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Review Paper

Deciphering RNA modifications at base resolution: from chemistry to biology

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Abstract

Nearly 200 distinct chemical modifications of RNAs have been discovered to date. Their analysis via direct methods has been possible in abundant RNA species, such as ribosomal, transfer or viral RNA, since several decades. However, their analysis in less abundant RNAs species, especially cellular messenger RNAs, was rendered possible only recently with the advent of high throughput sequencing techniques. Given the growing biomedical interest of the proteins that write, erase and read RNA modifications, ingenious new methods to enrich and identify RNA modifications at base resolution have been implemented, and more efforts are underway to render them more quantitative. Here, we review several crucial modification-specific (bio)chemical approaches and discuss their advantages and shortcomings for exploring the epitranscriptome.

Key words: epitranscriptome; RNA modifications; methylation; acetylation; method; sequencing

Introduction

The chemical diversity of RNA polymers is vastly expanded by more than 200 chemical modifications that can occur at the phosphate, ribose and nucleobases in living organisms [1]. These chemical tags fine-tune RNA–DNA, RNA–RNA and RNA–protein interactions, eventually affecting gene expression networks and cellular functions. Most of RNA modifications are incorporated during or after transcription and are often called post-transcriptional modifications. Borrowing from the language of epigenetics, and from the Ancient Greek *ἐπί* (epí, 'on top of'), RNA modifications are also called epitranscriptomic, to highlight that they carry another layer of information on top of the RNA sequence itself. In this review, we highlight methods developed to dissect this additional layer of information for a few base modifications with high biological impact.

Transfer RNAs (tRNAs) are the most chemically diverse RNA molecules. tRNAs have the advantage of being small (<100 nt), abundant in cells and carrying evolutionary conserved RNA modifications [2]. As a result, the study of tRNA modifications has driven forward technological advances in the last 6 decades. The state of the art for tRNA modification detection and quantification entails tRNA affinity purification with sequence specific probes, digestion with specific RNases and tandem mass spectrometry analysis [3]. Despite their abundance, full-length tRNAs remain difficult to sequence using RNA-seq techniques. The main culprit is the presence of specific tRNA modifications that create a roadblock for many commonly used reverse transcriptases (RTs) during the step of complementary DNA (cDNA) synthesis [4]. The two main strategies for overcoming this hurdle has been to either remove select tRNA modifications with appropriate eraser enzymes before RNA-seq library preparation [5, 6], or to use RTs, such as thermostable group II intron reverse transcriptases (TGIRTs) [7] that have the dual advantage of bypassing RNA modification roadblocks and of leaving a characteristic nucleotide misincorporation signature at RNA modification sites [8, 9].

Specific messenger RNAs (mRNAs) are significantly less abundant than specific tRNAs, making affinity purification

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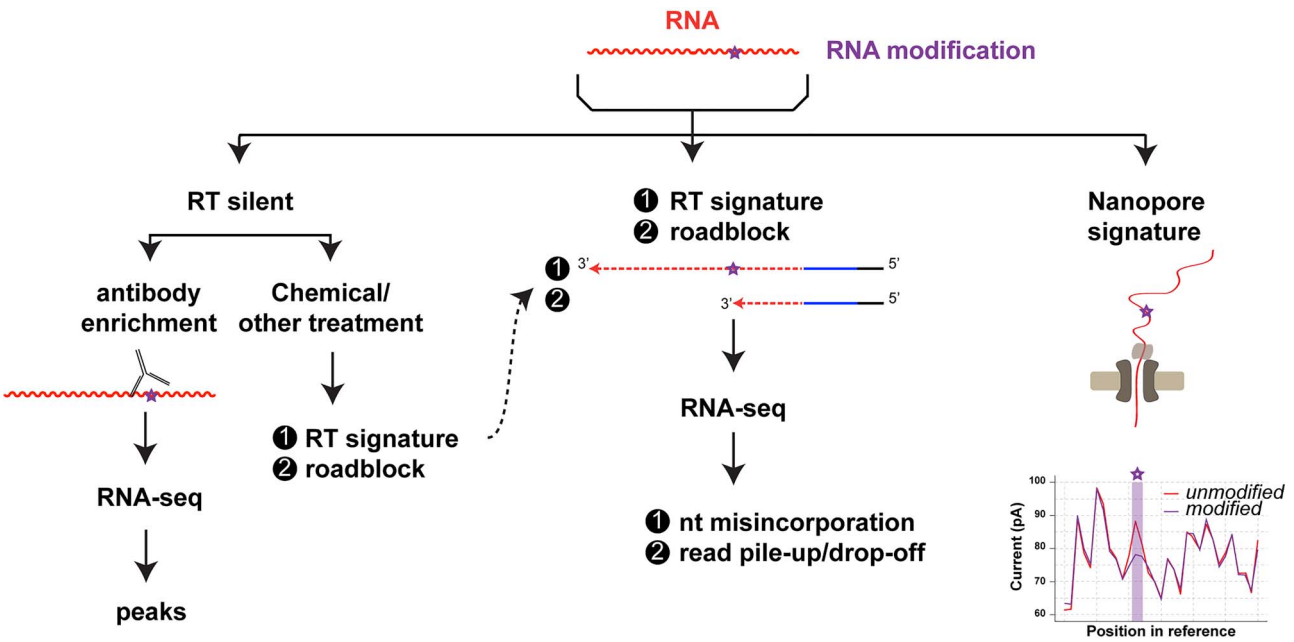

Figure 1. A given RNA modification may be detected by direct sequencing, such as the Oxford Nanopore Technology, or by SBS sequencing methods that involve an obligate RT into cDNA step. When the RNA modification does not change base pairing during RT (silent), the modification is enriched through specific antibodies, or 'unsilenced' through specific treatments. In the latter case, and when the RNA modification naturally changes the base pairing (RT signature) or creates a roadblock, it can inferred though mismatch to the reference or read pile-up or drop-off.

techniques coupled to tandem mass spectrometry unviable for detecting specific mRNA modifications at single nucleotide resolution level. Instead, modifications on specific mRNAs and other less abundant RNAs are most often inferred through either direct or indirect (sequencing-by-synthesis [SBS]) sequencing methods (Figure 1). Indeed, many RNA modifications are now detectable by a direct sequencing method from Oxford Nanopore Technology (Figure 1), which decodes changes to an electrical current as nucleic acids are passed through a protein nanopore to provide the specific RNA sequence [10]. While still in development, this technology is very promising for identifying RNA modifications and their combinations in intact RNA molecules, but due to space limitations, we will not review this method here, and refer the reader to an excellent review by Novoa *et al.* [10] as a starting point. In SBS methods, the RNA is first reverse-transcribed into cDNA with RTs prior to library amplification, and this step is crucial for detection of RNA modifications at the single nucleotide resolution level. RNA modifications that do not affect Watson–Crick base pairing are RT silent, i.e. they do not change the output sequence, while RNA modifications that change Watson–Crick base pairing can induce either a nucleotide misincorporation or an RT block at the RNA modification site during cDNA synthesis as mentioned above. In this case, the RNA modification site can be inferred from the mismatch to the reference sequence, or from read pile up/drop-off (Figure 1). RT silent modifications are most often enriched with specific antibodies prior to RNA-seq. While effective in identifying approximate RNA modification locations, such methods cannot precisely identify which sites in the RNA-seq peaks are modified, nor can they quantify the modification stoichiometry for each site. Remarkably however, RT silent RNA modifications can be 'unsilenced' through specific antibody crosslinking, chemical, editing or digestion methods to allow their identification at single-nucleotide resolution level (Figure 1).

N^6-Methyladenosine (m^6A)

Adenosine can be methylated on the exocyclic amino group ($-NH_2$) at the sixth position of the purine ring to form N^6-methyladenosine (m^6A). It is one of the most prevalent RNA modifications, especially in mRNA, where it is primarily written by METTL3/14 complex [11], and plays crucial roles in mRNA metabolism and associated cellular processes [12–14]. m^6A requires sophisticated methods for its site specific detection and quantitation, because it does not disrupt Watson–Crick base pairing and is silent during reverse transcription [15]. m^6A antibody-based enrichment and next-generation sequencing approaches (MeRIP-seq or m^6A-seq) were pioneered by Meyer *et al.* [16], and Dominissini *et al.* [17] in 2012, and have proved to be successful for transcriptome-wide m^6A mapping in many biological settings (reviewed in [18]). Another technique, termed site-specific cleavage and radioactive-labeling followed by ligation-assisted extraction and thin-layer chromatography (SCARLET) was developed by the Pan group and is useful for validating the precise location of the m^6A residue and its modification ratio one site at a time [19]. In addition, several metabolic tagging techniques using alkyne or allyl analogs of the methyl group donor S-Adenosyl-Methionine (SAM) that induce RT block [15] or misincorporation signature [20] have been reported. For example, the latter method uses Se-allyl-L-selenohomocysteine as SAM analog for in-cell experiments [20]. Once the allyl-handle is metabolically incorporated as N^6-allyladenosine at the position of m^6A by methyltransferases, through iodine induced cyclization, mismatch signatures at m^6A sites can be generated. The MAZTER-seq method takes advantage of the fact that the endoribonuclease mazF cuts immediately upstream of an ACA sequence but not (m^6A)CA [21], to detect and quantify the m^6A sites occurring within the (m^6A)CA motif. MAZTER-seq was successfully applied to profile and quantify m^6A at single-nucleotide resolution at 16–25%

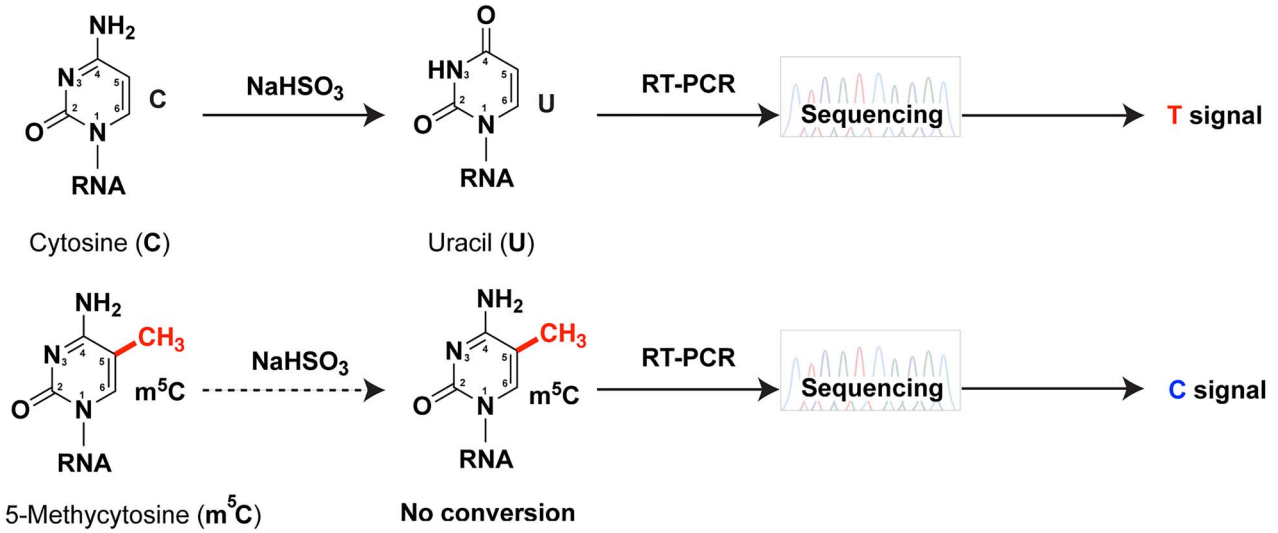

Figure 2. Chemistry behind bisulfite sequencing. Bisulfite deaminates cytosine (C) to uracil (U), giving rise to a T signal during sequencing. m^5C has an electron rich C5-C6 bond and is resistant to deamination, exhibiting a C signal.

of expressed sites in mRNA [22]. In another elegant method called DART-seq [23], Meyer fused the m^6A-binding domain YTH from the YTHDF2 m^6A reader to the deaminase domain (BE1) of APOBEC1 to induce a C to U editing in the invariable C residue of the m^6A writer consensus domain ($Gm^6AC \rightarrow Gm^6AU$) to enable antibody-free single nucleotide resolution m^6A mapping and quantitation. In [23], the cells were transiently transfected with an APOBEC1-YTH construct limiting its use to cell lines or model organisms engineered to express APOBEC1-YTH, however $Gm^6AC \rightarrow Gm^6AU$ editing post-RNA extraction with recombinant APOBEC1-YTH should be feasible and render m^6A mapping from low input samples—such as patient samples—finally possible.

5-Methylcytosine (m^5C)

Similar to DNA, RNA can be methylated on the carbon 5 of the pyrimidine ring of cytosines to form 5-methylcytosine or m^5C (Figure 2). m^5C is abundant in tRNA and rRNA, as well as in other RNA species, including mRNA where it may be the second most abundant base modification after m^6A [24]. Although RNA m^5C modification has been less studied than m^6A, it is clearly a very important modification carried out by eight different m^5C RNA methyltransferases (NSUN1-7, and TRMT1/DNMT2) in humans, most of which have important biological roles relevant to disease and cancer [25, 26]. m^5C is RT silent, but similarly to DNA, bisulfite treatment coupled to RNA-seq has been used to map m^5C at single nucleotide resolution [27–30]. Indeed, due to the electron rich C5-C6 double bond in the m^5C pyrimidine ring, bisulfite treatment converts C but not m^5C to U (Figure 2). Thus, upon bisulfite treatment, reverse transcription-polymerase chain reaction (RT-PCR) and sequencing, C gives rise to T, while m^5C stays C (Figure 2). However, bisulfite reaction suffers from RNA degradation and incomplete C to U conversion, especially in double stranded or highly structured regions (i.e. G-C clamps) [31], limiting m^5C mapping to abundant RNAs. Other methods have been developed to improve the sensitivity of m^5C detection. For example, in the Aza-IP method developed by Khoddami and Cairns [32], cells are metabolically labeled with the 5-azacytidine (5-azaC) C analog, which randomly gets incorporated into RNA instead of C. When this occurs at a target m^5C RNA site, a covalent bond (C–S) forms between the C6 position of the base and the sulfur atom of a cysteine residue in the catalytic pocket of specific RNA methyltransferases [32]. An alternative, UV-crosslink-free version of iCLIP named miCLIP (methylation-individual nucleotide resolution crosslinking and immmunoprecipitation) was developed by the Frye lab to identify NSUN2 m^5C RNA methyltransferase targets [33]. In miCLIP, NSUN2 is engineered to not release NSUN2 RNA targets due to irreversible covalent bond formation between the NSUN2-C271A mutant and the RNA catalytic intermediate. Thus, NSUN2 target RNAs could be enriched and identified through IP of the protein–RNA complex and RNA-seq of the recovered target RNAs [33]. Osmium reaction-based tagging of m^5C followed by liquid chromatography coupled to mass spectrometry (LC–MS) has also been reported [34, 35]. It takes advantage of the C5-methyl group that increases electron density along the C5=C6 double bond in m^5C and forms a stable m^5C–Os–ligand ternary complex (ligand=bipyridine). However, it suffers from structural problems and high reactivity toward m^5U (T) [34, 35].

Pseudouridine (Ψ)

Pseudouridine or Ψ is a highly abundant RNA modification catalyzed by a large number of Ψ-synthases (PUS, 13 different enzymes in humans), which break the nitrogen-carbon glycosidic bond between the ribose and U, rotate the base by 180° and form a carbon–carbon glycosidic bond instead [36–39]. This isomerization allows for an additional hydrogen bond donor in Ψ compared to U contributing to better thermodynamic (structural) stability and base-stacking [36–39]. Ψ detection is problematic for two reasons; firstly, it is an RT silent modification, and secondly, it is also a mass spectrometry silent modification due to Ψ having the same mass as U. [39]. However Ψ can be 'unsilenced' by chemical labeling with carbodiimides or carbodiimide derivatives that enable its detection by mismatch signatures during sequencing [39–43] or by using several tag-free or tag-on mass spectrometry based approaches [44–46] (Figure 3). Interestingly, a recent report also indicated base-skipping/deletion at/near the position of Ψ during bisulfite sequencing [27].

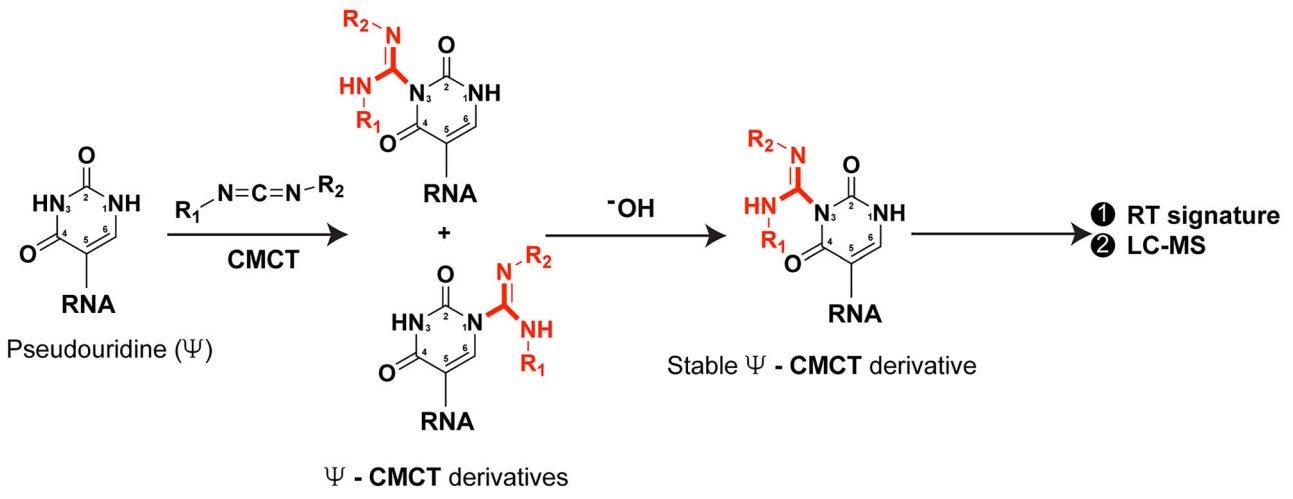

Figure 3. Labeling of Pseudouridine (Ψ) by carbodiimide CMCT (N-cyclohexyl-N′-β-(4-methylmorpholinium) ethylcarbodiimide p-tosylate) reagent. CMCT forms bulky derivatives which upon alkaline hydrolysis give rise to a single derivative exhibiting distinct RT signature. Using this chemistry, Ψ can be identified by sequencing and LC–MS. (R_1 = cyclohexyl group, R_2 = ethyl-4-methylmorpholinium group).

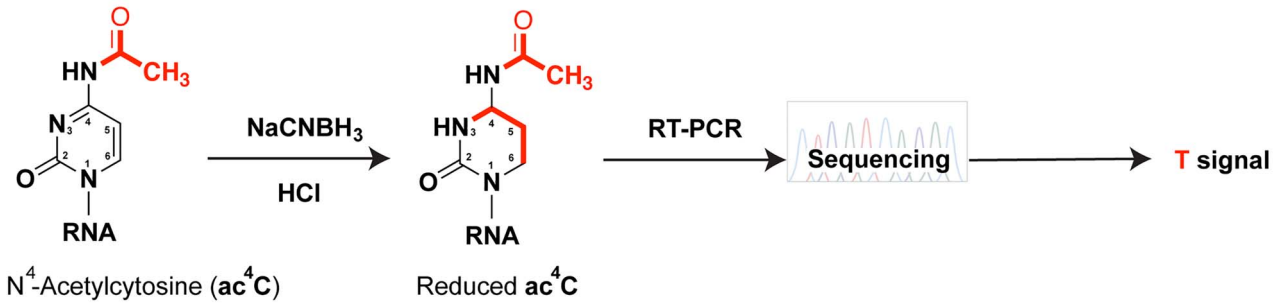

Figure 4. Sodium cyanoborohydride ($NaCNBH_3$) mediated reduction of ac^4C. In acidic media, the hydride reduces ac^4C to N^4-acetyltetrahydrocytidine, which generates a T signal instead of a C signal during sequencing.

N^4-acetylcytosine (ac^4C)

Cytosines can undergo acetylation on the exocyclic amino group ($-NH_2$) at the fourth position of the pyrimidine ring to form ac^4C in a reaction requiring ATP, Acetyl-CoA, the NAT10 acetyltransferase [47], and in specific cases the U13 snoRNA or the THUMPD1 adaptor protein [48]. Ac^4C stabilizes G–C interactions and fine-tunes mRNA translation efficiency and stability [49]. Ac^4C is also RT silent, and recently, two different approaches attempted to reveal the ac^4C epitranscriptome in human cell lines. The ac^4C antibody-based acRIP-seq approach identified numerous ac^4C sites in mRNA [49]. The other approach exploited the fact that sodium borohydride ($NaBH_4$) or sodium cyanoborohydride ($NaCNBH_3$) reduces ac^4C to give rise to T upon RT-PCR, whereas unmodified C remains C [50] (Figure 4). The sodium cyanoborohydride treatment coupled to RNA-seq mapped ac^4C in ribosomal RNA and a few tRNAs in human cells, but only mapped ac^4C to a few mRNAs—only when both NAT10 and THUMPD1 were overexpressed [51]. It was suggested that the ac^4C antibody suffers from non-specificity, however the sites of ac^4C identified by acRIP-seq disappeared in HeLa-NAT10-KO cells [49]. Thus, it is likely that the discrepancies between the two approaches come from yet to be identified differences, including differences in cell lines growth conditions and/or ratio of modification. This discrepancy could be solved by coupling the acRIP-seq to sodium cyanoborohydride treatment, which could concomitantly enrich the ac^4C modified RNAs and directly test the specificity of the antibody.

2′-O-methylation (Nm)

2′-O-methylation, i.e. methylation of the 2′-OH group in ribose, is one of the most common modifications observed in almost every class of RNAs, including mRNAs and piRNAs [52, 53]. Please note that the whole nucleotide is referred to as Nm, where N is any base. 2′-O-methylation disrupts hydrogen bonds due to the increase in lipophilic surface and thus has significant effects including stabilization of helical structures, protection of RNA from various endonucleases, or fine-tuning of RNA-protein interactions [52]. Due to the presence of 2′-OMe group, Nm is resistant to alkaline hydrolysis (Figure 5A) or periodate oxidation (Figure 5B). These properties have been coupled to RNA-seq to map Nm sites in transcriptome-wide scale [54–57]. However, given that Nm mapping relies on read pile-up or drop-off at the Nm site, instead of misincorporation, the confidence in the identified sites is weakened, while the rate of Nm modification is difficult to estimate. For example, Nm-seq identified 7412 sites in non-ribosomal RNAs species from HEK293 cells, with a consensus sequence of 10 nt present in 33% of all sites [57]. However, close inspection of this consensus sequence revealed that it is nearly identical to the 3′ adaptor used in the Nm-seq library, suggesting that a third of identified Nm sites may be due to a commonly observed mispriming artifact [58]. In spite of this, the rest of the reads may represent *bona fide* Nm sites, highlighting the need for more robust methods for systematic detection and validation of Nm sites. Indeed, internal Nm modifications may have important roles in gene expression regulation, as suggested

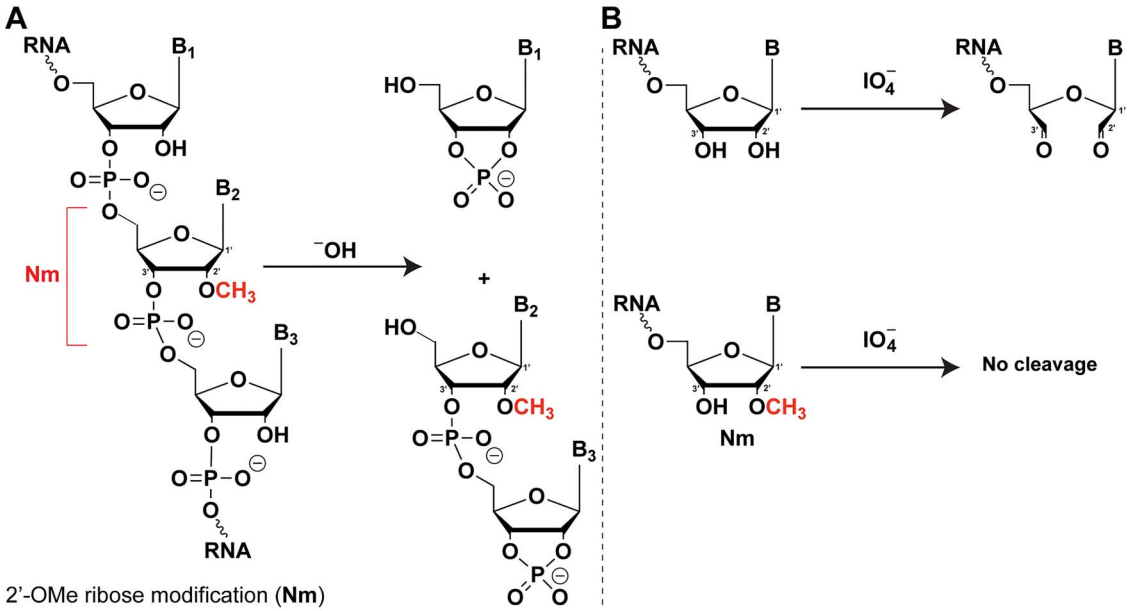

Figure 5. **(A)** Detection of Nm (2′-O-Me) by alkaline hydrolysis. Due to the presence of 2′-OH, RNA gets hydrolyzed in basic conditions, while 2′-OMe containing nucleotide is resistant to cleavage. **(B)** Detection of Nm (2′-O-Me) by periodate based oxidation. Periodate is capable of oxidizing 1,2-diol (2′,3′-OH groups), but not Nm due to the presence of 2′-OMe group.

by a recent study showing that similar to rRNA, Fibrillarin can be guided by snoRNAs U32A and U51 to modify a specific residue within the protein-coding region of peroxidasin to enhance the stability, but inhibit the translation of the mRNA into protein [59].

7-methylguanosine (m^7G)

Guanines can be methylated at the N7-position of the purine ring (m^7G) inducing a positive charge. The most widely known function of m^7G modification is related to it being an obligate feature of eukaryotic mRNA caps [60]. However, methylation of m^7G by METTL1 was recently mapped in internal sites in other RNA species [61–65] by exploiting an observation in the 1970's that sodium borohydride efficiently reduces m^7G in basic conditions, and the reduced base is prone to forming an abasic site in acidic conditions [66, 67] (Figure 6). Since the abasic site consists of an equilibrating mixture of the ring-closed acetal (major) and the ring-opened aldehyde (minor), it can be covalently bound with hydrazine probes (e.g. biotin-hydrazine) for enrichment and detection purposes [61–65]. Moreover, given that m^7G sites can be directly traced by the abasic site signatures generated during cDNA synthesis and sequencing [61–64], it is paramount to inspect that the sequence of the RNAs enriched by this method contain these abasic site signatures, in order to distinguish background RNAs from RNAs with *bona fide* m^7G sites.

N^1-methyladenosine (m^1A)

N^1-methyladenosine is a major modification primarily observed in conserved sites in tRNA and rRNA [68]. The m^1A nucleoside is positively charged and able of disrupting Watson–Crick base pairing. Consequently, m^1A creates RT roadblock and/or misincorporation signatures in ratios that are specific of the adjoining nucleotides and used reverse transcriptases [69–71]. These properties have been exploited to map m^1A at single nucleotide resolution in RNA-seq experiments performed with or without pre-enrichment with an m^1A antibody [5–9, 69–76]. The stoichiometry of m^1A modification in tRNA and rRNA is high, leading to excellent concordance of sites detected by mass spectrometry and RNA-seq [69–71], however the extent of m^1A in cytoplasmic mRNA has been debated [75], going from a dozen, to hundreds or thousands of sites identified [69–71, 74, 76]. While most of the cited publications agree that the m^1A antibody enriches sequences mapping to the 5′ ends of mRNAs, they diverge in the number of 'called' m^1A sites. This may be due to a combination of issues, including m^1A antibody specificity, accuracy of read mapping, and the ratio of misincorporation by RTs within the m^1A-antibody enriched sequences [75]. At least two publications have reported that the ratio of misincorporation is not linearly correlated to the ratio of m^1A modifications when using either TGIRT [69] or an RT specifically evolved from the human immunodeficiency virus RT catalytic domain (p66) to increase m^1A readthrough and misincorporation rate [71]. This problem may be the primary reason why, apart from a few overlapping called m^1A sites among datasets, there is variability in the numbers and identity of 'called' m^1A sites even within the same cell line. The case of m^1A exemplifies the difficulty of accurately identifying low stoichiometry RNA modification sites in RNA even in the advantageous situation of a modification that is not RT silent. Going forward, as previously proposed [18], evolution of new RTs with improved readthrough and higher misincorporation rate as in the elegant study from the He and Dickinson labs [71], should solve many of the issues related to the bioinformatic analysis of misincorporation signatures for detecting RNA modifications with low stoichiometry [77].

Remarks

Numerous (bio)chemical methods have been reported to date to identify/quantify a growing spectrum of RNA modifications. Here we described several methods targeting crucial modifications such as N^6-methyladenosine (m^6A), 5-methylcytosine (m^5C), pseudouridine (Ψ), N^4-acetylcytosine (ac^4C), 7-methylguanosine (m^7G), N^1-methyladenosine (m^1A) etc.

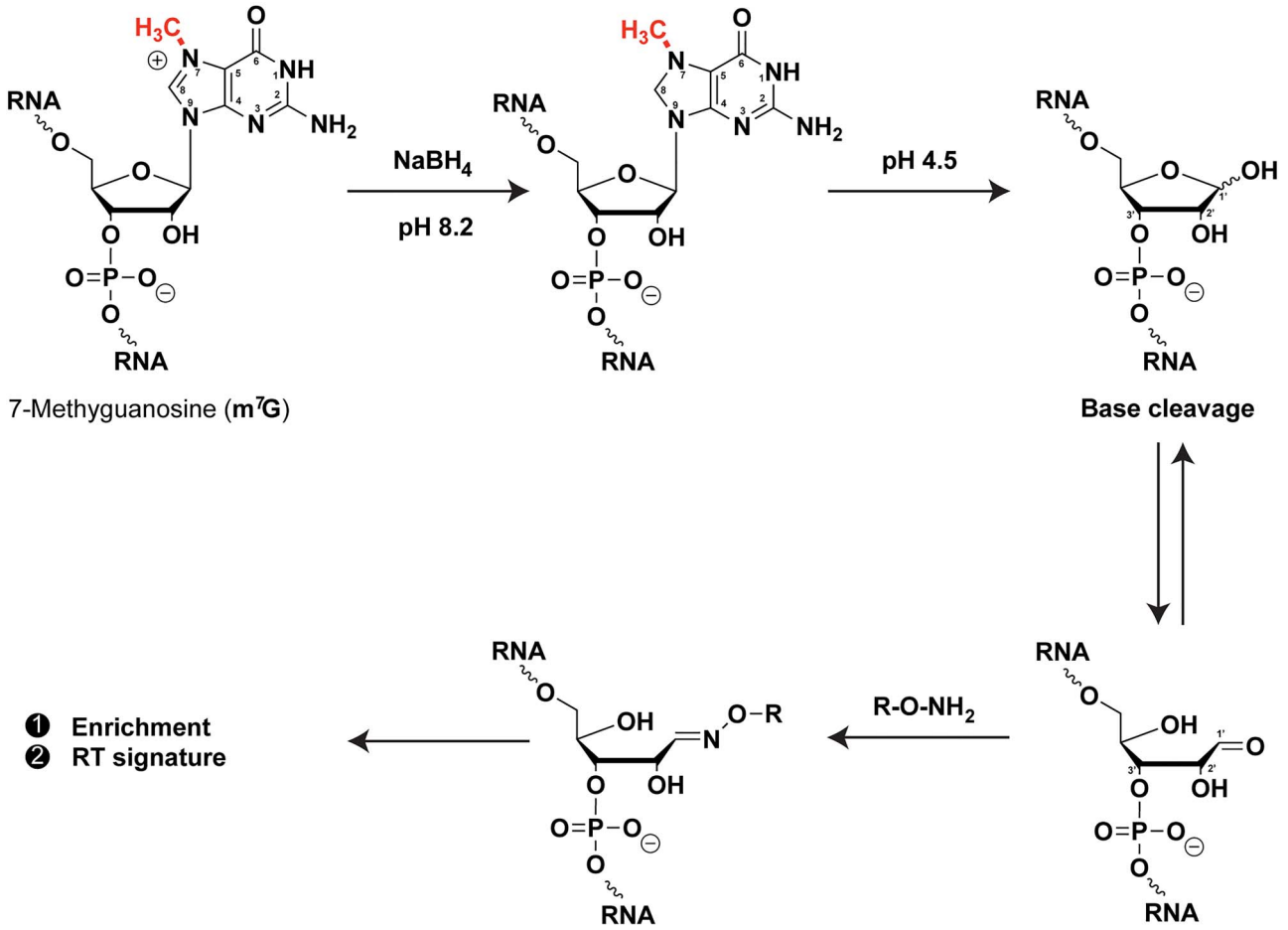

Figure 6. Sodium borohydride ($NaBH_4$) mediated detection of m^7G. $NaBH_4$ reduces m^7G and the reduced base is prone to cleavage forming an abasic site in acidic media. Abasic sites can be covalently linked with hydrazine probes (e.g. biotin-hydrazine) for enrichment and detection purposes. R = linker containing biotin moiety.

Antibody-based techniques require high input of RNA and often show background/non-specificity during enrichment. Thus, multiple controls and/or orthogonal approaches should be used to correlate/verify the results whenever possible. One should keep in mind this drawback before using any modification-specific antibody techniques.

LC–MS based methods are very sensitive for detecting modification. But impurity present in RNA samples or degraded RNA might give rise to 'false positive' results. Moreover, for low abundance RNAs (e.g. mRNA or miRNA), mass spectrometry dependent methods can be difficult to perform.

Reproducibility of results using available methods might vary from person to person (or lab to lab) depending on multiple crucial factors such as pH, reagent concentration, reaction/incubation time, solubility, salt concentration, temperature and RT used. RNA modifications are sensitive to environmental effects, e.g. heat shock, oxidative/reductive stress. Therefore, handing cells/tissues with proper care and proper experimental settings are extremely important to obtain reproducible results. Notably, epitranscriptomic profile or RNA modification levels may largely be context-specific and thus vary from one cell line to another cell line and from one organism to another.

Many of the techniques described here have been crucial for deciphering the roles of RNA modifiers in cellular processes involved in normal development and disease. However, many are laborious and often suffer from non-specificity, RNA degradation, background and structural problems, or inappropriate bioinformatic analysis. Moreover, RNA-friendly chemical reactions might require proper optimization to gain better target selectivity and yield. Thus, more extensive research, and more sensitive and accurate methods are still required to explore in depth the epitranscriptome and the panoply of proteins that write, read and erase them. This is paramount to elucidating their roles in disease and cancer, with the ultimate goal of targeting them for therapeutic benefit.

Summary points

- Detection at single nucleotide resolution level is paramount to uncover the molecular mechanism of action of RNA modifications.
- RNA modifications in rare RNAs are inferred through either direct or indirect sequencing methods.
- RNA modifications that affect base pairing can induce reverse transcription (RT) roadblock or nucleotide misincorporation, thus enabling detection at single nucleotide resolution.
- RNA modifications that do not affect base pairing are called RT silent, but can be 'unsilenced' through specific chemical or biochemical treatments to induce RT roadblock or nucleotide misincorporation.

Funding

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