Arraystar m6A Single Nucleotide Microarrays

Locate and quantify the exact m6A site at single nucleotide resolution

Highlights

Arraystar m6A Single Nucleotide Microarrays add invaluable benefits which are lacking for conventional m6A-seq analysis:

- Systematic m6A profiling independent of m6A-antibody immunoprecipitation based approaches.
- Precise detection of m6ACA at single nucleotide resolution.
- Quantifying m6A modification stoichiometry and abundance.
- Low demand for sample amounts, as low as 1 ug total RNA.
- Specialized pipeline to collect and annotate the quantifiable Single-, Poly-, or Clustered-m6ACA sites.

Introduction

Profiling m6A at single nucleotide has been challenging. Conventional methods such as m6A/MeRIP-seq have helped to make the analysis of m6A epitranscriptomics possible [1-5], but they cannot precisely identify which adenosines in a MeRIP-seq peak are actually modified, nor can they quantify the modification fraction for each site [6].

To address these challenges, Arraystar has developed m6A single nucleotide arrays that precisely locate the m6A modification at exact adenosine and quantify the stoichiometry of m6A modification fractions

	m6A Arrays	MeRIP-Seq
Quantification	• m6A stoichiometry as %Modified • m6A RNA abundance • Differential analysis of both %Modified and abundance	• Lack of modification stoichiometry • Differential analysis of abundance only
m6A-site resolution	Single-nucleotide	~ 100 nt
Starting RNA amount	>= 1µg total RNA	>= 120 µg total RNA
Poly(A) selection or rRNA removal	Not required	Required (scale-up needed)
RNA integrity demand	Tolerant	High

An orthogonal methodology for m6A detection

Due to the m6A-antibody cross-reactivity with other related modifications (e.g. m6Am)[1, 7-9], the assay specificity to m6A modification is limited. Also, in the absence of an orthogonal technique as an independent reference, the sensitivity of m6A-antibody based m6A profiling has not been systematically evaluated. For the first time, the microarrays based on methyl-sensitive MazF RNase now allow systematic m6A profiling independent of m6A-antibody immunoprecipitation based approaches such as MeRIP or miCLIP.

Single-nucleotide resolution for m6A site location

RNase MazF cleaves single stranded RNA 5′ immediate to unmethylated (ACA) sequence, but not methylated (m6ACA) (Fig. 1) [1, 10]. The MazF digested RNA fragments with cleaved (ACA) and uncleaved m6ACA are labeled with Cy5 color, whereas the input RNA fragments without MazF digestion are separately labeled with Cy3 color. These RNAs are then combined and hybridized with Arraystar m6A Single Nucleotide Arrays, to quantify the m6A modification stoichiometry and abundance for each site (Fig. 2).

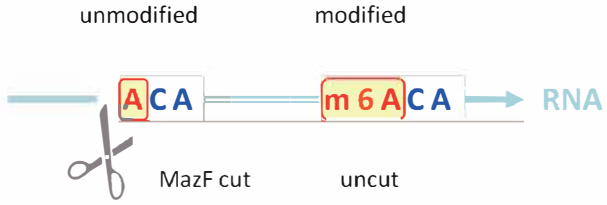


Fig 1. MazF enzyme cuts at unmethylated (ACA) sequence but not methylated (m6ACA).

m6A modification stoichiometry

The m6A stoichiometry, i.e. the fraction of m6A modification at that site, is critical for understanding its functional significance, answering questions about the m6A writing/reading/erasing, regulation, the dynamics in response to stimuli [3, 11, 12], and prioritizing the m6A sites for study. The fraction or percentage of m6A modification can be quantified by the two-color channel intensities at each site, addressing the long-standing unfulfilled needs in determining the dynamic m6A status.

Low RNA amount requirement

The microarrays use as low as 1µg total RNA. The highly sensitive and specific MazF enzymatically works well even on extremely low RNA amounts at nanogram or picogram level. m6A profiling can now be performed on rare samples, precious pathological specimens, particular histological sites, low yield sorted cells, or small animal models.

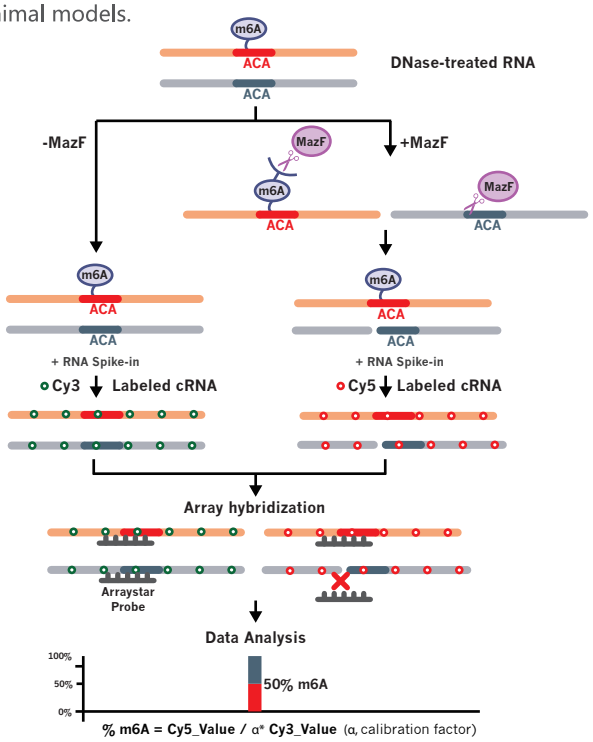


Fig 2. The workflow of Arraystar m6A Single Nucleotide Array.

Reliable collection and systematic annotations

Most m6A modifications occur in m6A motifs having a core (ACA) sequence, collectively referred to as m6ACA sites. For reliable collection of m6ACA sites, we have established a pipeline to discover all m6ACA sites that are quantifiable by array probes. An ACA site without another ACA present within 40 nt can be uniquely detected by a probe and is defined as quantifiable Single-ACA site. Multiple ACA sites closely located within a 20-nt region can be collectively detected by a probe and is defined as quantifiable Poly-ACA site. Additionally, when multiple Single-(ACA) or Poly-(ACA) are contained within a 500 nt region and the distance between them is < 100nt, they are merged as one Clustered-ACA region (Fig 3), which can be analyzed by integrating the signals from the individual probes of constituent Single- and Poly-ACA sites. Not all ACA sequences in the RNAs are modified by m6A. All quantifiable ACA sites are further mapped to the cataloged m6A sites in the miCLIP dataset[13-16] and the m6A consensus near the m6A-seq peak summits[17], which defines a high and ultra-high confidence Single-, Poly-, and Clustered-m6ACA sets.

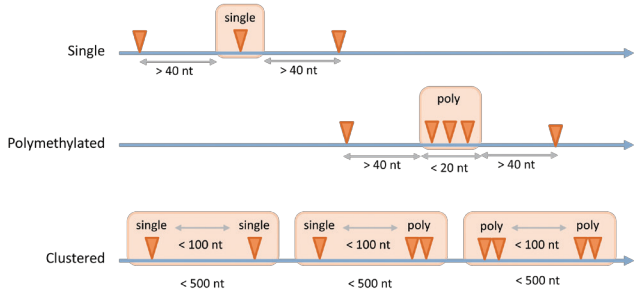


Fig 3. The collection pipeline of quantifiable Single-, Poly-, and Clustered-m6ACA sites.

The functional significance of Single-, Poly- and Clustered-m6A sites

The single-m6A modifications are often related to molecular functions in mRNA translation initiation or translation elongation dynamics. They also regulate noncoding RNA decay and activities.

Single-m6A site in 5′ UTR for cap-independent translation initiation of Hsp70 mRNA

A single-base m6A modification at in the 5′ UTR of Hsp70 mRNA is necessary and sufficient to promote its noncanonical cap-independent translation (Fig. 4) [18]. Under stress conditions, nuclear localized YTHDF2 reader protein protects the m6A from demethylation by FTO. Translation initiation factor (eIF3) can directly bind to the m6A and initiate the internal translation, thereby allowing cap-independent translation of HSP70 when the global cap-dependent translation is shutting down in the stressed cells.

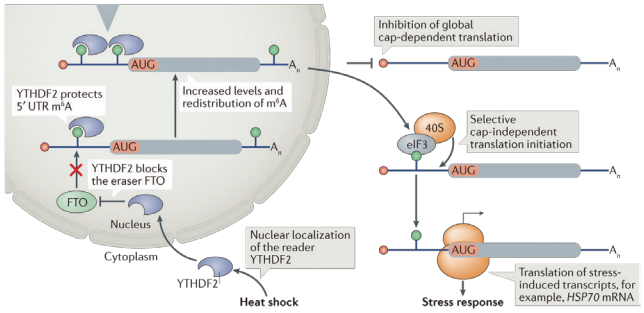


Fig 4. Molecular mechanism of cap-independent translation of HSP70 mRNA under heat shock stress by a single-base m6A modification [18, 19].

Single-m6A site in mRNA CDS affects mRNA translation dynamics

A single-base m6A modification in mRNA coding sequence (CDS) can affect the translation elongation dynamics [20]. An m6A in a codon can slow down the cognate-tRNA decoding and also act as a barrier/controller for tRNA accommodation and translation elongation. The m6A stalled translation may produce truncated proteins and have chain effects on co-translational protein folding, chaperoning, and localization signal recognition, all of which can potentially drive the protein for different fates or activities [21]. Conceivably, if m6A recodes a codon for another amino acid, it could lead to protein variants with an deviant function and substantial biological consequence even at low occupancy [20](Fig. 5).

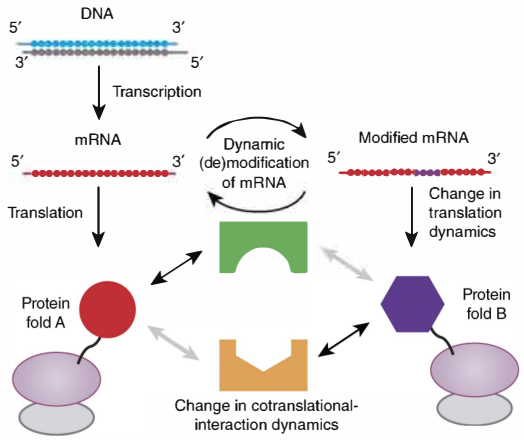


Fig. 5. The changed translation elongation dynamics can influence co-translational nascent protein folding or interaction with other partnering factors [20].

Single-m6A site regulating lncRNA decay

Single-base m6A sites (A917, A1025 and A1056) in linc1281 are required to decoy let-7 family miRNAs (Fig. 6) [22]. By sequestering these pluripotency-related miRNAs, the m6A modified linc1281 ensures the cells of mESC identity.

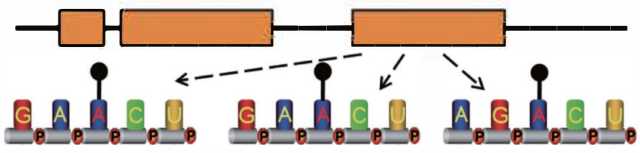


Fig. 6. m6A methylation of single-m6A sites in the last exon of linc1281 is required for the lncRNA to bind let-7 miRNAs and act as a competing endogenous RNA (ceRNA) to regulate mESC differentiation [22].

Poly- or Clustered-m6As partition phase separation of mRNAs into subcellular compartments

Multiple m6As packed in clusters are most prominent in mRNA degradation to shape the half-life of mRNAs in cytoplasm. The mRNA metabolic fates are destined by the m6A readers targeting them into cytoplasmic compartments by phase separation. Polymethylated regions are also the substrates for heavy m6A modification required for lncRNA activities.

Polymethylated or clustered m6As, but not singly methylated m6A, can partition the mRNA population into different subcellular compartments via liquid phase separation (Fig. 7) [23]. Cytosolic YTH-DF1, 2, 3 readers spontaneously partition themselves into liquid phases both in vitro and in cells. Polymethylated mRNAs act as a multivalent scaffold for YTHDF binding, which are carried into different phase-separated subcellular compartments, such as P-bodies, stress granules or neuronal RNA granules for different metabolic fates. For example, polymethylated mRNAs stored in stress granules are translationally repressed. Polymethylated mRNAs targeted to P-bodies by YTH-DF2 are degraded [24]. A singly methylated m6A does not have sufficient YTHDF binding affinity for this to occur[23].

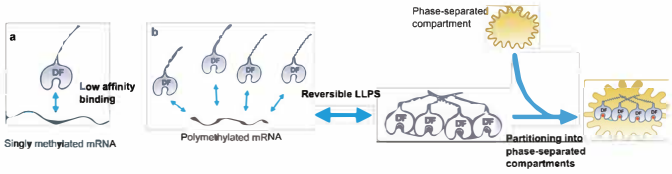
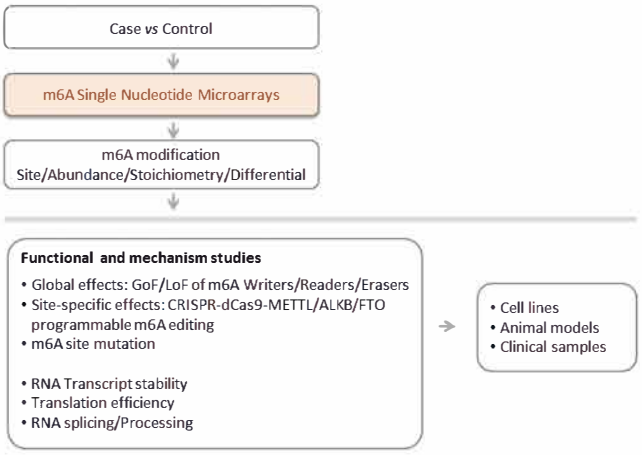


Fig. 7. Polymethylated m6As bind cytosolic YTH-DF1,2,3 readers at multivalent affinity and are separated into subcellular compartments for the metabolic fates.

m6A function and mechanism research roadmap



m6A Single Nucleotide Microarray Specifications

	Human	Mouse	Rat
Array Format	8 × 15K		
Probe length	60 nt		
Total number of distinct probes	14,321	14,319	14,581
M6ACA site sources	miCLIP dataset [13-16]; RMbase database [17]		
Single ACA site	11,237	11,120	11,499
Multiple ACA sites	3,084	3,199	3,082
Cluster ACA sites	693	279	2,614

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