. sapiens*	SVM + XGBoost	K-mer nucleotide composition, accumulated nucleotide frequency, and nucleotide chemical property
Random forest (RF)	NmSEER V1.0[167]	HeLa and HEK293 transcriptome	RF	One-hot encoding
	NmSEER V2.0[167]	HeLa and HEK293 transcriptome	RF	One-hot encoding, position-specific dinucleotide sequence, and K-nucleotide frequency encoding
	NmRF[168]	*H. sapiens and S. cerevisiae*	RF	Light gradient boosting-assisted and incremental feature selection
	Pichot *et* al's[169]	*H. sapiens*	RF	Sequence features, calculated RiboMethSeq scores and score-related values per position
Neural network (NN)	Deep-2'-O-Me[170]	*H. sapiens*	CNN	rna2vec
	iRNA-PseKNC[171]	*H. sapiens*	SVM + CNN	Nucleotide binary encoding
	DeepOMe[172]	*H. sapiens*	CNN + BLSTM	Nucleotide binary encoding
	BERT2OME[173]	*H. sapiens*	BERT + CNN	rna2vec

Table 5.

A comparison of nanopore-based tools for the detection and quantification of RNA modifications

Category	Tool	RNA modification	Analytic criteria	Methods for the model	>Dataset used	ROC score
Differential analysis	Tombo[199]	m^5C	Signal intensity	Kolmogorov-Smirnoff, Mann-Whitney, or Student's t-test	in vitro E. coli RNAs, or IVT	N/A
	ELIGOS[190]	m^6A	Base calling error	Fisher exact test + Benjamini-Hochberg correction	Curlcakes & IVT (in vitro transcribed) data	Variable
	DiffErr[191]	m^6A	Base calling error	G-test + Benjamini-Hochberg correction	Arabidopsis cells	N/A
	DRUMMER[192]	m^6A	Base calling error	G-test + Bonferroni correction	A549 cells	N/A
Bayesian approach	Yanocomp[193]	m^6A	Signal intensity	GMM	Arabidopsis cells	N/A
	xPore[194]	m^6A	Signal intensity	GMM	HEK293T cells	0.86
	Nanocompore[189]	m^6A	Signal intensity	Kolmogorov-Smirnov test or bivariate GMM with a logistic regression	MOLM13 cells	0.989–0.995
Machine learning	NanoNm[43]	Nm	Signal intensity	XGBoost	mRNA in Human cells, rRNA in human, Yeast and Fly	0.90–0.98
	nanoRMS[198]	Psi, Nm	Signal intensity	K-means/ KNN	Yeast strains	N/A
	MINES[187]	m^6A	Signal intensity	RF	HEK293T cells	0.54–0.76
	nanoDoc[195]	m^6A	Signal intensity	Deep One-Class model with transfer learning	E. coli & yeast rRNA	0.96
	Penguin[204]	psi	Signal intensity	SVM/RF/NN	HEK293 cells	0.85–0.95
	nanom6A[186]	m^6A	Signal intensity	XGBoost	Curlcakes data	0.97
	EpiNano-SVM[185]	m^6A	Base calling error	SVM	Curlcakes data	Variable
	mAFiA[196]	m^6A	Signal intensity	Linear logistic model	Synthesed short RNA oligos, Human Cells Arabidopsis	0.86–0.97
	Nm-nano[205]	Nm	Signal intensity	XGBoost + RF	*H. Sapiens* cells	0.89–0.932
Deep learning	DENA[188]	m^6A	Signal intensity	Bi-LSTM	Arabidopsis cells	0.90–0.97
	m6Anet[206]	m^6A	Signal intensity	Multiple Instance Learning	HCT116 cells, multiple species	0.83
	nano-ID[191]	5EU	Signal intensity	NN	K562 cells	0.94
	TandemMod[203]	Mutiple	Signal intensity	CNN + Bi-LSTM	Curlcakes data, multiple species	0.95–0.99
	CHEUI[202]	m^6A and m^5C	Signal intensity	CNN	Curlcakes data	0.92–0.95
	NanoSPA[201]	Psi and m^6A	Signal intensity, Base calling error	Feed forward neural network	Curlcakes data	0.97–0.99