1 **Antibody-free enzyme-assisted chemical labeling for detection of** 2 **transcriptome-wide N^6 -methyladenosine**

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9 **Abstract**

10 The inert chemical property of RNA modification N^6 -methyladenosine (m^6A) makes it very
11 challenging to detect, and all of the transcriptome-wide m^6A detection methods rely on m^6A -
12 antibody immunoprecipitation. However, their results are dependent on the quality and
13 specificity of antibodies. Although the endoribonuclease-based single-base m^6A sequencing is
14 antibody-free, it maps only 16~25% sites. Here, we present an antibody-free, FTO-assisted
15 chemical labeling method termed m^6A -SEAL for m^6A detection. We applied m^6A -SEAL to
16 profile m^6A landscapes in human and plant, which had good overlaps with antibody-based
17 results and displayed the known m^6A distribution features in transcriptome. Comparison with
18 all available m^6A sequencing methods and specific m^6A sites validation by SELECT, we
19 demonstrated that m^6A -SEAL has good sensitivity, specificity, and reliability for
20 transcriptome-wide detection of m^6A . Given its tagging ability and FTO's oxidation property,
21 m^6A -SEAL enables many applications like enrichment, imaging, and sequencing techniques to
22 drive future functional studies of m^6A and other modifications.

23 **Introduction**

24

25 *N*⁶-methyladenosine (m⁶A) is the most prevalent chemical modification in mRNA and lncRNA
 26 in eukaryotes¹. This epitranscriptomic mark has essential regulatory functions in RNA
 27 processing and metabolism, and so-called writer, eraser, and reader proteins for the mark have
 28 been identified. m⁶A is installed by an m⁶A writer complex—the core subunits have been
 29 identified as METTL3, METTL14, and WTAP in human^{2,3}—and is erased by the AlkB family
 30 dioxygenases (e.g., FTO and ALKBH5 in human)^{4,5}. m⁶A is read by m⁶A-binding proteins, and
 31 such reading has been shown to affect RNA processing in a way that regulates cell physiology<sup>6-
 32 11</sup>. Moreover, aberrant m⁶A methylation has been associated with various diseases¹²⁻¹⁵.
 33 Therefore, mapping of the transcriptomic distribution of m⁶A can deepen our basic
 34 understanding of epitranscriptomic metabolism on cellular physiology and can potentially
 35 define novel targets for fighting diseases.

36 Due to the inert reactivity of the methyl group of m⁶A, transcriptome-wide m⁶A detection
 37 methods must rely on m⁶A-antibody immunoprecipitation (m⁶A-IP). m⁶A-seq (also termed
 38 MeRIP-seq) is the first developed and presently most widely used method for m⁶A
 39 detection^{16,17}: it combines m⁶A-IP of fragment mRNAs with high-throughput sequencing to
 40 locate m⁶A sites within RNA segments of approximately 200 nucleotides. More recently
 41 developed m⁶A detection methods have adapted protocol advantage steps from cross-linking
 42 immunoprecipitation RNA target identification methods (e.g., iCLIP and PAR-CLIP) to
 43 develop the miCLIP (or m⁶A-CLIP) and PA-m⁶A-seq methods¹⁸⁻²⁰, which both can map m⁶A
 44 marks at higher resolution than m⁶A-seq based on antibody crosslinking (via either UV or 4-
 45 thiouridine). Another alternative m⁶A detection method is called m⁶A-LAIC-seq²¹; this relies
 46 on m⁶A-IP of full-length poly(A)⁺ RNA and quantifies methylated transcripts vs. non-
 47 methylated transcripts in the immunoprecipitated RNA population. More recently, an antibody-
 48 free m⁶A-seq method (m⁶A-REF-seq and MAZTER-seq) using RNA endoribonuclease MazF
 49 drew single-base resolution m⁶ACA map on transcriptome; but it can only identify 16~25% of
 50 m⁶A sites^{22,23}. Notably, most of these methods are dependent on the specificity of anti-m⁶A
 51 antibodies, thus emphasizing the strong desirability of developing transcriptome-wide and
 52 antibody-free methods to facilitate the routine drawing of m⁶A atlases for many cell and even

organismal contexts. However, it is a very challenging to develop a chemical-assisted sequencing method for m⁶A detection due to the inert chemical property of m⁶A.

Here, we present a dithiolsitol (DTT)-mediated thiol-addition chemical reaction that transfers unstable N⁶-hydroxymethyladenosine (hm⁶A) to more stable N⁶-dithiolsitolmethyladenosine (dm⁶A). Combination with FTO's enzymatic oxidation of m⁶A on RNA to hm⁶A, we developed FTO-assisted m⁶A selective chemical labeling method (termed as m⁶A-SEAL) for transcriptome-wide detection of m⁶A. m⁶A-SEAL identified 7,785 m⁶A sites in human HEK293T and 12,297 m⁶A sites in rice seedling, which had good overlaps with antibody-based results and displayed the known m⁶A distribution features in transcriptome. Comparison with all available m⁶A sequencing methods, m⁶A-SEAL-unique sites have the same m⁶A distribution profiles along transcripts as the common sites, however, the unique sites from all antibody-based m⁶A-seq methods display distinct features. Eight specific sites (five m⁶A sites and three unmethylated sites) were validated by SELECT method, in line with m⁶A-SEAL results, but not other m⁶A-seq methods. These results reveal that m⁶A-SEAL is a reliable and robust method for transcriptome-wide detection of m⁶A.

Results

FTO-assisted selective chemical labeling of m⁶A in RNA. The inert chemical property of m⁶A makes it very challenging to develop a chemical-assisted sequencing method for m⁶A detection. To overcome this challenge, we developed a FTO-assisted m⁶A selective chemical labeling method (m⁶A-SEAL) (Fig. 1). Consider that RNA demethylase FTO oxidizes m⁶A to hm⁶A in 5 min and further slowly oxidizes this hm⁶A to N⁶-formyladenosine (f⁶A)²⁴. These two intermediates are not stable in aqueous solution (only ~3-hour half-life). Fundamentally, our strategy coupled FTO's enzymatic oxidation of m⁶A marks on RNA to hm⁶A with a dithiolsitol (DTT)-mediated thiol-addition reaction to install a free sulfhydryl and generate substantially more stable N⁶-dithiolsitolmethyladenosine (dm⁶A)-marked RNA species from biological samples (Fig. 1a). Having overcome the limited reactivity of m⁶A marked RNA species, we could exploit the free sulfhydryl group present on dm⁶A to install a variety of tags (e.g., biotin and fluorophores) through reaction with methanethiosulfonate (MTSEA), thereby easily

facilitating applications including detection, sequencing and imaging (Fig. 1b).

We initially explored a variety of chemistries for the modification of hm⁶A using a model system based on chemically synthesized hm⁶A single nucleoside and synthetic m⁶A-modified RNA oligonucleotide. Inspired by the aminomethylation product in formaldehyde induced DNA-protein crosslinking²⁵, we quickly narrowed our attention to the use of sulfhydryl reagents to convert the unstable hemiaminal structure of hm⁶A into a more stable thiol-aminal structure, and found that DTT performed very well for derivatizing hm⁶A nucleoside in aqueous solution (Supplementary Fig. 1 and Supplementary Note 1). Other sulfhydryl reagents were also able to react with hm⁶A single nucleoside (Supplementary Fig. 2). Given the additional attractive benefit of a second free sulfhydryl provided by DTT, which should facilitate subsequent functional tagging for downstream applications, we chose DTT for further study. We used mass spectrometry and NMR to confirm the chemical structures of dm⁶A produced by our DTT treatment of hm⁶A nucleoside (Supplementary Note 2). Presumably, the thiol-addition reaction on hm⁶A is accomplished via [1, 2] addition of imine, but detailed mechanism remains to be fully elucidated (Supplementary Fig. 3).

Optimization showed that the yield of the DTT-mediated thiol-addition on hm⁶A nucleoside reached as high as 80% under mild acidic condition (~pH 4.0) at 37 °C over 3 h (Supplementary Figs. 1 and 4). We evaluated the stability of the dm⁶A nucleoside at various pH conditions. Our finding that dm⁶A had a much longer half-life (182 h) in neutral aqueous solution than hm⁶A (3 h, as previously reported²⁴) (Supplementary Fig. 5) supported the basic stability-enhancing concept of our method's design and suggested that the two-reaction process was likely practicable with biological samples.

Having optimized the conversion of dm⁶A from hm⁶A single nucleoside, we next conducted experiments with synthesized 9mer RNA oligonucleotide (model RNA) bearing m⁶A modification at the 5' end. Initial testing with the model RNA identified the optimal FTO oxidation and DTT-mediated thiol-addition reaction conditions to obtain the highest yield of the conversion of dm⁶A from m⁶A. Importantly, our choice to position the m⁶A mark at the 5' end in the model RNA ultimately facilitated precise HPLC-based quantification of the products from two reactions: the FTO-catalyzed oxidation of m⁶A to hm⁶A and the DTT-

mediated thiol-addition of hm⁶A to dm⁶A. Specifically, the nucleoside at the 5' end of model RNA can be selectively released via nuclease P1 treatment in neutral pH condition within 15 min, which efficiently reduced the degradation of the hm⁶A intermediate during routine RNA digestion procedures. In the optimized two-step reactions, 0.5 nmol of FTO was used to treat with 1 nmol of model RNA for 5 min at pH 7 and 37 °C, generating 60% of hm⁶A formation converted from m⁶A (Supplementary Fig. 6), a yield similar to a previously reported study of FTO kinetics²³. After ethanol precipitation purification, the purified RNA was subsequently treated with 200 mM of DTT for 3 h at pH 4 and 37 °C, generating 83% of dm⁶A formation from hm⁶A (Fig. 2a and Supplementary Fig. 7).

m⁶A-SEAL sensitively and specifically enriches m⁶A-modified RNA. After validation of m⁶A-SEAL on model RNA, we next tested m⁶A-SEAL on human RNA to optimize the reaction conditions and examine its sensitivity and specificity. First, purified poly(A)⁺ RNA from HEK293T cells was chemically fragmented into 100 nt molecules at 95 °C, which were then treated with FTO (at a variety of concentrations to facilitate optimization). We found that a five-minute incubation at 37 °C with 0.2 μM of FTO resulted in the highest yield (~50%) of hm⁶A using 300 ng of poly(A)⁺ RNA in 100 μL of demethylation reaction, these hm⁶A can be further converted to dm⁶A (yield 69%) under the treatment of DTT (Fig. 2b and Supplementary Fig. 8). Importantly, we detected no noticeable degradation for the fragmented poly(A)⁺ RNA upon this mild acidic condition (Supplementary Fig. 9), emphasizing that the protocol would not substantially affect the quality of RNA and sequencing libraries. The successfully DTT labeled poly(A)⁺ RNA was subsequently reacted with MTSEA-biotin, a commercial thiol-reactive biotin reagent. The labeling of biotin was confirmed with a dot-blot assay (Fig. 2c). No free dm⁶A was detected in LC-MS/MS analysis of the RNA sample following the MTSEA-biotin labeling step indicated very high biotin labeling efficiency (Supplementary Fig. 10).

To test the sensitivity and specificity of m⁶A-SEAL for sequencing applications, we next conducted several spike-in RNAs (Supplementary Note. 3) in poly(A)⁺ RNA pool to perform m⁶A-SEAL sequencing (m⁶A-SEAL-seq). The high-throughput sequencing results showed that our method facilitated strong enrichment for the spike-in m⁶A-modified sequences (for both singly and multiply m⁶A modified species), which is comparable to MeRIP (Fig. 2d).

Importantly, we detected no cross-reactivity for unmodified sequence (Fig. 2d). Therefore, these results support impressive sensitivity and specificity for m⁶A-SEAL based evaluation of m⁶A distribution in biological samples. These spike-in experiments also showed that a five-minute reaction time and 0.2 μM FTO were ideal to obtain the highest enrichment efficiencies of the spike-in m⁶A-modified sequences (Supplementary Fig. 11).

m⁶A-SEAL identifies transcriptome-wide m⁶A sites in human cells. We subsequently performed m⁶A-SEAL-seq for poly(A)⁺ RNA from HEK293T cells. We detected a total of 14,274 m⁶A peaks within human genes in two biological replicates, and classified the overlapping 7,785 peaks as high confidence sites for subsequent analysis (Supplementary Fig. 12). Several known m⁶A sites in transcripts were consistently identified in two biological replicates of m⁶A-SEAL (Fig. 3a). Note that sequencing on different platforms (and with a variety of analysis modes) yielded satisfactory data in each case (Supplementary Table. 1). The m⁶A sites identified by m⁶A-SEAL-seq revealed strong enrichment for the well-known canonical m⁶A motif RRACH (R=purine, H=A/C/U) (Fig. 3b). The center of m⁶A peaks were highly enriched at the consensus sequence compared to the negative control peaks (Fig. 3c), supporting the specificity of m⁶A-SEAL. The distribution of m⁶A within transcriptomes revealed strong enrichment in the vicinity of stop codons (Fig. 3d), which echoed a typical m⁶A distribution pattern mapped using MeRIP-seq^{16,17}. Mapping m⁶A sites to exons revealed that ~89% of m⁶A-modified exons were longer than 400 nt compared to the negative control peaks ($p < 2.2 \times 10^{-16}$, Wilcoxon test; Fig. 3e), in line with previous finding that m⁶A preferentially locates within long exons^{16,20}.

Closer examination of the distribution of the identified m⁶A sites within regions of transcriptome revealed that the majority of m⁶A (83.1% of 7,785 confident m⁶A sites) were present on coding transcripts with a preference within the coding sequences (CDS) and 3' untranslated region (3'UTR) (Fig. 3f). 33 m⁶A sites near transcription start sites (TSS, < 150 nt) were regarded as potential N⁶,2'-O-dimethyladenosine modifications at the cap +1 position (termed cap m⁶A_m; Fig. 3f), suggesting that our method could capture m⁶A and cap m⁶A_m sites simultaneously. 1,222 m⁶A sites (15.7%) belongs to non-coding RNAs, including lncRNA, pri-miRNA, snoRNA, and snRNA (Fig. 3f).

m⁶A-SEAL confirms that UGUAAHH is a plant-specific m⁶A motifs. Having established proof-of-concept for global m⁶A profiling from biological samples, we were next eager to deploy m⁶A-SEAL-seq to explore unsolved scientific questions in the exciting basic research area of epitranscriptomics cellular regulation. MeRIP-seq cannot identify the canonical m⁶A motif RRACH in *Oryza sativa* (rice); instead, a different motif sequence was found but not ascribed with high confidence because of the potential for non-specific antibody binding and the absence of experimental confirmation^{27,28}. Considering that our m⁶A-SEAL is a chemically covalent cross-linking method for mapping m⁶A sites, which eliminates the non-specific binding, we addressed this unsettled scientific question by performing m⁶A-SEAL-seq in poly(A)⁺ RNA samples isolated from 60-day-old rice leaves.

12,297 high confidence m⁶A peaks were identified in two biological replicates (Supplementary Fig. 13a). Metagene profiling and the distribution within the non-overlapped segments of transcripts of the m⁶A sites identified by m⁶A-SEAL showed that m⁶A predominantly occurs within 3'UTR (Supplementary Fig. 13b,c), in consistent with the previous MeRIP-seq result in rice²⁷. To identify m⁶A motifs, we clustered all confident m⁶A peaks in HOMER (Hypergeometric Optimization of Motif Enrichment) software to search motifs. We did not find the canonical m⁶A motif RRACH in rice; instead, we identified a non-canonical motif UGUAAHH (H=A/C/U) with highest significance (Supplementary Fig. 13d). Note that similar motifs were previously found in rice panicles (UGWAMH, W=U or A; M=C or A; H=U, A, or C)²⁷ and Arabidopsis (URUAY, R=G or A; Y=U or A)²⁹. To further confirm it, we calculated the enrichment of m⁶A-SEAL identified m⁶A peaks to the centre of UGUAAHH and RRACH motifs, which showed that m⁶A peaks were strongly enriched at the centre of the UGUAAHH motif but not the RRACH motif (Supplementary Fig. 13d).

m⁶A-SEAL determines m⁶A sites more reliably compared to all available m⁶A sequencing methods. To further evaluate the reliability and performance of m⁶A-SEAL, we compared it with all available m⁶A sequencing methods. We firstly compared our m⁶A-SEAL results identified in HEK293T and rice with MeRIP-seq results. To do this, we used the published MeRIP sequencing data for HEK293T (GSE29714)² and our own constructed MeRIP sequencing data in 60-day-old rice leaves, ~70% of the m⁶A peaks in HEK293T and ~59% rice

peaks from m⁶A-SEAL-seq positionally overlapped with peaks from MeRIP-seq (Fig. 4a). We categorized all m⁶A sites from m⁶A-SEAL and MeRIP into three groups for further analysis: common sites (overlapped m⁶A peaks between m⁶A-SEAL and MeRIP), m⁶A-SEAL-unique sites (not identified in MeRIP), and MeRIP-unique sites (not identified in m⁶A-SEAL). Motif search analysis by HOMER revealed that in HEK293T m⁶A-SEAL-unique sites and MeRIP-unique sites had the same m⁶A motif identified in common sites; however, in rice m⁶A-SEAL-unique sites contained the same motif as common sites while MeRIP-unique sites can not identify any significant motif (Fig. 4b). Metagene profilings showed that m⁶A-SEAL-unique sites both in HEK293T and rice had the same m⁶A distribution feature as common sites, however, MeRIP-unique sites presented a distinct feature (Fig. 4c). We next compared our m⁶A-SEAL results with the published miCLIP results in HEK293T, an antibody and CLIP-based single-base m⁶A sequencing method¹⁸. m⁶A sites identified by either cross-linking-induced truncation sites in miCLIP (miCLIP-CITS) or cross-linking-induced mutation sites in miCLIP (miCLIP-CIMS) had 40~50% of overlap with that of m⁶A-SEAL (Supplementary Fig. 14). Further metagene profilings revealed that both miCLIP-CITS-unique sites and miCLIP-CIMS-unique sites exhibited distinct m⁶A distribution features from the common m⁶A sites (Fig. 4c). These results suggest antibody-based m⁶A sequencing methods might have non-specific antibody binding effect.

To further demonstrate the reliability of m⁶A-SEAL, we picked eight sites—five m⁶A sites and three unmethylated sites—from m⁶A-SEAL results for validation using SELECT method we developed previously (Fig. 4d and Supplementary Fig. 15). SELECT is an elongation and ligation-based qPCR amplification method for detection of m⁶A position and fraction in mRNA/lncRNA at single-nucleoside resolution³⁰. SELECT method confirmed these five m⁶A sites and three unmethylated sites, which were consistent with m⁶A-SEAL results (Fig. 4e). SELECT validation results showed that all antibody-based m⁶A sequencing methods had high false positive rates. In total eight sites, MeRIP had three false positives and one false negatives, (Fig. 4d,e and Supplementary Fig. 15). The antibody-based single-base m⁶A sequencing methods (miCLIP and m⁶A-CLIP) displayed low ability for mapping m⁶A sites and high false positive rates. In total eight sites, miCLIP-CIMS and miCLIP-CITS together identified four

m⁶A sites, but only half of them were positives; m⁶A-CLIP identified three m⁶A sites, but one of them was false positives (Fig. 4d,e and Supplementary Fig. 15). m⁶A-REF-seq as an antibody-free and endoribonuclease-based single-base m⁶A detection method exhibited good reliability on ACA motif, however, it was powerless to map other m⁶A-modified motifs (for instance, m⁶A-REF-seq can not identify m⁶A-modified ACU motif in *MALAT1* 2577 site (chr11: 65500338)) (Fig. 4e and Supplementary Fig. 15). Collectively, m⁶A-SEAL as a chemical labelling method has higher reliability for detection of m⁶A sites.

Discussion

The epitranscriptomic modification m⁶A regulates numerous biological processes and diseases. It is desirable to develop a chemical-assisted m⁶A sequencing to reduce the non-specific antibody binding effect. In this work, we report an FTO-assisted m⁶A selective chemical labeling method (m⁶A-SEAL) for transcriptome-wide detection of m⁶A. This method combines two reactions in mild conditions: FTO-assisted oxidation of m⁶A to yield an unstable intermediate hm⁶A and DTT-mediated thiol-addition reaction to stabilize and enable functionalization of this intermediate. m⁶A-SEAL has multiple advantages: it is an antibody-free covalent cross-linking method, requires only commercial available reagents (Note that FTO protein is commercial available or can be easily purified in laboratory; DTT is a common biochemical reagent.), and used available bioinformatics tools for data processing. We demonstrated that this cost-effective, antibody-free method has good sensitivity, specificity, and reliability for transcriptome-wide detection of m⁶A.

Transcriptome-wide m⁶A sites in both human HEK293T and rice identified by m⁶A-SEAL had good overlaps with MeRIP results and displayed the known m⁶A distribution features in transcriptome. Compared with all available m⁶A sequencing methods, m⁶A-SEAL-unique sites performed the same m⁶A distribution feature as common sites, however, MeRIP-unique sites and miCLIP-unique sites showed distinct m⁶A distribution profiles. Single-base m⁶A sites validation by SELECT showed that all antibody-based m⁶A sequencing methods read unmethylated sites as m⁶A sites, further confirming the non-specific antibody binding leads to false positives. The ΔC_T values of SELECT between FTO treatment (+FTO) and without FTO

treatment (-FTO) indicate the m⁶A methylation level³⁰. SELECT results showed that the methylation fraction of m⁶A 2605 site on *TRIM32* transcript (chr9: 116700186) is the lowest among these five identified m⁶A sites. All antibody-based m⁶A sequencing methods failed to map this site, but m⁶A-SEAL succeed, indicating m⁶A-SEAL has higher sensitivity. m⁶A-REF-seq is an FTO-assisted endoribonuclease MazF-dependent single-base m⁶A sequencing method that only reads m⁶A sites in ACA motif²². Note that the three unmethylated sites and one *MALAT1* m⁶A site belong to non-ACA motif. m⁶A-REF-seq is powerless to detect these sites, however, m⁶A-SEAL can distinguish whether these sites are modified or not.

Cap m⁶A_m is a new identified reversible RNA modification in human and mouse poly(A)⁺ RNA³¹⁻³⁴. m⁶A-SEAL identified 33 m⁶A sites near TSS, which could be potential cap m⁶A_m sites. As known that FTO can oxidize cap m⁶A_m to hm⁶A_m and FTO demethylates cap m⁶A_m *in vitro* with a higher efficiency over that of internal m⁶A³¹, by optimization of the FTO oxidation conditions, the procedure of m⁶A-SEAL could be used for detection of cap m⁶A_m modification. Additionally, FTO also oxidizes DNA N⁶-methyldeoxyadenosine (6mA) to N⁶-hydroxymethyldeoxyadenosine (6hmA)^{4,24}. DNA 6mA is a new emerging epigenetic modification with low abundance³⁵⁻³⁹. m⁶A-SEAL could be optimized to be a potential sensitive and reliable method for detection of DNA 6mA modification.

hm⁶A is an unstable modification that is present in poly(A)⁺ RNA isolated from human cells and mouse tissues²⁴. Its ~3-hour half-life is comparable to the average half-life of mRNA in mammalian cells⁴⁰; whether hm⁶A plays a biological function in cells is unknown because it is hard to be detected and studied. Without FTO oxidation step, DTT-mediated hm⁶A labeling method could be adapted to detect this unstable modification in transcriptome-wide manner. Furthermore, m⁶A-SEAL converts the inert m⁶A to dm⁶A with a free sulfhydryl, which can be installed a variety of tags (e.g., biotin and fluorophores) through reaction with MTSEA. Here we have demonstrated one application that we installed biotin tag on m⁶A for enrichment and sequencing. We anticipate that m⁶A-SEAL method could be modified to image cellular m⁶A modification by installation of fluorophores on cellular m⁶A.

In summary, we developed m⁶A-SEAL method, which couples FTO's enzymatic oxidation of m⁶A to the unstable intermediate hm⁶A with DTT-mediated thiol-addition reaction to

generate a more stable dm⁶A with a sulfhydryl group that facilitates simple installation of functional application tags like biotin. We demonstrated that m⁶A-SEAL is a robust and reliable method for detection and functional studies of m⁶A. Considering FTO's oxidation property on multiple modifications and the tagging ability of dm⁶A, m⁶A-SEAL with optimization could be adapted to detect other modifications and image cellular m⁶A modification.

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Author contributions

G.J. and Y.W. conceived the project and designed the experiments; Y.W. performed the experiments and data analysis with the help of S.D., Q.Y., and Y. X.; G.J. and Y.W. wrote the manuscript.

Additional information

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Figure Legends

Fig. 1 | FTO-assisted selective chemical labeling of m⁶A. **a**, FTO oxidation of m⁶A to hm⁶A and a DTT-mediated thiol-addition reaction to convert unstable hm⁶A to the more stable dm⁶A. **b**, Schematic diagram of m⁶A-SEAL. m⁶A in RNA can be sequentially converted to hm⁶A and dm⁶A. The free sulfhydryl group on dm⁶A reacts with MTSEA to install a variety of tags (e.g., biotin and fluorophore), thereby easily facilitating applications including affinity purification, sequencing, and imaging.

Fig. 2 | m⁶A-SEAL on model RNA and human poly(A)⁺ RNA. **a**, Upper Panel: HPLC chromatograms showing the formation of dm⁶A on model RNA after FTO oxidation-assisted and DTT-mediated thiol-addition reactions, and the dm⁶A standard. 1 nmol of m⁶A-modified model RNA was treated with 0.5 nmol of FTO for 5 min at pH 7 and 37 °C, followed by incubation with 200 mM DTT for 3 h at pH 4 and 37 °C. Lower Panel: MS/MS profile of dm⁶A nucleoside. **b**, LC-MS/MS chromatograms showing the formation of hm⁶A and dm⁶A on HEK293T poly(A)⁺ RNA after FTO oxidation and FTO oxidation-assisted and DTT-mediated

thiol-addition reactions, respectively. 300 ng of HEK293T poly(A)⁺ RNA was treated with 0.2 μM of FTO for 5 min at 37 °C in 100 μl of reaction mixture, followed by incubation with 200 mM DTT for 3 h at pH 4 and 37 °C. The chromatograms of A, U, C, and G are scaled to the left y axis, and the chromatograms of m⁶A, hm⁶A, and dm⁶A are scaled to the right y axis. **c**, Dot-blot assay confirming biotin labeling of m⁶A on HEK293T poly(A)⁺ RNA. A 3' biotinylated 15mer RNA oligo was used as a positive signal control. **d**, Enrichment of spike-in RNA sequences detected by m⁶A-SEAL-seq and MeRIP-seq. Values represent fold enrichment of IP over input (n=2), normalized to non-m⁶A-modified spike-in. 10% m⁶A, reverse transcribed RNA with 10% of m⁶ATP; single m⁶A, synthetic RNA with a single m⁶A site.

Fig. 3 | m⁶A-SEAL-seq uncovers the transcriptome-wide m⁶A methylome in HEK293T cells. **a**, Representative views of m⁶A-SEAL-seq identified typical m⁶A peaks on mRNA (*TPT1* and *BSG*). Peaks are highlighted in gray, which contain SCARLET identified m⁶A sites²⁶. Transcript architecture is shown below with thin and thick parts respectively representing UTR and CDS. **b**, Canonical RRACH motif identified in m⁶A-SEAL-seq. **c**, Density plots showing the distance of the centre of m⁶A peaks/control peaks between the nearest consensus sequence RRACH. **d**, Metagene profile illustrating the distribution of m⁶A sites identified by m⁶A-SEAL across mRNA segments. **e**, Density plots showing the distribution of m⁶A/control peaks according to their located exon length. **f**, Pie chart presenting the fraction of m⁶A sites identified by m⁶A-SEAL-seq in each non-overlapping RNA segment. **g**, Repeats families (y-axis) ranked by the number of elements containing intergenic m⁶A sites (x-axis).

Fig. 4 | Comparison of m⁶A methylome in human HEK293T cells and rice detected by m⁶A-SEAL-seq and all available m⁶A-seq methods. **a**, Overlap of m⁶A peaks identified by m⁶A-SEAL-seq and MeRIP-seq. **b**, Human and rice m⁶A motif enriched in common m⁶A sites (identified in both m⁶A-SEAL and MeRIP-seq), m⁶A-SEAL-unique sites (not present in MeRIP), and MeRIP-unique sites (not present in m⁶A-SEAL). **c**, Metagene profile of the distribution of common m⁶A sites (identified in both m⁶A-SEAL and MeRIP-seq (or miCLIP)) and respective unique m⁶A sites across human and rice mRNA segments. **d**, Representative view of m⁶A sites on mRNA *PNN* identified by m⁶A-SEAL, MeRIP, and miCLIP. **e**, SELECT validation of eight individual sites detected by different m⁶A-seq methods in HEK293T. Eight

425 sites are validated as three unmethylated sites and five m⁶A sites by SELECT. Bar plots
 426 showing the relative threshold cycle (C_T) of qPCR for detecting the m⁶A site (normalized by
 427 input control site). Data are presented as means ±SD, n=2 biological replicates ×3 technical
 428 replicates. The m⁶A sites of MeRIP, miCLIP-CIMS, miCLIP-CITS, m⁶A-CLIP, and m⁶A-REF-
 429 seq are obtained from the literatures.

Methods

Synthesis of model RNA oligonucleotides. 9mer model RNA containing m⁶A (5'-m⁶ACUGACUAG-3') was synthesized on ABI Expedite 8909 nucleic acid synthesis system using commercially standard RNA phosphoramidites and m⁶A phosphoramidite (Glen Research) in DMT-ON model. The phosphate backbone of the first three bases on 5-terminal and the last three bases on 3-terminal were thiolated by Beaucage reagent (Glen Research) in order to prevent RNase degradation. Purification of oligo nucleotide is carried out by Glen-Pak cartridges (Glen Research).

Expression and purification of recombinant human FTO protein. Protein expression was performed as previously described with minor modifications⁴¹. Briefly, human N-truncated human FTO protein (NP_001073901.1, 31-505aa) was subcloned into pET28a vector. Plasmid was transformed into *Escherichia coli* BL21(DE3) and grew on LB agar plate with 50 mg/L kanamycin. After being inoculated to 1 L LB medium that contained 50 mg/L kanamycin, bacteria were cultured under 37°C, 220 r.p.m in shaker until OD₆₀₀ reached 1.0. The expression of protein were induced by 0.5 mM IPTG at 16°C for overnight. Cell pellets were harvested and resuspended in 20 mL Buffer A (10 mM Tris pH 7.9, 150 mM NaCl) and sonicated on ice. Lysate was centrifugated for 30 min at 13000g and 4°C. Supernatant was collected and filtered by 0.22 µm filter (Millipore) then loaded on Ni-NTA column (GE Healthcare). After washed by 20 mL Buffer A and then 20 mL 8% Buffer B, protein was eluted by Buffer B (10 mM Tris pH 7.9, 150 mM NaCl, 500 mM imidazole). The collected fraction was then purified by MonoQ ion exchange column(GE Healthcare, Buffer A: 10 mM Tris pH 7.9, 3 mM DTT. Buffer B: 10 mM Tris pH 7.9, 1M NaCl) to remove non-specific nucleic acids binding (0-100% Buffer B gradient) and subsequently purified by Superdex 75 gel-filtration column (GE Healthcare, 10 mM Tris pH 7.9, 150 mM NaCl and 3 mM DTT). Protein was concentrated into 10 mg/mL and 20% glycol was added. Aliquots of protein were frozen by liquid N₂ then stored in -80°C.

Human Cell culture and isolation of poly(A)⁺ RNA. HEK 293T/17 cell (CRL-11268, ATCC) was cultured in DMEM medium (Corning) that contains 10% FBS (Cellmax) and 1% Penicillin Streptomycin (Corning) under the condition of 37 °C and 5% CO₂. Cells were washed once by PBS and harvested for the isolation of total RNA using TRIzol reagent (Magen). Poly(A)⁺ RNA

was isolated from total RNA using oligo(dT)₂₅ Dynabeads (Thermo Fisher Scientific). RNA concentration was measured by Nanodrop UV-Vis Spectrophotometer (Thermo).

Rice sample preparation and isolation of poly(A)⁺ RNA. Rice (Nipponbare) seeds were soaked in water in the dark at 30 °C for two days. After that, seedlings were grown for 28 days at 28°C in a soil seed bed. The humidity was 70% with 12h light/12h dark. Then the seedlings were transplanted into pots and grown for 60 days in a greenhouse under normal condition. Rice leaves were harvested and frozen by liquid N₂, then disrupted using the TissueLyser II (QIAGEN). Total RNA was extracted by TRIzol reagent (Magen) from homogenized samples. Poly(A)⁺ RNA was isolated from total RNA using oligo(dT)₂₅ Dynabeads (Thermo Fisher Scientific).

FTO oxidation of m⁶A to hm⁶A in model RNA. Oxidation of 9-mer model RNA (5 μM) was performed in 300 μM of (NH₄)₂Fe(SO₄)₂·6H₂O, 2 mM of L-ascorbic acid, 300 μM of α-KG, 100 mM pH=7.0 HEPES, 2.5 μM FTO at 37 °C. After 5 min treatment, the reaction was quenched by 5 mM EDTA and then frozen in liquid N₂ immediately for HPLC analysis.

FTO oxidation of m⁶A to hm⁶A in poly(A)⁺ RNA. Poly(A)⁺ RNA was fragmented by Magnesium RNA fragmentation module (NEB). In poly(A)⁺ RNA oxidation assay, the reaction was performed in 300 μL aliquots of aqueous solution containing 300 μM of (NH₄)₂Fe(SO₄)₂·6H₂O, 2 mM of L-ascorbic acid, 300 μM of α-KG, 100 mM pH7.0 HEPES, 0.2 μM FTO and 1μg Poly(A) RNA. After the FTO treatment at 37 °C for 5 min, RNA was purified by RNA Clean & Concentrator-5 column (Zymo Research). A large volume of FTO oxidation reaction can be concentrated by lyophilization before column purification.

DTT labeling of hm⁶A-modified RNA produced by FTO oxidation. hm⁶A-modified RNA converted from m⁶A by FTO oxidation was treated by 200 mM freshly prepared DTT at 37 °C for 3 h in acidic aqueous solution (100 mM HEPES, pH 4.0). The product RNA was purified by ethanol precipitation.

Biotinylation of DTT-labeled RNA. After ethanol precipitation, DTT treated RNA was washed by 75% ethanol and dissolved in 200 μL biotinylation buffer that contains 100 μM of MTSEA-XX-biotin (Biotum), 100 mM HEPES (pH 7.0), 1 mM EDTA and 20% DMF. The

reaction was performed at 25 °C and 800 r.p.m in ThermoMixer for 1h. The product RNA was purified by phenol-chloroform extraction or by RNA Clean & Concentrator-5 column purification.

Determination of the formation efficiencies of hm⁶A and dm⁶A in model RNA using HPLC analysis. After FTO oxidation and DTT labeling, 5' nucleoside in model RNA for each step was released by 1 U of nuclease P1 (Wako) at 37 °C and 800 r.p.m in ThermoMixer (eppendorf) for 15 min, then analyzed by HPLC (Agilent 1260 Infinity) using C18 column (5 µm, 150×4.6 mm, Bonna-Agela Technologies).

Determination of the formation efficiencies of hm⁶A and dm⁶A in poly(A)⁺ RNA using LC-MS/MS analysis. After FTO oxidation and DTT labeling, 200 ng Poly(A)⁺ RNA was digested by 1 U of nuclease P1 (Wako) in 50 µL water solution at 42 °C for 1 h, followed by the addition 1 U of shrimp alkaline phosphatase (rSAP, NEB). The mixture was incubated at 37 °C for an additional 1 h. Digested samples were filtered through 0.22-mm syringe filters prior to UPLC-MS/MS analysis. The nucleosides were separated by UPLC (Shimadzu) equipped with a ZORBAX SB-Aq column (Agilent) and detected by MS/MS using a Triple Quad 5500 (AB SCIEX) mass spectrometer in positive ion mode by multiple reaction monitoring. Nucleosides were quantified using the nucleoside-to-base ion mass transitions of m/z 268.0 to 136.0 (A), m/z 245.0 to 113.1 (U), m/z 244.0 to 112.1 (C), m/z 284.0 to 152.0 (G), m/z 282.0 to 150.0 (m⁶A), m/z 298.1 to 136.0 (hm⁶A), m/z 435.0 to 148.1 (dm⁶A). Standard curves were generated by running a concentration series of pure commercial nucleosides and our synthesized nucleoside standards. Concentrations of nucleosides in samples were calculated by fitting the signal intensities to the standard curves.

Dot-blot assay. 1 µg biotin-labeled poly(A)⁺ RNA was spotted on Hybond-N⁺ membrane (Amersham), followed by crosslinking twice under 1500 mJ 254 nm UV (UVP). After dried by air, the membrane was blocked by 1% BSA at room temperature for 1 h and then washed by 1×PBST buffer for three times. HRP-streptavidin (Invitrogen, 1:15000 dilution in PBST) was used to incubate with the membrane at room temperature for 1 h. After washed by PBST for four times, the signals were visualized by Immobilon Western Kit (Millipore) and imaged by Luminescent Imaging Workstation (Tanon 5200).

m⁶A-SEAL-seq and library construction. 5 µg of poly(A)⁺ RNA spiked with 500 pg spike-in RNAs were chemically fragmented into 100-150 nt. The fragmented RNA was treated with FTO oxidation, DTT-mediated thiol-addition reaction, and biotin labeling according to the protocols described above. 50 ng biotin-labeled RNA was saved as input, the rest was proceed to affinity enrichment. 20 µL Dynabeads MyOne Streptavidin C1 (Invitrogen) was washed twice by 200 µL 0.1 M NaOH to remove RNase contamination, then washed with DEPC water to neutral pH. The beads were resuspended in 100 µL binding solution containing 10 µL of high salt wash buffer (100 mM Tris pH 7.5, 10 mM EDTA, 1 M NaCl, 0.05% Tween 20) and 90 µL DEPC water, and incubated with the biotinylated RNA for 1 h. The beads with biotinylated RNA were washed three times with 1 mL high salt wash buffer. 50 µL of 100 mM DTT was used to release the biotinylated RNA at 37 °C for 15 min on ThermoMixer (800 rpm). After collecting the supernatant, the second elution was performed with 50 µL of 100mM DTT at 50 °C for 5 min to completely release the RNA. The twice eluted RNA were combined and purified by ethanol precipitation. Library construction was performed using NEBNext Ultra II RNA Library Prep Kit for Illumina according to the manufacturer's protocol. Human libraries were sequenced on Illumina HiSeq XTen platform with pair-end model (PE150). Rice libraries were sequenced on Illumina NextSeq500 platform with sing-end model (SR75).

FTO-assisted SELECT method. Poly(A)⁺ RNA was treated with FTO following previously published method³⁰. FTO-treated and untreated polyA-RNA were mixed with 800fmol Up Primer, 800fmol Down Primer and 1pmol dTTP in 1X CutSmart Buffer (50 mM KAc, 20 mM Tris-HAc, 10 mM MgAc₂, 100 µg/ml BSA, pH 7.9 at 25°C). The primers and RNA were annealed by incubating at the following condition: 90°C for 1min; (-1°C/6s)x 41 cycles; 48°C, hold. A 5 µL mixture of 0.001U Bst 2.0 WarmStart DNA Polymerase in 1x CutSmart Buffer was added in the former mixture. The reaction was incubated at 48°C for 20min, and kept at 35°C. Subsequently, a 10 µL mixture containing 0.5U SplintR ligase and 10nmol ATP was added to the final volume 20 µL. The reaction mixture was incubated at 35°C for 15min, denatured at 95°C for 5min, and then kept at 4°C. The quantitative real-time PCR (qPCR) was performed with 2µL reaction mixture as the DNA template. Data was analyzed with QuantStudio™ Real-Time PCR Software v1.3. The detected results of m⁶A site were

corrected by neighboring control site.

Data pre-processing. Reads were trimmed by cutadapt (v1.18) to remove adaptor and reads shorter than 12bp. Trimmed reads were mapped to GRCh38 human genome combining spike-in sequences using HISAT2 (v2.1.0)⁴² Conversion of output sam/bam files were performed by SAMtools⁴³ Transcripts from alignment were quantified, assembled and then merged to create a uniform set of transcripts and by StringTie⁴⁴. More precise quantification for each sample was performed by StringTie to obtain FPKM of each gene.

Identification of m⁶A peaks in transcriptome. Aligned reads were subjected to model-based analysis of ChIP-seq (MACS2) algorithm (v2.1.0)⁴⁵ for peak-calling. The option “--nomodel” and “--slocal 200” was used to call m⁶A peaks and to avoid miss of peaks that shadowed by other adjacent significant peaks. The q-value and enrichment threshold for peaks were 0.05 and 1 respectively. After peak-calling, peaks whose FPKM less than 1 will be filtered to obtain high-confident m⁶A peaks. Overlapped peaks between different samples were found by BEDtools⁴⁶ *intersect* function.

Motif search. m⁶A peaks were subjected to *De novo* motif search by *findMotifsGenomes.pl* script of HOMER⁴⁷ (v4.10) Output motif length was restricted in 5,6 and 7.

Metagene profile. Metagene profile was drawn by R package Guitar⁴⁸. Briefly, human annotation GTF file was downloaded from Ensemble (www.ensembl.org). Genomic TxDb object was built from GTF file through *makeTxDbFromGFF* function. Transcriptomic landmarks was built from Genomic TxDb object via *makeGuitarCoordsFromTxDb* function. Peak file was read by *system.file* function and imported as Granges by *import.bed* function. Metagene distribution profile was drawn by transcriptomic landmarks and imported peaks through *GuitarPlot* function.

Enrichment profile of m⁶A-seal around MeRIP-seq peaks and different motif. Enrichment profile was drawn by deepTools⁴⁹. Briefly, m⁶A-SEAL enrichment score file (bigwig file) was generated from IP and Input bam files through *bamCompare* function. MeRIP-seq region files are bed files from peak-calling, UGUAMM and RRACH region files are generated from rice transcriptome via *scanMotifGenomeWide.pl* script in HOMER package. After that, enrichment

signal distribution matrix was calculated by enrichment score files and region files through *ComputeMatrix* function. Enrichment profile and heatmap was generated by enrichment signal distribution matrix through *plotProfile* and *plotHeatmap* function.

Code availability. Custom Bash, Perl and R codes used for analysis are available on request

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a **b**

- - - - ➔ Affinity purification
- - - - ➔ Sequencing
- - - - ➔ Imaging

a **c** **b** **d**