

Detection of N⁶-methyladenosine modification residues (Review)

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Abstract. Among a number of mRNA modifications, N⁶-methyladenosine (m⁶A) modification is the most common type in eukaryotes and nuclear-replicating viruses. m⁶A has a significant role in numerous cancer types, including leukemia, brain tumors, liver cancer, breast cancer and lung cancer. Although m⁶A methyltransferases are essential during RNA modifications, the biological functions of m⁶A and the underlying mechanisms remain to be fully elucidated, predominantly due to the limited detection methods for m⁶A. In the present review, the currently available m⁶A detection methods and the respective scope of their applications are presented to facilitate the further investigation of the roles of m⁶A in biological process.

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1. Introduction

Gene regulation via DNA and protein modifications has been intensively studied. However, the knowledge on RNA modifications remains insufficient (1). Among all mRNA modifications, N⁶-methyladenosine (m⁶A) modification is the most common type in eukaryotic RNA and occurs in three to five sites per transcript on average (2-4).

m⁶A was discovered in the 1970s in a wide range of cellular mRNAs (4-6). Methylation occurs at the sixth position of nitrogen atoms of adenosine at the post-transcriptional level with S-adenosylmethionine serving as the methyl donor for m⁶A formation, which is termed m⁶A modification (7-9).

The reversible activity of the m⁶A modification is regulated by the combined action of methylase and demethylase (10). The m⁶A methylase complex consists of at least five 'writer' proteins (11-14), among which methyltransferase like 3 (METTL3) protein serves a central role. METTL14 protein supports METTL3 protein structurally (15), WT1-associated protein (WTAP) regulates the recruitment of the m⁶A methyltransferase complex to mRNA targets (12) and the RNA-binding motif protein 15 serves a role in helping this complex move towards the appropriate m⁶A sites (16). Vir like m⁶A methyltransferase associated (VIRMA) is also a component of this m⁶A methylase complex; however, its molecular function remains largely unknown (13). METTL16 is a newly discovered m⁶A methyltransferase that primarily methylates m⁶A sites in the 3'-untranslated region (3'-UTR) of RNA. When METTL16 is knocked down, the level of m⁶A in the cell decreases by ~20% (17). Two reported demethylases that reverse m⁶A modification are FTO (18) and alkB homolog 5 (ALKBH5) (19). It has been confirmed that m⁶A levels increased following the knockdown of FTO and ALKBH5 expression (18,19). The identified 'readers' of m⁶A are YT521-B homology (YTH) domain-containing proteins, including YTHDF1-3, YTHDC1 and YTHDC2, which participate in the translation (20), stabilization (21), splicing (22) or nuclear export (23) of mRNA. The affinity of 'readers' has been reported to be higher compared with unmethylated mRNA for m⁶A-methylated mRNA (24).

m⁶A bases cannot be detected directly by sequencing because the m⁶A modification does not change the base pairing properties and cannot be distinguished from regular bases by reverse transcription (25,26). Previously, new methods have

been developed to identify m⁶A modification in cells, which are based on immunoprecipitation or selective RNA chemistry to isolate modified RNA fragments and combined with high-throughput sequencing (3,25,27). Dot blot technology is frequently used to observe changes in m⁶A. However, dot blots cannot determine the quantitation and precise location of m⁶A (28). RNA photo-crosslinkers, quantitative proteomics and electrochemical immunosensor methods may be applied to detect the presence of m⁶A in cells; however, they cannot precisely determine the m⁶A modification sites (29,30). The newly developed methylated RNA immunoprecipitation sequencing (MeRIP-seq) method combines m⁶A antibody immunoprecipitation and deep sequencing to identify the m⁶A residues in 100-200 nucleotide RNA segments (3,25). However, this approach is complicated as m⁶A often appears in clusters since multiple different m⁶A-containing fragments generate overlapping reads, which can result in large peaks spanning several m⁶A residues (2). Thus, the summit of these peaks may not accurately reflect the positions of m⁶A residues. By contrast, when using the m⁶A individual-nucleotide-resolution cross-linking and immunoprecipitation (miCLIP) technique, identification of m⁶A residues is not influenced by the peak shapes (31). Furthermore, identification of m⁶A residues by miCLIP is not restricted to a specified subset of DRACH (D=A, G) motifs (2,3,13). Therefore, miCLIP can properly identify m⁶A residues (31).

High-performance liquid chromatography (HPLC) or mass spectrometry are applied in some of the abovementioned methods to detect specific modified RNA or RNA bases (3,29). Isolated RNA is fragmented into nucleosides prior to analysis, which may change the original RNA structure. Dot blot technology, which does not require fragmentation, is used as an alternative method to detect specific modifications. However, dot blot technology is limited in that RNA samples cannot be separated by the size, and hence, it is impossible to distinguish the targeted specific RNA in the RNA samples (32,33). Notably, HPLC can be used alone to measure m⁶A modification rate (18).

The abovementioned methods and specific references are provided in Table I (18,26,27,29-51). In the present review, the most recent information on the currently established m⁶A detection methods is presented and discussed.

2. Functions of m⁶A modifications

m⁶A is the most common type of RNA residue modification in eukaryotes (2-4). However, its specific biological roles remain largely unknown. Studies have indicated that the dynamic regulation of m⁶A has a significant impact on the control of gene expression (20,21). m⁶A-seq has revealed that m⁶A predominantly exists on exons and the 3'-UTR of mRNA (2). METTL3, FTO and ALKBH5 have been identified to serve significant roles in biological regulation, including development, metabolism and fertility (18,19).

FTO, an m⁶A demethylase, is recognized as a major obesity factor. It belongs to the ALKB enzyme family, which oxidatively demethylates m⁶A on mRNA (32,52). ALKBH5 is another mammalian m⁶A demethylase, and is associated with certain mRNA-processing factors in nuclear speckles, which may influence RNA export and metabolism (19).

Table I. Methods for detecting m⁶A residues.

Method	(Refs.)
Semi-quantitative	
Dot blot technology	(32,33)
Methyl sensitivity of MazF RNA endonucleases	(34)
Immune-northern blot	(35,36)
Quantitative	
RNA photo-crosslinkers and quantitative proteomics	(29)
Electrochemical immunosensor method	(30)
Support vector machine-based method	(37)
Detection of precise locations	
HRM	(38)
LAIC	(27,39)
SCARLET	(39-41)
MeRIP-seq	(42-46)
MiClip	(31,39,47)
DNA polymerase for direct m ⁶ A sequencing	(26,48)
HPLC	(18,49-51)

m⁶A, N⁶-methyladenosine; miCLIP, m⁶A individual-nucleotide-resolution cross-linking and immunoprecipitation; HRM, high-resolution melting analysis; SCARLET, site-specific cleavage and radioactive labeling followed by ligation-assisted extraction and thin layer chromatography; LAIC, level and isoform characterization; MeRIP-seq, methylated RNA immunoprecipitation sequencing; MiClip, m⁶A individual-nucleotide-resolution cross-linking and immunoprecipitation; HPLC, high-performance liquid chromatography.

ALKBH5-deficient mice have differential expression of genes involved in the p53 signaling pathway and spermatogenesis (19), suggesting a global role for m⁶A in human health. METTL3 is expressed in all human tissues and is highly expressed in the testis (10,11). It has been identified that METTL3 can participate in tumor growth and progression by regulating the cell cycle of cancer cells (53,54). WTAP was primarily recognized as a protein associated with Wilms' tumor and has been indicated to exhibit a significant role in cell cycle progression (12). WTAP also participates in RNA splicing and results in embryonic defects (12). Cooperation between the RNA m⁶A methyltransferase complex and the demethylases establishes a reversible regulation of RNA m⁶A modifications. Taken together, RNA m⁶A modifications exhibit significant roles in the molecular mechanisms of gene biology, as illustrated in Fig. 1.

3. Methods used to analyze m⁶A modifications

Dot blot. Dot blot (or slot blot) technology is used in molecular biology as a semiquantitative or quantitative detection method for DNA, RNA and protein samples, and is predominantly used semiquantitatively in m⁶A analysis (32,33,55-57). Compared with western blotting, northern blotting or Southern blotting methods, dot blot technology has a simpler operation with a similar working principle. The biomolecules are not subjected

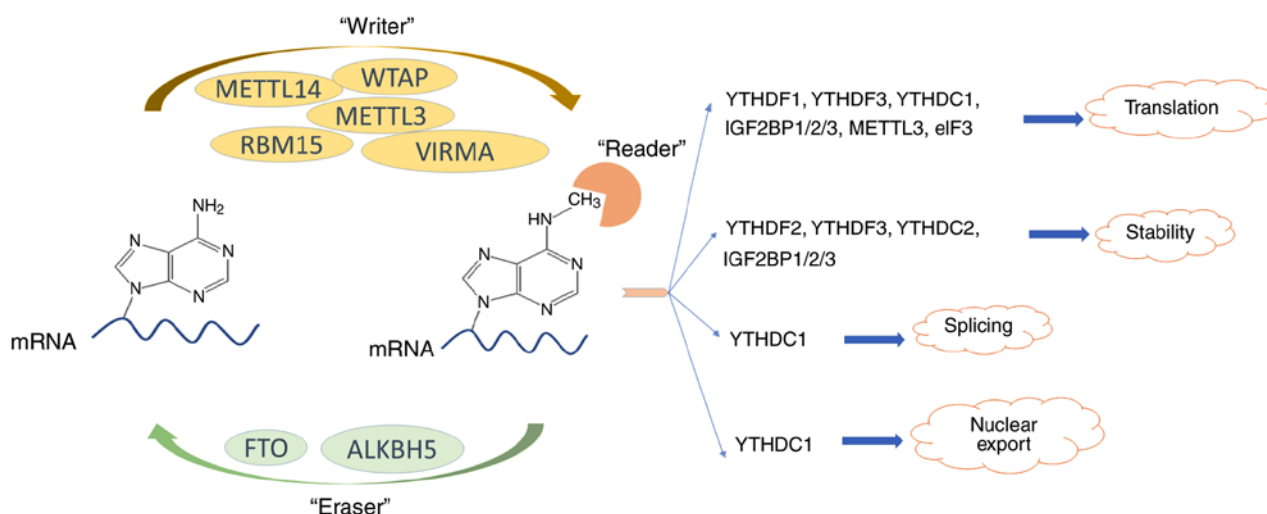


Figure 1. Roles of the N⁶-methyladenosine Writer, Eraser and Reader complexes in regulating mRNA. RNA m⁶A modifications serve important roles in the molecular mechanisms of gene biology. METTL, methyltransferase like; WTAP, WT1-associated protein; VIRMA, Vir like m⁶A methyltransferase associated; RBM15, RNA binding motif protein 15; YTHD, YT521-B homology; IGF2BP1/2/3, insulin like growth factor 2 mRNA binding protein 1/2/3; eIF3, E74-like factor 3.

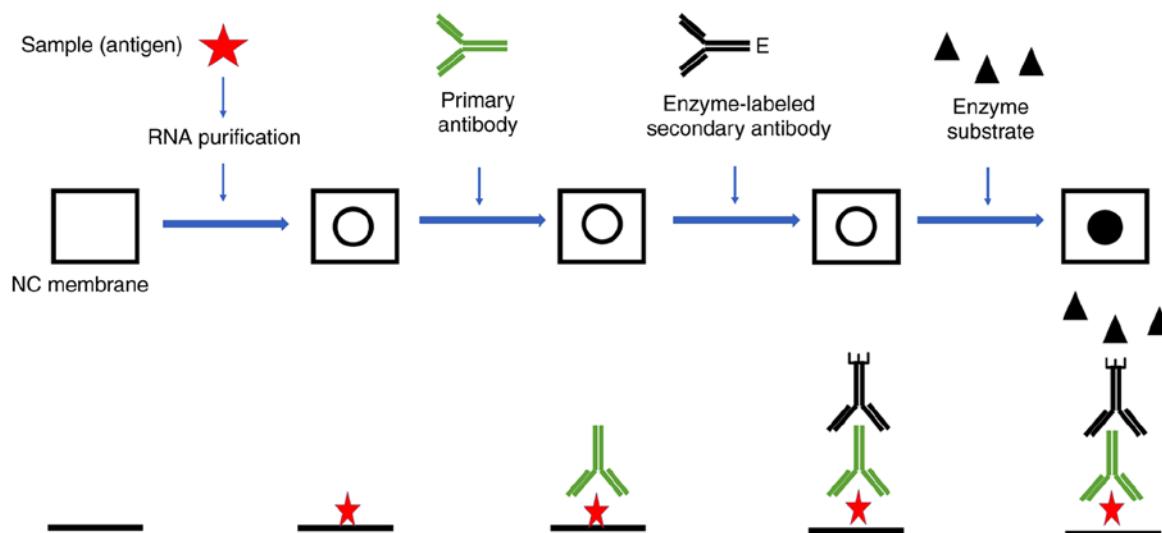


Figure 2. Schematic diagram of dot blot technology. Samples containing a mixture of RNAs are directly applied to a NC membrane. The membrane is then dried and detected with antibodies. The filled circle represents a detected signal. NC, nitrocellulose filter.

to electrophoretic separation prior to detection with the dot blot method. Instead, samples containing a mixture of RNAs are directly applied to a membrane through an apparatus with circular templates that form a dot when the sample is applied. After applying a vacuum to embed RNAs and dry the membrane, biomolecules are detected with antibodies. When detecting RNA, the DNA must be removed prior to loading to eliminate its influence on m⁶A (32,33). A schematic diagram of the determination of m⁶A modification residues by dot blot is presented in Fig. 2.

Dot blot technology markedly saves time, since it does not require chromatography, gel electrophoresis or complex gel blocking procedures (32,33). However, regarding m⁶A detection, dot blot technology is only able to verify the presence of m⁶A or compare the amounts of m⁶A between different groups (32,33). Li *et al* (32) improved the traditional dot blot

method to measure the global m⁶A abundance in the transcripts of four acute myeloid leukemia cell lines. However, this method is still not able to quantitate or precisely determine the location of m⁶A.

Methyl-sensitive MazF RNA endonucleases. The regulatory enzymes of m⁶A have been reported to contribute to tumorigenesis (58-60). While the significance of m⁶A has been confirmed, no convenient approach has been developed to analyze m⁶A methyltransferase and demethylase activities or to monitor the inhibitors of these activities.

The *Escherichia coli* toxin MazF exhibits endoribonuclease activity specific against ACA sequences and is susceptible to m⁶A. MazF is the first enzyme discovered with the ability to specifically cleave RNA containing m⁶A, and is used to study activities of m⁶A demethylase and methyltransferase (34).

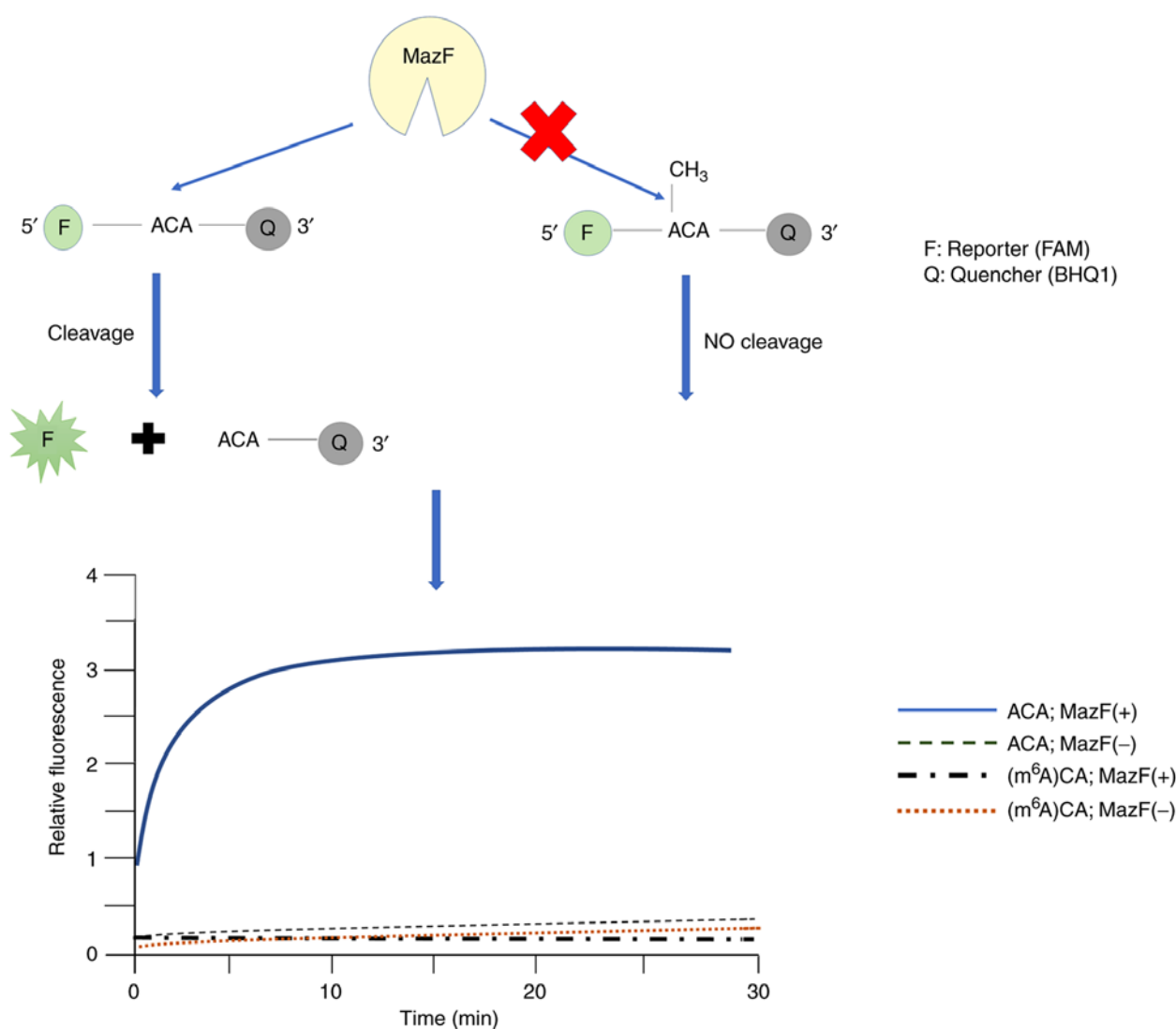


Figure 3. Schematic diagram of fluorescence resonance electron transfer-based MazF assays. RNAs are cleaved by MazF at the 5'-ACA-3' site in single-stranded RNAs and detected by fluorescence assay. m⁶A, N⁶-methyladenosine; A, adenine; C, cytosine; BHQ1, black hole quencher-1.

Furthermore, MazF has an application for monitoring the inhibitors of m⁶A methyltransferase and demethylase. RNA cleavage by MazF may be detected by polyacrylamide gel electrophoresis and the fluorescence-resonance energy transfer-based plate assay (61). A schematic diagram of the determination of m⁶A modification residues by methyl-sensitive MazF RNA endonucleases is presented in Fig. 3.

At the current stage of development, the MazF cleavage methods are restricted regarding the evaluation of m⁶A, as MazF is only able to cleave the 5'-ACA-3' site in single-stranded RNA, frequently occurring in endogenous RNAs. However, MazF is not able to cleave the 5'-ACA-3' site in double-stranded RNA (61), and thus, it may not accurately determine the presence of m⁶A in structured RNA.

Immuno-northern blot. The immuno-northern blot combines a northern blotting experimental program and m⁶A-binding antibody, which is different from conventional northern blot techniques that use DNA probes. Immuno-northern blot does not require RNA fragmentation prior to analysis and the RNAs are separated based on their molecular weights (35). Thus, the

detection of m⁶A modifications by immuno-northern blot is applied in various types of RNA (35).

In brief, RNAs are separated in a denaturing acrylamide gel or an agarose gel and then transferred onto nylon membranes. The RNA strands on the membranes are exposed to ultraviolet (UV) irradiation for cross-linking, followed with incubation with primary antibodies against m⁶A modifications, corresponding secondary antibody and chemiluminescent detection (35).

4. Quantification of m⁶A modifications

RNA photo-crosslinkers and quantitative proteomics. The regulatory role of mRNA predominantly depends on the interaction between mRNA and RNA-binding proteins to regulate RNA splicing, stability, localization and translation (62). Photo-crosslinking technologies are diffusely applied to stabilize direct protein-RNA interactions (63). These technologies depend on the tendency for UV-induced photochemistry of nucleobases, which are natural or derivatives containing sulfur or halogen substituents. Photo-affinity labels, including

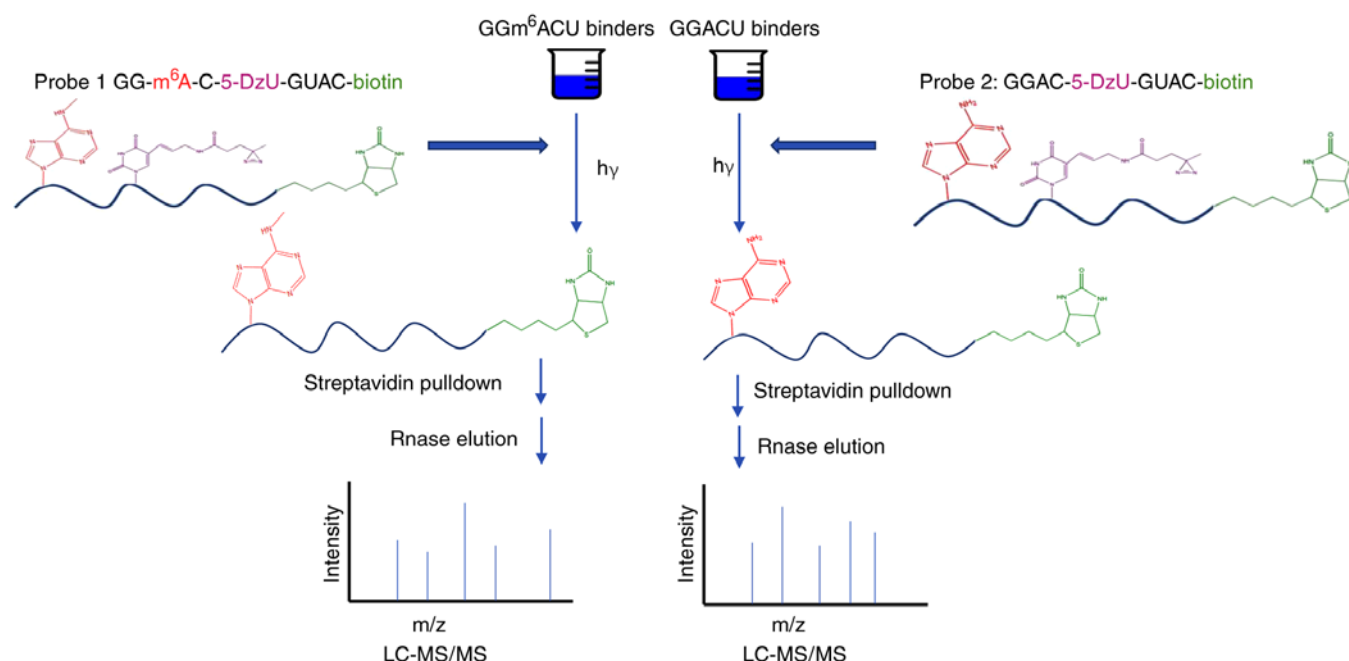


Figure 4. Schematic diagram of RNA photo-crosslinkers and quantitative proteomics. RNA probes are required to contain the following: i) m⁶A molecules; ii) a photo-crosslinker that is efficient and does not influence the protein-RNA interactions; and iii) streptavidin as an affinity handle for protein enrichment. m⁶A, N⁶-methyladenosine; LC-MS/MS, liquid chromatography-mass spectrometry/mass spectrometry; hv, irradiation with light.

diazirine (64) or benzophenone (65), are not widely used in the analysis of protein-RNA interactions; however, they may be activated by longer wavelengths and provide more efficient crosslinking.

Arguello *et al* (29) developed a chemical proteomics approach based on photo-crosslinking of the RNA base and diazirine, which was highly efficient in quantitatively analyzing protein-RNA interactions regulated by m⁶A modification. By using this method, novel m⁶A 'readers' have been discovered. To isolate m⁶A readers with photosynthetic and quantitative proteomics, RNA probes are required to contain the following: i) m⁶A molecules; ii) a photo-crosslinker that is efficient and does not influence the protein-RNA interactions; iii) streptavidin as an affinity handle for protein enrichment. A probe was prepared that contains the sequence GGm⁶ACU, the common recognition pattern of the m⁶A site in mammalian cells. This sequence is indispensable for binding the YTH-domain proteins (66,67). The probe was validated by known m⁶A RNA 'readers', including YTHDF1 (20), YTHDF2 (21), YTHDF3 (3,22,68) and YTHDC1 (22). A schematic diagram of the detection of m⁶A modification residues by the RNA photo-crosslinkers and quantitative proteomics technologies is presented in Fig. 4. We hypothesize that the requirement of the synthesis of the sensor may be a limitation regarding these techniques.

Electrochemical immunosensor method. The majority of the abovementioned analytic methods are difficult to perform and expensive. The electrochemical immunosensor method was developed to provide convenience and high sensitivity (30). An anti-m⁶A antibody has been used to detect m⁶A by targeting m⁶A-5'-triphosphate (m⁶ATP). The detection and capture of m⁶A relies on silver nanoparticles and SiO₂ (Ag@SiO₂) nanospheres with amine-polyethylene glycol 3-biotin. Ag@SiO₂

nanospheres were prepared to amplify signals. Phos-tag-biotin was prepared to link m⁶ATP and Ag@SiO₂ through two types of specific interaction between phosphate group of m⁶ATP and phos-tag, biotin and streptavidin, respectively. Experiments for evaluating this strategy indicated that the immunosensor has acceptable reproducibility and specificity with a wide linear range and a low detection limit. A schematic diagram for the determination of m⁶A modification residues by the electrochemical immunosensor method is presented in Fig. 5.

The efficacy of the detection of the m⁶A content using the electrochemical immunosensor method had been verified in human cell lines (30). It also provides a technological basis for the detection of RNAs and DNAs with the advantages of convenience, low cost, and high specificity and sensitivity.

Vector method to detect m⁶A sites. High-throughput next-generation sequencing-based technology for identifying m⁶A sites based on where adenosine is methylated has not been applied in most species. In recent years, a vector machine method was developed to identify m⁶A sites in *Arabidopsis thaliana* (37). When combining anti-m⁶A antibodies and high-throughput sequencing, Luo *et al* (69) obtained thousands of m⁶A peaks for *A. thaliana*, including 'common' m⁶A peaks. Since the RRACH motif, where R resembles purine, A stands for m⁶A and H resembles a non-guanine base (69), was identified in most of the m⁶A peaks, Chen *et al* (37) collected segments containing RRACH at the center of the 'common' m⁶A peaks and proposed a model that may accurately identify specific m⁶A sites with high accuracy.

If the model is adapted to other plant species other than *A. thaliana*, this vector machine-based method may be used for the detection of m⁶A and other post-transcriptional modifications in these other plants as well.

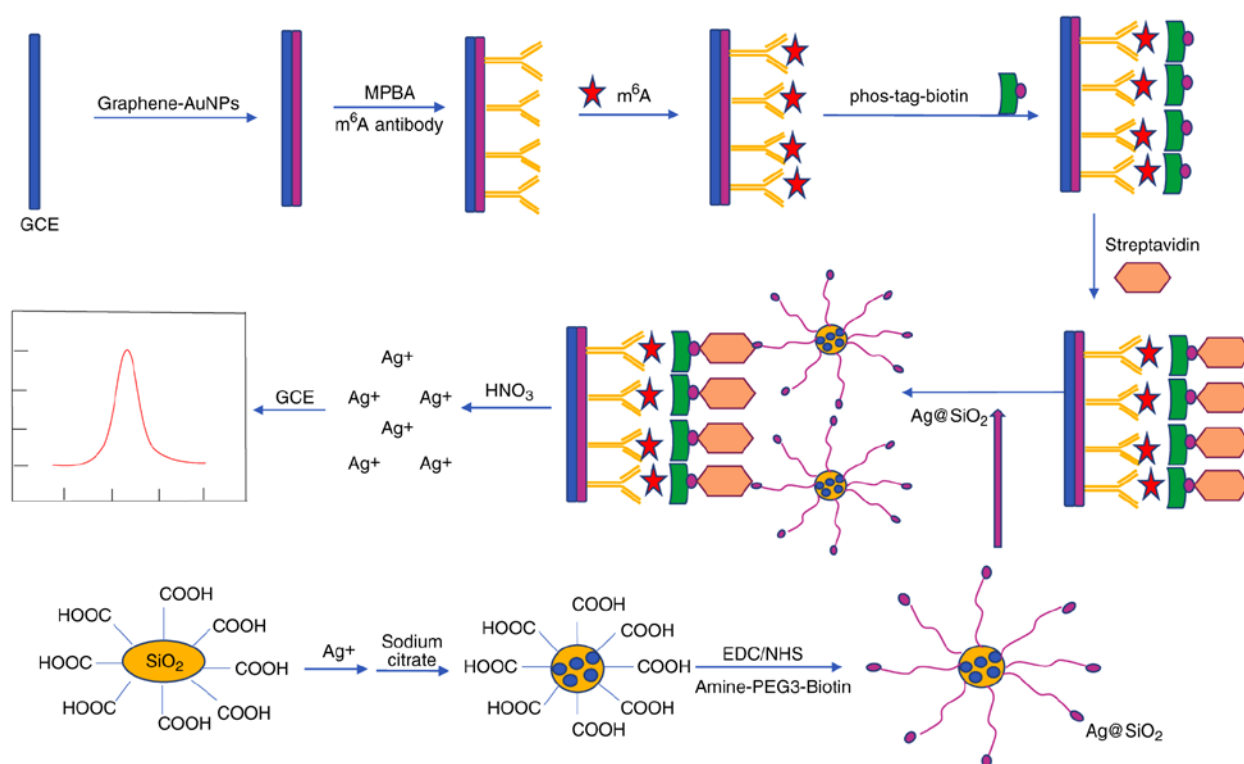


Figure 5. Schematic diagram of the electrochemical immunosensor method. Ag@SiO₂ nanospheres are prepared to amplify signals. Phos-tag-biotin is prepared to link m⁶ATP and Ag@SiO₂ through two types of specific interaction between the phosphate group of m⁶ATP and phos-tag, biotin and streptavidin. m⁶A, N⁶-methyladenosine; Ag@SiO₂, silver nanoparticles and SiO₂; GCE, glassy carbon electrode; MPBA, P-mercaptobenzenboric acid; EDC, 1-ethyl-3-(3'-dimethylaminopropyl) carbodiimide; NHS, N-hydroxysuccinimide.

5. Methods to determine m⁶A residue locations

HRM. HRM analysis is a simple method to detect m⁶A modification residues at a specific location in RNAs (38). HRM may be applied to high-throughput measurement. The resulting HRM curves of the samples of RNA mixtures change steadily from 100% of methylated RNA to 100% of unmethylated RNA (38). As presented in Fig. 6, the detection of m⁶A modification residues by the HRM method relies on the modified nucleoside position at a particular site of RNA and is followed by rapid screening for conditions or genes necessary for analysis of that modification (38).

According to the specificity of the oligonucleotide probe hybridization, bulk cellular RNA, as opposed to purified specific RNA, has been designed for detecting m⁶A at a pre-defined position (38). In addition, partial non-ribosomal target enrichments may be easily accomplished using commercially available kits.

A possible application for this method would be to screen knockout/knockdown strain libraries to identify genes contributing to the formation of a specific m⁶A nucleoside. Another possible application is to detect the presence of a particular m⁶A nucleoside under different growth or environmental conditions (2,3). HRM analysis may help to elucidate the dynamic events that result in the modification of certain RNAs.

m⁶A level and isoform-characterization sequencing (m⁶A-LAIC-seq). For thorough investigation of the m⁶A epitranscriptome, Molinie *et al* (27) invented the m⁶A-LAIC-seq

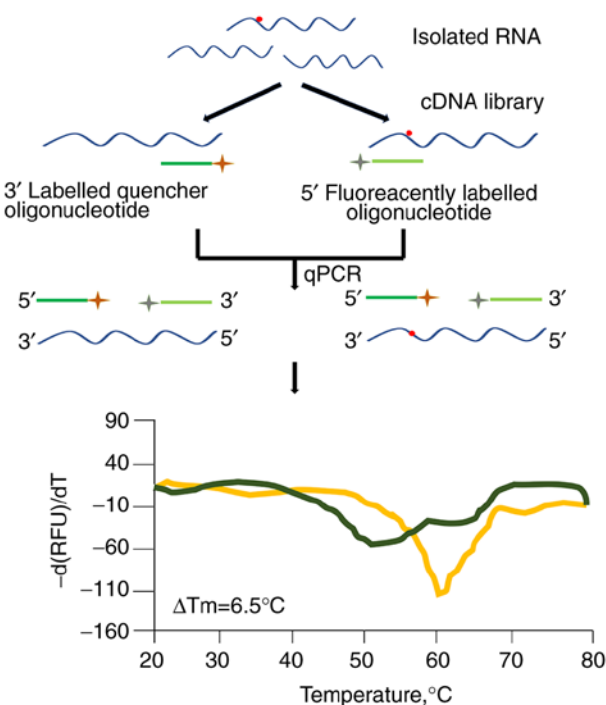


Figure 6. Schematic diagram of high-resolution melting analysis. The green and orange curves corresponded to the different types of strains (wild-type or gene mutation, respectively). qPCR, quantitative polymerase chain reaction; cDNA, complementary DNA; T_m, melting temperature.

technique. Combined with RNA IP whole-transcriptome sequencing, m⁶A-LAIC-seq may quantify m⁶A contents,

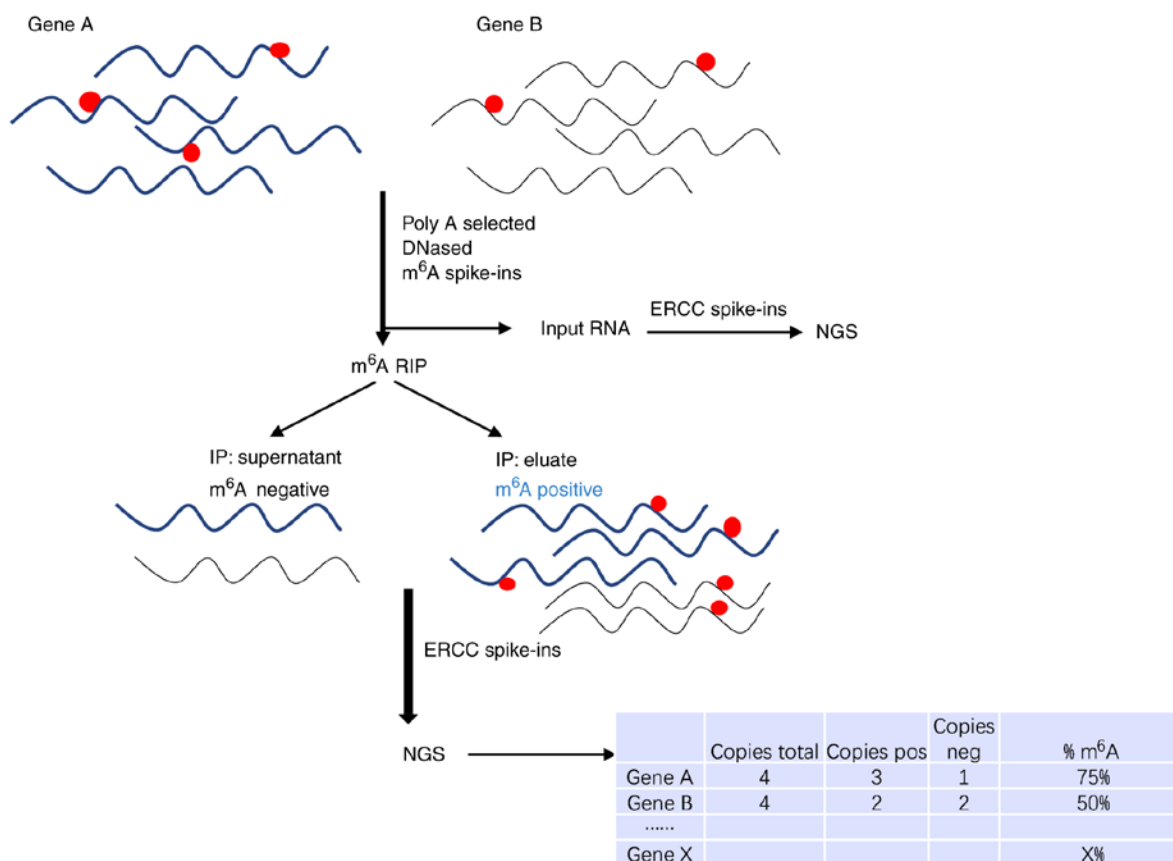


Figure 7. Schematic diagram of m⁶A-level and isoform-characterization sequencing. Poly A RNAs bind with anti-m⁶A antibody. A vast excess of antibody, as a second round of anti-m⁶A RIP on the m⁶A-negative fraction recovers no further RNA. m⁶A levels per gene are quantified by the ratio of RNA abundance as follows: Eluate/(eluate + supernatant). m⁶A, N⁶-methyladenosine; poly A, polyadenylated; IP, immunoprecipitation; RIP, radioimmunoprecipitation; NGS, next-generation sequencing; ERCC, External RNA Controls Consortium; pos, positive; neg, negative.

with spike-in RNAs as an internal standard. A schematic diagram for the determination of m⁶A modification residues by m⁶A-LAIC-seq is presented in Fig. 7. The results demonstrate a quantitative road map to which genes are the most or the least likely to be influenced by m⁶A-dependent regulatory networks. This method may determine the m⁶A levels in each gene but cannot stoichiometrically analyze the methylation of a single modified nucleotide. m⁶A-LAIC-seq complements m⁶A-seq identification of methylation sites and helps to expand the understanding of the biology of m⁶A.

Site-specific cleavage and radioactive labeling followed by ligation-assisted extraction and thin-layer chromatography (SCARLET). To elucidate the dynamic biological functions of m⁶A, Liu *et al* (40) provided the SCARLET method that directly measures the precise location and the status of m⁶A modification at any candidate site of mRNA/lncRNA at a single nucleotide resolution. In addition to m⁶A, SCARLET may be used to observe other RNA modifications, including 5-methylcytosine, pseudouridine and 2'-O-methyl ribonucleosides. SCARLET is available to study the biological functions of RNA modifications with general experimental equipment and materials. A schematic diagram for the determination of m⁶A modification residues by SCARLET is presented in Fig. 8.

The feasibility of using the SCARLET method has been confirmed in HeLa cell RNA samples, which produced similar

results compared with previously reported m⁶A sites in HeLa samples. Using this method, a minimally modified m⁶A site that was not precisely determined in a preceding study was also identified, demonstrating that SCARLET is able to easily resolve the ambiguity of modification sites (70).

MeRIP-Seq. Although RNA methylation has been identified and verified in the 1970s, the relevant modification mechanism, regulatory means and biological significance have not been clarified due to technical limitations. The recent emergence of MeRIP-Seq technology makes it possible to study m⁶A methylation at the transcriptome level by high-throughput sequencing (2,3).

MeRIP-Seq is a combination of ChIP-Seq and RNA-Seq that is able to elucidate global mRNA m⁶A sites in mammalian cells. MeRIP-seq is a novel type of IP-seq technology, in which the known ChIP-seq, photoactivatable ribonucleoside-enhanced crosslinking and IP (a more mature sequence), is applied. MeRIP-seq has been successfully used to detect whole-genome m⁶A modifications (42-46). The major principle of this technique is as follows: The anti-m⁶A antibody is incubated with randomly interrupted RNA fragments using a co-IP method resulting in an m⁶A modification fragment that is precipitated and sequenced. Concurrently, a control sample is run that eliminates the background during antibody capture (2).

Successful preparations of each library should be evaluated prior to massive parallel sequencing. To identify and localize the m⁶A sites at a transcriptome-wide level by m⁶A IP,

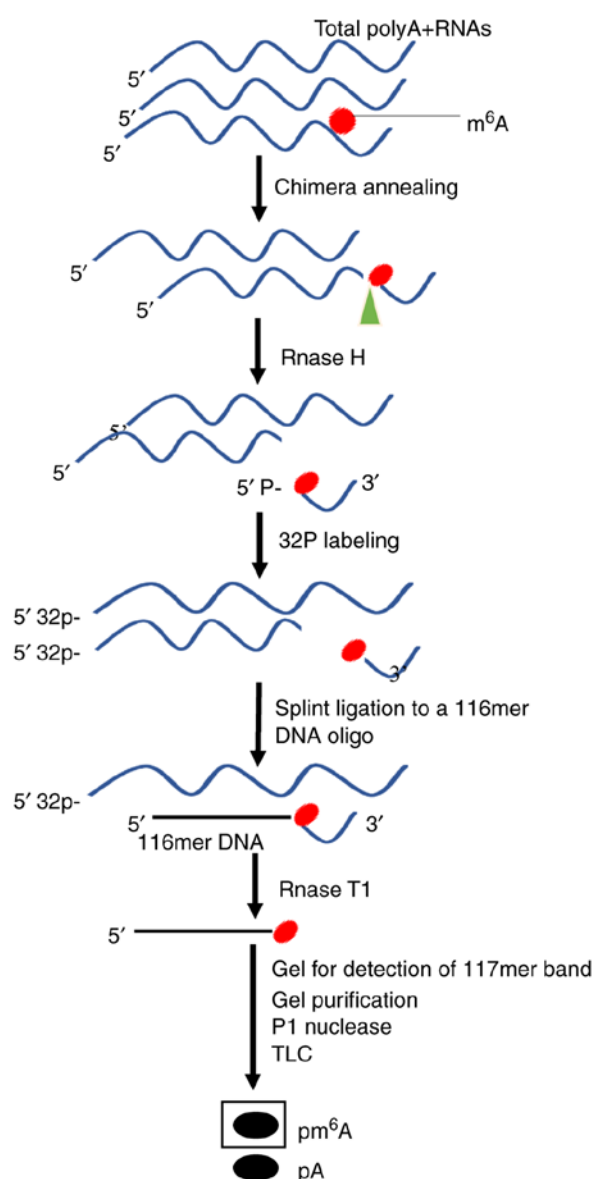


Figure 8. Schematic diagram of the site-specific cleavage and radioactive labeling followed by ligation-assisted extraction and thin-layer chromatography technique. m⁶A, N⁶-methyladenosine; TLC, thin layer chromatography; polyA, polyadenylated; pm⁶A, 32P-labeled N⁶-methyladenosine.

fragmentation of poly(A)⁺-selected RNAs (input) is required prior to IP with anti-m⁶A antibodies. The input and m⁶A IP RNAs are then separately processed for next-generation sequencing (2). Zhang *et al* (48) first proposed a Bayesian statistical model, BaySeqPeak, to analyze MeRIP-Seq data to help discover methylation site signals in the transcriptome. A reference transcriptome was prepared, which contains a single, non-intron splice variant of each gene. The resultant reads were then specifically compared with the reference to identify m⁶A sites with a low false-detection rate (3). A schematic diagram for the determination of m⁶A modification residues by MeRIP-Seq is presented in Fig. 9.

MiClip. It has been reported that m⁶A residues may be located by producing unique signature mutations with anti-m⁶A antibodies and UV crosslinking techniques (31). m⁶A residues were mapped with two antibodies, one of which translates C

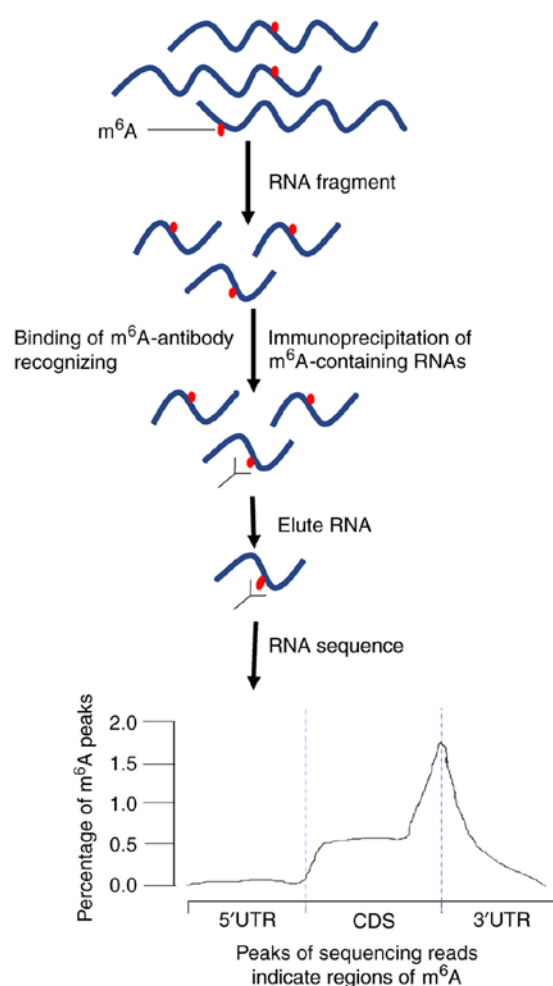


Figure 9. Schematic diagram of m⁶A/methylated RNA immunoprecipitation sequencing. Fragmentation of poly(A)⁺-selected RNAs (input) is required prior to IP with anti-m⁶A antibodies. The input and m⁶A IP RNAs are then separately processed for next-generation sequencing. m⁶A, N⁶-methyladenosine; UTR, untranslated region; IP, immunoprecipitation.

to T to detect single and clustered m⁶A residues, and the other antibody that produces truncations is used to determine the position of m⁶A sites and detect m⁶A residues concurrently. A schematic diagram for the determination of m⁶A modification residues by miClip is provided in Fig. 10.

Identification of m⁶A residues by direct detection is superior to that by bioinformatics predictions from MeRIP-Seq peaks (31). The reliability of bioinformatics prediction depends on the characteristics of the m⁶A peak. MeRIP-Seq can accurately predict m⁶A residues only with a single clear peak of a single m⁶A residue and is limited to the centrally-located DRACH motif (31). m⁶A residues usually cluster in mRNAs, resulting in multiple MeRIP-Seq peaks (2). Identification of m⁶A residues using miCLIP technology is not influenced by peak shapes and not confined to the centrally-located DRACH motif. Therefore, miCLIP can correctly identify m⁶A residues.

A DNA polymerase for direct m⁶A sequencing. At present, RNA samples are prepared by antibody-based enrichment of m⁶A residues prior to sequencing, as m⁶A modifications are usually lost after reverse transcription (25,26). The indirect detection may lead to a higher error rate, pushing the

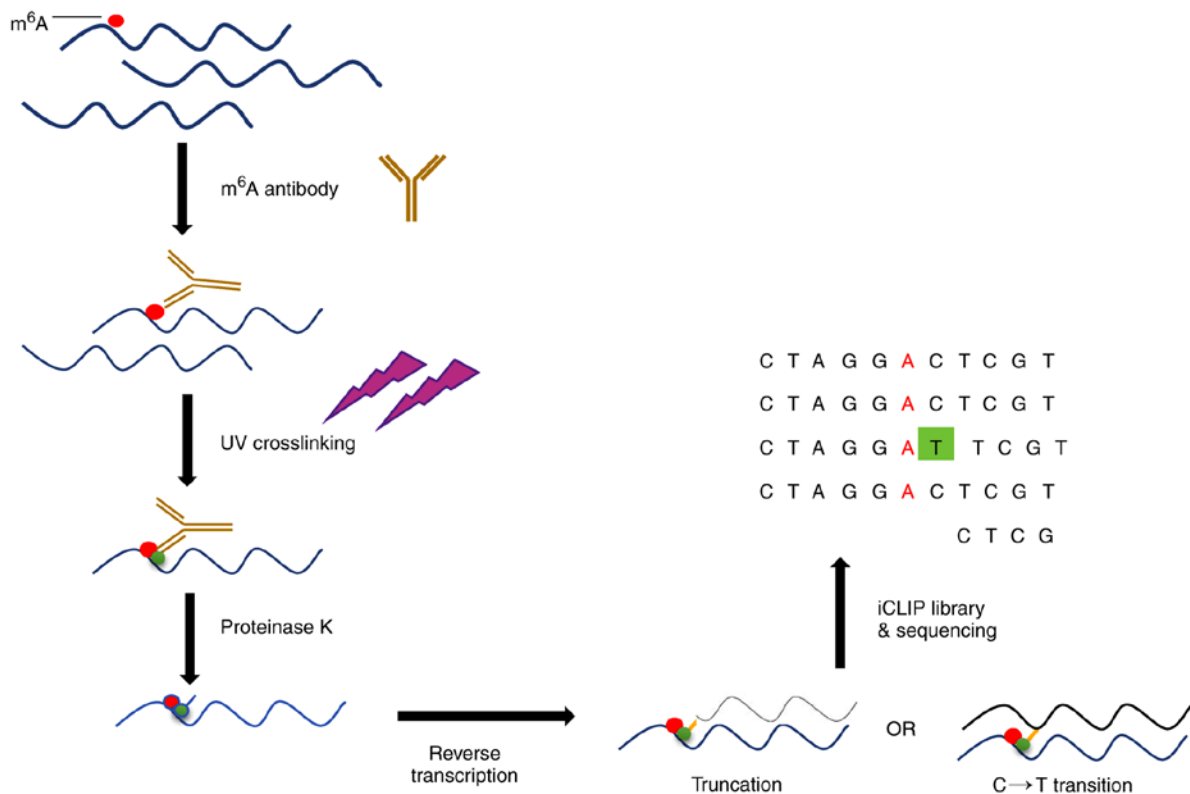


Figure 10. Schematic diagram of miCLIP technology. m⁶A residues can be mapped by generating unique signature mutations with m⁶A-specific antibodies and UV crosslinking, followed by analysis by sequencing. miCLIP, N⁶-methyladenosine individual-nucleotide-resolution cross-linking and immunoprecipitation; UV, ultraviolet; m⁶A, N⁶-methyladenosine; iCLIP, individual nucleotide resolution CLIP.

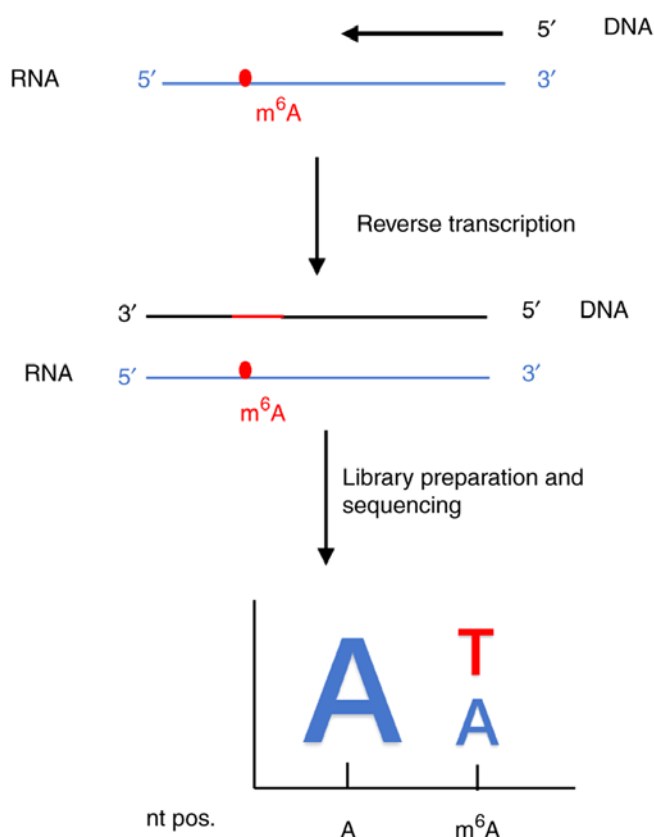


Figure 11. Schematic diagram of a DNA polymerase for direct m⁶A sequencing. This is based on the reverse transcriptase variants exhibiting RT-signatures as a response to encountering a specific RNA modification. m⁶A, N⁶-methyladenosine; nt pos, nucleotide position; RT, reverse transcription.

generation of novel DNA polymerase to sequence m⁶A directly (26). In this light, Aschenbrenner *et al* (26) developed a screening method to develop a reverse transcription-active KlenTaq DNA polymerase variant for labeling N⁶-methylation residues. A schematic diagram for the determination of m⁶A modification residues by the DNA polymerase for direct N⁶-methyladenosine sequencing is presented in Fig. 11.

HPLC. The development of HPLC occurred only 30 years ago; however, the development of this separation analysis technology is very rapid, and is now widely used, including in the detection of m⁶A modification (50). HPLC was firstly used to detect m⁶A modification in nine DNAs (50). Subsequently, Rana and Tuck (49) applied HPLC in the detection of m⁶A modification in a T7 RNA transcript coding for mouse dihydrofolate reductase by separating m⁶A from adenine, cytosine, uracil and so on. In a study by Jia *et al* (18), HPLC helped observe the changes of the m⁶A ratio in mRNAs following FTO treatment to better understand the function of FTO. The development of this *in vitro* methylation assay opened the door for studies investigating m⁶A levels in specific mRNAs or learn the biological significance of m⁶A modification.

6. Discussion

Detection of RNA modifications and study of their functions are emerging fields of research. The potential role of m⁶A modifications in regulating molecular and physiological processes in several organisms, particularly in RNA stability, splicing, transport, localization and translation, is valued (19,20,52,21,70-73).

With the discovery of 'reader' proteins, the downstream molecular mechanisms of m⁶A modifications are gradually being clarified (21). There is strong evidence that m⁶A methylation is associated with RNA splicing and that 'readers' of m⁶A reduce the stability of RNA transcripts (21). After removal of m⁶A, a second class of proteins may bind to the RNAs, which may be affected by changes of the RNA secondary structure from m⁶A additions (74). Thus, the physiological effects of m⁶A modifications should be observed at multiple levels, including the tissue level, the pathway level, the cellular level and the molecular level (75). An increasing number of studies have suggested a link between m⁶A RNA modifications with cancer and other similar disease-associated processes (32,53,54,73,74-76). Detection of m⁶A modification *in vitro* can help identify the precise regulatory forms and synergistic roles of m⁶A modifications in cancer and other diseases (32,53,54,74-76). Certain m⁶A methylases, m⁶A demethylases or downstream genes have become prognostic factors for different cancer types (77-79). Certain abovementioned methods, including MeRIP-seq, could help identify the downstream genes and mutation sites (54,80). Therefore, detection of them *in vitro* may help diagnose and predict the final progress. The present review provides an overview of these methods to drive the elucidation of the biological roles of m⁶A and encourage their further development.

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Availability of data and materials

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Authors' contributions

JWe and HY designed the study and revised the article. WZ and JWa reviewed the literature and wrote the article. ZX, MC, QH, CP and MG reviewed the literature and revised the article. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no conflict of interests.

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