The impact of RNA modifications on cellular function via alterations to structure and macromolecular interactions

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CHALLENGE

RNA structure and RNA modifications are aspects central to RNA function. They have both been widely recognized for decades to be important for regulatory RNAs like tRNAs and rRNA, where the structure of RNA is relatively well-characterized. When looking at mRNA and non-coding RNA, a much less complete picture emerges, where RNA structure is often neglected and the importance of RNA modifications are just starting to emerge (1,2). The basic RNA structure is often depicted as a linear strand of nucleotides, sometimes with a hairpin to illustrate some degree of structure. A recent perspective article (3) very accurately highlighted the need for progression of the RNA community, as well as for the entire scientific community, towards appreciating the complexity of RNA tertiary structure, the three-dimensional arrangement of RNA building blocks that includes helical duplexes and triple-stranded structures, and the importance of structure for the molecular functions of RNA, including mRNA.

In this paper we challenge the community to take on an ambitious effort to understand RNA structure on a transcriptome-wide level to gain new insights into the dynamics of RNA and its interaction with other macromolecules, e.g., DNA, RNA, and proteins (**Figure 1**). We emphasize the need to develop more high-throughput tools for identification of the RNA modifications currently known to occur on mRNAs, in order to obtain nucleotide-precision and cell-state-dependent mapping of each modification of the transcriptome. Tools like chemical and enzymatic conversion of specific RNA modifications and stronger direct RNA sequencing analysis pipelines are needed to meet this goal. In addition, a coordinated effort by the community to align data standards and data processing to a uniform format will be essential for driving this ambitious effort forward. RNA modifications and their impact on mRNA structure should then be assessed for their ability to mediate RNA-protein interactions (as well as interactions with RNA and DNA) with experimental and computational approaches. We encourage the community to incorporate these data into a global model that can predict RNA structure, mRNA-protein

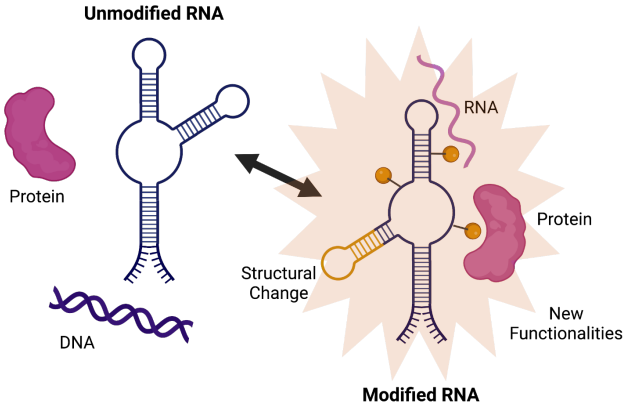

Figure 1. RNA modifications dictate RNA structure, intermolecular interactions, and function. Created with Biorender.com.

interactions, and how these are affected by differential RNA modifications. We anticipate an “AlphaFold for RNA”, which will enlighten research into functional RNA elements, the importance of RNA modifications on basic mRNA function, and the dynamics of mRNA structure and function within the cell.

STATE OF THE ART

The diversity of RNA modifications is demonstrated by reported functions for a diverse subset of the roughly 170 known modifications (4), including N^6-methyladenosine (m^6A), N^6, 2′-O-dimethyladenosine (m^6Am), 5-methylcytosine (m^5C), 5-hydroxymethylcytosine (hm^5C), inosine (I), pseudouridine (Ψ), N^1-methyladenosine (m^1A), 2'-O-methylation (Nm), N4-acetylcytidine (ac^4C), N7-methylguanosine (m^7G), dihydrouridine (D), and many others (5). Different chemical modifications play distinct regulatory roles. The m^6A modification influences RNA stability, splicing, translation, localization, and RNA secondary structure (6–9). m^5C in mRNA influences mRNA export, RNA stability, and translation, and in tRNA is essential for maintaining structural stability and translational fidelity (10–15). Inosine preferentially exists in double-stranded RNA (dsRNA) and affects codon recoding, splice-site choice, microRNA (miRNA) biogenesis, and targeting efficiency (16–18). Ψ is required for proper rRNA folding, tRNA structure stabilization, and snRNP (small nuclear ribonucleoprotein) biogenesis (19–27). In addition, introducing Ψ into mRNA increases protein production and alters translation (24,28,29). m^1A at position 58 in tRNA is conserved and vital for stabilizing tRNA tertiary structure, and m^1A in mRNA influences translation (30–35). Nm is essential for accurate and efficient protein synthesis (36–39). Internal m^7G increases mRNA translation efficiency and augments miRNA biogenesis (40,41). Finally, ac^4C in mRNA promotes translation, and ac^4C in rRNA can affect rRNA biogenesis (42–46).

Additional features of RNA modifications have been observed for mRNA. In addition to the presence or absence of an RNA modification, the percentage of a particular transcript that is modified plays a significant role on the functional consequences of the RNA modification. For example, a modification that leads to mRNA decay is unlikely to have a significant biological effect if it affects a small percentage of the transcriptome. As modifications can affect mRNA structure and/or the recruitment of RNA binding proteins, modifications of a fraction of the transcripts at any specific site would generate two distinct mRNA species that differ in their structures or the proteins that bind to them. Hence, changing the stoichiometry of modifications may represent another mechanism to generate functional diversity of mRNA.

While current RNA modification profiling methods can map the modification locations, they do not quantify the relative fraction of modified and unmodified RNA for a given transcript or phase modifications along a single RNA strand. High-throughput methods for site-specific quantification of all RNA modifications and determination of the stoichiometry of modifications will considerably advance our understanding of the functional roles of RNA modifications.

Existing Technologies - Deficiencies

1. Quantification of global levels of RNA modifications

Several methods to quantify RNA modifications have been established, including two-dimensional thin-layer chromatography (2D-TLC), dot blot, and liquid chromatography–mass spectrometry (LC–MS). These approaches can be used to quantify the modification abundance in specific RNA species. However, they require significant amounts of RNA, which limits their utility for rare or difficult-to-acquire samples. This is especially true in the case of 2D-TLC and LC-MS, which require dedicated and expensive equipment not readily available in most laboratories around the world.

2. Methods to obtain positional information of RNA modifications.

Various methods have been developed to determine and quantify precise position of RNA modifications.

1. Primer Extension: The principle of this technology is that a reverse transcriptase (RT) can reach the 5'-end of RNA and produce full-length cDNA only if certain RNA modifications are not encountered. In the presence of modifications such as m^1A, Ψ, and m^1G, RT extension is blocked upstream of the modified site. The RT products are separated by polyacrylamide gel and the terminal position of the truncated cDNA determines the position of the RNA modification. Although very sensitive, this approach has major limitations. Prior knowledge of the modification type and sequence information of the target RNA are required. Also, the technology can only be used for modifications that block reverse transcription and cannot be applied to "silent" modifications like m^6A or m^5C.

2. qPCR-based approach: Semiquantitative PCR or quantitative (q)PCR-based approaches depend on the fact that modified nucleotides impede reverse transcriptase extension. To date, several semiquantitative PCR- or qPCR-based approaches have been developed and have successfully detected Nm, Ψ and m^6A in diverse RNA species (47–52). The use of qPCR approaches has been accelerated by the development of RT enzymes engineered to facilitate modified nucleotide detection. For instance, an engineered thermostable KlenTaq DNA polymerase variant possesses reverse transcription activity and can discriminate Nm at normal dNTP concentrations. The combination of this engineered DNA polymerase with qPCR has achieved expeditious quantification of Nm (47). In addition to the engineered thermostable KlenTaq DNA polymerase variant, two other DNA polymerases, Tth and Bst, also have reverse transcriptase activity and exhibit distinct capacities for extension when encountering m^6A residues versus A residues, which allows locus-specific detection of m^6A (48,50,53).

3. RNase H approach: In this method, purified RNA is cleaved by RNase H into two halves at the 5' end of the nucleotide of interest. Cleavage specificity is achieved by annealing with a specific 2′-O-methyl RNA–DNA chimeric oligonucleotide. The 3'-half of the RNA is then purified, and its 5′ terminus is further labeled with phosphorus-32 (^{32}P). Finally, the oligonucleotides are completely digested into single nucleotides and subsequently resolved by thin layer chromatography (TLC). This method has some major limitations since it requires prior knowledge of the sequence information to target a single specific nucleotide, and it cannot be used to quantify the modification status in a *de novo* manner. In addition, the requirement of radioactive reagents and complicated analytical procedures limit wider application of this method.

4. Electrospray Ionization-Mass Spectrometry (ESI-MS) approach: Isolated RNA samples are first digested into 5–15 nucleotide fragments by selective endoribonucleases, such as RNase T1, RNase A, and RNase U2. The oligonucleotides are then separated by HPLC into sequence ladders. These sequence ladders are further generated through ESI-MS and used for sequence reconstruction and modification identification. The major limitation of this approach is the costly requirement for highly sensitive ESI-MS. In addition, given the detection principle, the required starting material is large; thus, this method is only suitable for abundant RNA species.

3. Next-generation sequencing methods

Several next-generation sequencing (NGS) methods have been developed for mapping RNA modifications transcriptome wide. The methods can be divided into chemical-assisted sequencing technologies, antibody-based (immunoprecipitation) technologies, enzyme- or protein-assisted technologies, and direct sequencing technologies. Examples of these technologies validated by published methods are described below.

1. Chemical-assisted sequencing: To discriminate modified nucleotides from unmodified nucleotides, various groups have used (1) biotin tags to enrich modified transcripts; (2) altered base-pairing features to induce misincorporation or truncation in reverse transcription; and (3) chemical-induced cleavage followed by specific adaptor ligation. More recently, an approach termed GLORI was developed that uses glyoxal and nitrite-mediated deamination of unmethylated adenosines and enables accurate and quantitative determination of m^6A sites (54).

2. Antibody-based technologies: This method uses antibodies specific for modifications like m^6A or m^7G to immunoprecipitate modified RNA followed by fractionation and NGS (5).

3. Enzyme- or protein-assisted sequencing: DART-seq uses the cytidine deaminase APOBEC1 fused to the m^6A-binding YTH domain to deaminate nucleotides next to m^6A-modified nucleotides (55). This technology has also been used at single-cell resolution. The *E. coli* endoribonuclease MazF cleaves an unmethylated 5'-ACA-3' motif, but not the 5'-m^6ACA-3' motif, when followed by NGS. This method can map the presence of m^6A at single-nucleotide resolution (MAZTER-seq) within a subset of m^6A sites. The related evolved TadA-assisted N^6-methyladenosine sequencing (eTAM-seq) approach relies on an optimized bacterial deaminase specific for unmodified A bases and was shown to enable m^6A profiling, including from very limited RNA input amounts (56).

4. Direct RNA sequencing: Several approaches have been described using nanopore direct RNA sequencing technology coupled with artificial intelligence (AI)-based data analysis and modification identification (57–60). Nanopore sequencing has also been used to detect pseudouridine semi-quantitatively (61). This analysis was facilitated by construction of hybrid synthetic RNA templates with base modifications at specific locations. An engineered nanopore structure with a reactive site was shown to be able to discriminate among fourteen different nucleotide monophosphates containing known RNA modifications, showing that highly accurate detection of modified bases is feasible, although this system is not yet configured for sequencing of RNA strands (62).

The NGS approaches have expanded over the last couple of years at the bulk mRNA level. Major limitations are the absence of efficient and widely applicable methods for single-cell modification sequencing as well as the limited expansion of NGS technology on modifications of other RNA species (rRNA, tRNA, circRNA, etc.). Finally, the AI algorithms for nanopore technology and the resolution and noise deconvolution methods for using nanopore for modification-sequence mapping still need refinement, as they can only be applied for a few of the known modifications.

SHORT-TERM GOALS

There are important technological components that must be included in any plan for studying RNA modifications. These include additional investments in sequencing methods using a diversity of approaches, the availability of standard materials for benchmarking, standard operating procedures, and appropriately organized repositories for data. However, additional near-term, foundational tasks must also be undertaken to progress to long-term goals. We must:

1. Create affordable, reliable benchmarking materials that are widely available coupled to accepted community standards for data analysis downstream.
2. Develop easy, affordable, and accessible techniques for quantifying modifications such as N^6-methyladenosine (m^6A) and the other known mRNA modifications.
3. Mine microbial systems for novel enzyme potential.

4. Develop libraries of enzymes for modification-specific reactions–a reverse transcriptase for each modification, endonucleases (toxin/antitoxin) to facilitate single-molecule labeling, and/or RNA-targeting CRISPR/Cas systems.
5. Explore methodologies for structural assessment of modified RNAs (e.g., cross-linking and immunoprecipitation (CLIP)-seq and selective 2'-hydroxyl acylation analyzed by primer extension and mutational profiling (SHAPE-MaP) approaches for modified RNAs).

Proposed methods to address our short-term goals:

1. *Quantification of global levels of RNA modifications*: To address our goal of creating fast, reliable detection of RNA modifications, we need to develop easy, highly quantitative, reproducible, affordable, and expandable methods. Our proposal would be to start with methods for global quantification of a single, abundant modification like m^6A on mRNA, and then expand to other modifications as technology and community standards mature. For an easy, quantitative, and affordable method to quantify m^6A on mRNA, we believe that efforts should focus on qPCR-based approaches (e.g., immuno-PCR), since these are easy to use, highly quantitative, require minimal starting material so they can be used with rare or difficult to obtain samples, and can be adopted by laboratories around the world. Independent of the approach that is ultimately successful for this purpose, we will need clear and robust experimental and data standards that are agreed upon and adopted by the user community.

2. *Positional Information and Next Generation Sequencing approaches*: To obtain the tools to specifically map and sequence all RNA modifications, we propose several tasks. **(A)** We see large potential in mining bacterial systems and/or enzymes used by bacteria as defense mechanisms against other pathogens to identify RNA-targeting enzymes that recognize and modify (or protect) specific RNA motifs harboring individual modifications, like those implemented in eTAM-seq. Another example comes from the bacterial (*Escherichia coli, Mycobacterium tuberculosis*) toxin-antitoxin systems, which include the endoribonuclease - MazF - capable of recognizing m^6A-modified motifs on mRNA (63–66) and also cleaving rRNA (67–69) and certain tRNAs (70). Similar approaches have been used on a global RNA-seq level with techniques like MORE-seq, which determines target sites of MazF (67). **(B)** Recently, two approaches to map and quantify single-base m^6A in the mammalian transcriptome have been introduced. GLORI-seq (54) and eTAM-seq (56) use enzyme-assisted adenosine deamination to discriminate methylated from unmethylated adenosines at a single-nucleotide resolution. These techniques, although they avoid the false positive signals obtained by MazF and the limited MazF cleavage to ACA motifs, cannot discriminate between m^6Am and m^1A, are more expensive than traditional MeRIP-seq and are limited to m^6A. Development of similar enzyme-assisted approaches that discriminate between other modified and unmodified nucleotides at single-base resolution will significantly enhance our understanding of the biological importance and role of RNA modifications in mammalian systems. Finally, **(C)** we need to perform a systematic search for reverse transcriptases or DNA polymerases with RT activity (e.g. KlenTag and Marathon RT (71)) to discover enzymes that can be engineered to detect individual RNA modifications; and **(D)** pursue discovery of RNA-specific CRISPR-Cas systems capable of recognizing and preferentially editing or cleaving modified versus unmodified RNA. The recent discovery of the Class VI CRISPR-Cas system effector, Cas13, shown to target RNA (72–74) makes this approach a promising option.

3. *Single-cell NGS for RNA modifications*: We propose to employ the enzyme-assisted sequencing described above (e.g., MAZTER-seq), by using newly discovered enzymes from bacterial systems (see point 2 above) to cleave single-cell RNA on certain modifications, depending on the recognition specificity of the enzyme. The fragmented RNA could then be barcoded, or adaptor ligated, and sequenced at a single cell level. Since this is still a quite laborious technology with a significant limitation

for the number of cells that can be sequenced, we propose to develop recombinant enzymes as fusion enzymes with endoribonuclease activity and tagmentation capabilities (e.g., MazF-Tn5). These enzymes should be able to generate tagmented, modified RNA fragments that could be pooled and sequenced based on limitless combination of indexes and primers used for tagmentation, a technology reminiscent of combinatorial indexing.

Achieving these goals will require coordinated efforts from many laboratories and disciplines, using multiple complementary or orthogonal methods and approaches pursued in parallel to address the range of modifications in mRNA and their effects on structure and interactions. While we propose initial, proof-of-principle studies focused on mRNA and well-characterized modifications such as m^6A, the methods we establish here should be expandable into other subsets of RNA–tRNA, rRNA, lncRNA–as well as to other RNA modifications.

LONG-TERM GOALS

The challenge of defining the impact of RNA modifications on RNA cellular function is a big task and will likely advance our understanding of RNA-modification biology in multiple directions. These will be punctuated by a series of long-term goals over the next 5-10 years, namely to:

1. Develop single-cell (or low-abundance) modification sequencing to study structure at the single-cell level.
2. Determine impact of RNA modifications on interactions of RNA with proteins (including readers, writers, and erasers of RNA modifications), other RNAs, and DNA sequences.
3. Predict RNA structure based on modifications and sequence, including through use of molecular dynamic simulations and machine learning, and experimental approaches that probe RNA structure.
4. Assess impact of structure on function.

Proposed methods to address our long-term goals.

1. Develop single cell (or low abundance) modification sequencing to study base modifications at the single cell level.

Existing technologies. The advent of single-cell RNA-seq (scRNA-seq) technology has brought about a lowered detection limit for rare RNA species, and facilitated new, previously unimaginable investigations. In conjunction with additional molecular techniques, scRNA-seq can now be coupled to chromatin accessibility, surface protein expression, and even spatial information (75–77). Techniques like the recently released RiboMap have even allowed translation to be monitored in a large subset of genes at spatial resolution across individual cells (78). These applications of scRNA-seq are representative of the potential of sequencing individual RNAs in a single cell and provide encouragement for ultimately reaching the level of the single-cell or even single-molecule epitranscriptome. In fact, a recent example goes so far as to measure m^6A levels *in vivo*, in single large cells (e.g. embryonic stem cells, zebrafish zygotes, and mouse oocytes), suggesting that, in principle, our goal is not so far from reality (79). The recent emergence of third-generation sequencing technologies like Oxford Nanopore direct RNA sequencing is groundbreaking, but it is clear that we still have room to grow. Early work has facilitated sequencing of individual RNAs, albeit with relatively high starting concentrations of RNA; expanded our understanding of RNA processing; and has even allowed detection of some modifications, including m^6A (60,80,81). Unfortunately, error rates remain relatively high, secondary structure of the RNA can be a challenge, and the sensitivity is still lacking in most use cases (5,82–84); however, the recent release of new, direct RNA-seq chemistry has started to address some of these issues (Oxford Nanopore Technologies). Bioinformatic strategies for processing

scRNA-seq and direct RNA sequencing have greatly improved, but to understand RNA modifications of a single molecule within a single cell, work remains (85).

Specific Actions. The first long-term goal we propose is to advance technology to be able to sequence modifications in individual cells and/or from low concentrations of RNA input. The added value for such an advance is obvious, as increased sensitivity of scRNA-seq will certainly increase our ability to detect RNA modifications, particularly rare or novel modifications that may only be observable in disease states. To achieve this goal, more reliable library preparation methods will likely be required, in addition to novel bioinformatic approaches to "deconvolute" the data. We can imagine applications in conjunction with rapidly emerging AI technologies, which we suspect will greatly impact our day-to-day routines in the 5-to-10-year time span. We also anticipate that increases in sensitivity and reliability of Oxford Nanopore direct RNA sequencing, and other platforms, will be required to reach this long-term goal. A major push in the field is already underway, but refined efforts towards defining best practices and providing benchmarking materials will be essential in providing a robust solution. For this purpose, better spike-in controls for modified RNAs will be needed to optimize approaches and more accurately identify RNA modifications in single cells and/or low abundance samples.

Reliance on short-term goals. The improvement of scRNA-seq and direct RNA-seq to detect RNA modifications in low abundance samples like single cells will be built on the foundation established in our short-term goals. It will be essential to develop easy, affordable, and accessible techniques for quantifying modifications such as m^6A as a first use case. We appreciate that the solution to reliable, robust single-cell, single-molecule modification sequencing may require completely novel technology. We expect many advances in this direction to continue to come from mining of microbial systems, which have been repeatedly shown to carry vast potential. As work on understanding microbial communities continues, we expect that novel enzyme activities will be identified, for example, from non-model organisms, particularly the understudied fungi. The development of libraries of enzymes for modification-specific reactions will also likely facilitate new approaches, as has occurred with the examples discussed above.

2. Determine impact of RNA modifications on interactions of RNA with proteins (including readers, writers, and erasers of RNA modifications), other RNAs, and DNA sequences.

Existing technologies. RNA modifications have been studied for decades to great success. Unfortunately, our knowledge begins to wane with the role of RNA modifications in the interactions between a modified RNA and other macromolecules. Examples certainly exist, but the tools to understand these interactions on a large scale remain relatively absent. For example, we know that editing by proteins like adenosine deaminase acting on RNA (ADAR) can result in inosine-containing RNAs with altered innate immune activities (86,87). Modified tRNAs were shown to fit more snuggly into the ribosome, suggesting alterations to interactions between the modified tRNA and ribosomal proteins/RNA (88–90), and even mRNA methylation marks influence binding of downstream factors, including m^6A core machinery and additional peripheral factors (8,91). These examples hint at regulation of biological function, but we also know that RNA modifications can influence RNA structure. Again, the best examples likely come from study of tRNA modifications, where installed modifications have been shown on numerous occasions to stabilize tRNA tertiary structure (92). RNA modifications are also thought to influence RNA:DNA hybrids after damage and during R-loop formation, again hinting that a systemic description of the rules of interaction of the epitranscriptome with all cellular macromolecules holds vast potential in advancing our molecular understanding of the influence of RNA modifications on cellular interactions and function (93).

Specific Actions. To systematically define the influence of RNA modifications on intermolecular interactions, the field will need to strive for several key advances. First, a robust set of biochemical assays must be established and thoroughly tested to facilitate measurement of interactions between individual modified

RNAs and other macromolecules. These initial biochemical assays should be extended to broad-scale analysis, likely requiring efforts to improve large-scale synthesis of modified RNAs for massively parallel reporter assays. Additional efforts in high-throughput synthesis of modifications at many sites simultaneously followed by structure elucidation will also be essential. It will be important to develop targeted RNA editing platforms that can be tunable to further introduce marks and determine structures.

Reliance on short-term goals. To succeed with the second long-term goal, we will need to develop easy, affordable, and accessible techniques for quantifying modifications as described above. The short-term goals described are necessary advances to robustly characterize the importance of modifications in intermolecular interactions. Again, as above, we see potential in mining microbial systems for novel enzyme potential, and we suspect that additional novel solutions will be found within the vast microbial pangenome. Many other tasks must also be completed, but we see value in developing tools that will further facilitate this long-term goal, for example by developing libraries of enzymes for modification-specific reactions, endonucleases to facilitate single-molecule labeling, and RNA-targeting CRISPR/Cas systems that could be used for reading out modified RNAs or even intermolecular interactions.

3. Predict RNA structure based on modifications and sequence, including through use of molecular dynamic simulations and machine learning.

Existing technologies. In recent years, Cryo-EM technology and structural probing methods have greatly improved our understanding of tertiary interactions in RNA structure (94), yet in many cases RNA prediction remains the logical first step in understanding potential function. As such, a wide array of RNA prediction tools has been developed to address this particular challenge (Mfold, ViennaFold/RNAfold, many others) (95,96). These tools tend to rely on thermodynamic models, such as Turner's nearest-neighbor model to predict secondary structures by focusing on nearest-neighbor loops (97). These can then be combined to find the minimum-free energy structure using the Zuker algorithm (98). As we continue to obtain more experimentally validated structures, these predictions are more frequently based on reference molecules, which has improved prediction (99,100). The inclusion of advanced computational approaches is also pushing our prediction capabilities forward, for example by leveraging deep learning and neural networks (97,101–104). For the most part, the role of modifications in influencing tertiary, or even secondary, structure, has been difficult to incorporate into these predictions, but some efforts have been made (reviewed in (105)), including prediction of structures with well-characterized modifications like m^6A (106).

Specific Actions. To successfully predict RNA tertiary structure for RNAs containing modifications, a much larger reference set of experimentally validated RNA structures is needed. As with AlphaFold for proteins, the use of AI can allow for valid, informative predictions with enough input, but our current cohort of RNA structures remains far too limited to saturate the complexity of RNA structures, especially RNA molecules containing modified nucleotides. In achieving this goal, we will continue to need organized, curated databases of high-quality structures to efficiently integrate large amounts of data into better learning predictions. In fact, some amazing examples of RNA structure and dynamics have already been predicted (107), but these extremely computationally heavy predictions already push our computational power to the limit, suggesting additional advances are necessary. With recent successes in quantum computing, we expect that this technology, or even another currently unappreciated approach, will lead to sufficient resources for better predictions in the future. Although beyond the scope of this paper, performing such energy intensive computational activities should be developed with climate neutral strategies in mind.

Reliance on short-term goals. The recent progress in approaches like CLIP-seq (108) and SHAPE-MaP; (109,110) have facilitated a more rapid assessment of RNA structure in the laboratory, but additional approaches to more rapidly assess these structures in conjunction with RNA modifications are certainly needed. Our short-term goal in exploring additional methodologies for structural assessment of modified

RNAs will be essential to improving predictions. Clearly, a better toolbox of benchmarking materials will also facilitate the structural determination of modified RNAs, allowing for the field to move closer to a state where an AlphaFold-like solution will be able to robustly, rapidly predict all RNA molecules.

4. Assess impact of structure on function (as controlled by modifications).

Existing technologies. The fourth long-term goal strives to provide a resolution for the overall goal of understanding the functional consequences of RNA editing on RNA function, particularly during disease. RNA modifications are already known to control normal cellular homeostatic processes (111), contribute to regulation or dysregulation of cellular processes as during cancers (112), and even influence the outcome of infection, both by regulating host immune processes and pathogen virulence (113). Modifications of tRNAs control translational fidelity (114), editing marks installed by ADAR and APOBEC3 proteins control autoimmunity and antiviral activity (115,116), and ribosomal RNAs are severely dysfunctional in the absence of modifications resulting in devastating human diseases (Reviewed in (117)). If we focus only on the m^6A RNA modification as one example, we already know a lot (118). This prolific mark has clear roles in the function of mRNAs as well as noncoding RNAs, but also contributes to regulation of housekeeping RNAs like rRNA. These functions collectively contribute to regulation of chromatin architecture, transcriptional regulation, and genome stability (119). In fact, m^6A methylation can also directly influence protein binding, for example by preventing binding of proteins to particular sites. A clear example of this regulation comes from *C. elegans*, where environmental growth conditions trigger the METT-10 writer to modify the 3' splice site of S-adenosylmethionine synthetase to prevent binding of the essential splicing factor U2AF35 and splicing (120). Many other examples show similar regulation of RNA modifications across biology, yet despite this clear importance, in most situations, RNA editing is barely considered in regulation.

Specific Actions. Should our dream come to fruition, we expect to have the tools to produce a detailed description of RNA modifications on a single molecule of RNA in a single cell, which would facilitate the elucidation of cellular consequences of such modifications. Clearly a tall order, smaller steps again must be taken prior to reach this comprehensive understanding. We believe that striving to define a single, simple system more fully (e.g., a cell type selected from Encode Tier 1) will ultimately facilitate our larger goals. By perturbing such a system (e.g., with nutrient deprivation, genetic manipulation, temperature change, infection), we expect to understand the dynamics of modifications and the cellular consequences of RNA modifications. We expect that advances along the way will push analyses like these in many directions, beyond single cell types to tissues or even whole organisms. By establishing workflows and techniques for a complete description of RNA modifications impact on structure to control function in one cell type, we expect to facilitate easier investigations more broadly.

Reliance on short-term goals. Clearly the final long-term goal will rely on input from many of the other pieces of our approach. The success of the short-term goals, especially the creation of easy, affordable, and accessible techniques for quantifying modifications such as N^6-methyladenosine (m^6A) will be critical in reaching a fully described cellular example of RNA modification impact on function. At the moment, it is not exactly clear how to achieve such a goal, but we expect that advancements in enzymatic studies, including novel activities mined from microbes, will facilitate our success.

FUTURE TECHNOLOGIES

Several technologies already exist that can be leveraged for the success of this project. These include NMR, Cryo-EM, X-Ray Crystallography, multiple sequencing strategies (including mass spectrometry, nanopore sequencing, and affinity capture methods), and a variety of bioinformatics approaches (e.g., machine learning) as described above. An important task to realize these goals will be to establish a

strategy to incorporate existing technologies more robustly, support collaborations, promote the scientific exchange of ideas and foster development of new technologies as well. The technology does not exist to fully address our stated challenge, but we can at least partially envision the advances required to reach these goals. We will certainly need alternative enzyme activities, which we can envisage being revealed through exploration of the microbial world or synthetic efforts to evolve or design new functionalities in existing enzymatic scaffolds. Among these activities, we anticipate the need for enzymes capable of barcoding modified RNA. Many laboratories and companies are already exploring this space, and we are cautiously optimistic that we are on the cusp of a revolution in RNA enzymology. Although we expect new functionalities, and likely entirely new solutions, we do expect advances to continue from current workhorses, particularly with the rapid advancement in technologies surrounding the use of CRISPR-Cas-like approaches to specifically add/remove modifications. Synthetic biology tools and programmable RNA editing technologies may help us to establish/generate standardized reagents (reviewed in (121,122)).

We believe that the strategic, specific, and reliable installation of RNA modifications on synthetic RNA molecules is a technology that is being advanced in many ways, but still needs additional effort to realize a robust method to create useful standards for the types of experiments we are proposing. Synthesis of long RNAs with multiple modifications introduced at specified positions at proper stoichiometry would be a big advantage but remains nearly impossible. There are some promising leads in this direction, for example with techniques relying on enzymatic splint ligation (123). With all these gains in the wet lab, we will also need to keep pace with our computational infrastructure.

If Moore's law continues to hold true, we expect to see advances in computing power, as well as cleverly designed databases to support sharing of information on RNA modifications, structures, and interactions with appropriate metadata. Many discussions are currently underway, but key among them will be resources to maintain databases over long stretches of time, especially as computational techniques and supercomputing capabilities balloon the amount of data collected from each experiment. Finally, we expect that optimization of experimental design may also result in improvements in data output. As just one example, new approaches are constantly being explored to increase depth of coverage as sequencing the same sample deeper does not always reveal new information (e.g., modification sites or unique RNA molecules). We therefore expect that additional approaches will be developed to extract information from the datasets we have in hand, as well as to improve collection in new experiments.

SOCIETAL RELEVANCE

Stakeholders: Depending upon where any biomedical research falls on the spectrum of basic to clinical, stakeholders in a research endeavor can include collaborators, peer reviewers, research institutions, funding agencies, clinicians and professional associations, patients, and policy makers (124–126). Collaborators can be actively engaged in a research endeavor, provide an important resource, or act in an advisory capacity. Peer reviewers, whether their purview is study protocols, manuscripts, or applications for funding, are engaged as stakeholders in upholding research rigor and ethical conduct. Research institutions, whether for-profit or not-for-profit entities, are a fundamental driver of research innovation and development. These institutions are often early incubators of patentable technologies developed from research. Research institutions, particularly those with graduate programs and professional schools, invest heavily in equipment, facilities, and personnel in support of the research activities of their faculty (127). Biomedical and clinical research, especially those studies involving clinical trials, can be expensive. Much of this research is funded by federal agencies such as the National Science Foundation, the National Institutes of Health, the U.S. Departments of Energy and Defense but organizations such as foundations and philanthropies support smaller research studies too. Patients are increasingly seen as stakeholders in research involving their illnesses and medical care. Patient input provides important information for the development of essential aspects of a clinical study such as a study's design and outcome measures. The

importance of patient stakeholder engagement has been recognized and is oftentimes mandated or strongly endorsed by research funders and journals (128). Policy makers include government agencies at the federal, state, and local levels as well as other funders of research. Policies based upon the interpretation of outcomes of major research endeavors can have a significant impact on public health (for example, vaccine development for COVID-19). Policy makers also determine research priorities and funding levels.

Ultimately, the more heavily invested - intellectually or financially - a collaborator, an institution, a funder, etc. is in a research project, the greater the stake that individual or group may feel they have in the success of that project.

*Beneficiaries: The b*eneficiaries of biomedical research would be wide ranging, from basic researchers, to patients, to local and national governments, in terms of potential economic impact. In the short term, other basic researchers would benefit the most quickly from improved technologies in RNA structure prediction, molecular interaction detection, and direct RNA sequencing. For other groups pursuing clinical applications, the benefit will come in the long term after requisite steps such as developing strategies to promote or block RNA modification-directed processes, clinical trials, and new drug preclinical and clinical development. In all cases, near-term and long-term benefit is dependent on the dissemination and adoption of any novel, cutting-edge technologies that are developed (129).

Workforce, Educational, and Societal Considerations: There is disagreement within the scientific community on the “benefit” of research and development. Some express strong views that science and technology progress, when enabled by regressive science policy practices, may make life harder for people, especially those already marginalized by class, gender, race, occupation, and location. An example cited is that scientific innovation such as new therapeutics mainly benefits those that can afford it (130). Others argue that public investment in science translates to public use of science–a “benefit”. One such study analyzed the scientific literature and identified positive correlations between funding in a research area and scientific use. This extended to public use as well (131). At minimum, stronger public engagement in research planning and in the discussion of research outcomes may increase public perception of the importance and positive impact of RNA research on human health and medicine.

Workforce development, interdisciplinary training, and diversity and inclusion are certainly critical need areas discussed at multiple, recent scientific venues including a May 2022 workshop organized by the NIH Institutes, NHGRI and NIEHS, entitled “Capturing RNA Sequence and Transcript Diversity, From Technology Innovation to Clinical Application” (132). The following were among several relevant points captured in the executive summary and report for the workshop.

1. Support of undergraduate and graduate-level training, particularly at minority-serving institutions, could help improve diversity within the biomedical research community.
2. Training in both experimental methods (“wet lab”) and computational biology and bioinformatics (“dry lab”) is critical for the advancement and widespread adoption of RNA technologies.
3. Easily accessible, quality, low (or no)-cost, online training resources on technology use and data analysis could be a considerable driver in technology adoption.
4. Enhancing career pathways for all groups, including those underrepresented in biomedical research, is important. Considerations include identifying current successful career enhancement approaches that could be scaled up, and determining resources and strategies needed to address ongoing barriers to advancement.

CONCLUDING REMARKS

Anticipated Outcome

The end goal of the project is to understand how RNA modifications affect RNA function, including processing and interactions with other macromolecules on a transcriptome-wide scale. The completeness of addressing this challenge can be measured by the RNA community's ability to establish standards and common analysis pipelines to allow for a high degree of collaboration. This can also be supported by the level of usage of public data and sharing of data between groups. To accomplish this goal, we need an even higher degree of open data policies and data sharing between laboratories working in all aspects of the biology of RNA modifications.

We can assess the progress of single-cell technologies directly and in parallel follow the depth and accuracy possible for mapping and quantification of the various RNA modifications. A more challenging aspect to assess is the prediction of RNA structure and the influence of RNA modifications on structure and interactions with macromolecules. While the field is expected to continuously progress, when can we really claim that we have reached our goal? Certainly, when tertiary structure can be predicted from primary sequence, and the impact of a subset of the predominant mRNA modifications are faithfully incorporated in predictions that are subsequently experimentally validated, then we have come a long way. Adding the ability to predict dynamic interactions with proteins accurately and reliably, especially proteins involved in RNA processing on a large scale, will bring us across the finish line.

The four long-term goals proposed will have tremendous impact on the RNA community, if accomplished, and will allow an unprecedented insight into the dynamics of RNA function and biological importance of RNA modifications. The development of single-cell knowledge of RNA modification identity and stoichiometry will reveal novel regulatory functions for subsets of RNA transcripts previously thought to be identical based on sequence information. With the addition of RNA modification information, we are likely to appreciate new flexible and dynamic conformations in these RNAs, in part mediated by differential RNA modification, which can mediate a multitude of previously unappreciated functions.

The ability to predict tertiary structure of RNA based on sequence and modification pattern will be a breakthrough for RNA research and will help bring RNA as an attractive molecule into several other disciplines. The ability to faithfully predict dynamic and context-dependent protein interactions will reveal new details of molecular biology with interest for RNA biologists, biochemists, protein researchers, and cell biologists, whereas an approach to predict RNA interaction with small molecules on a high-level would revolutionize the potential of RNA medicine and provide new candidate targets for a plethora of diseases.

Conclusion

The Human Genome Project revealed the blueprint for building a human; however, the DNA sequence is just the beginning. We now must strive to understand the role of RNA in this construction process, and fully elucidate the function of RNA in the cell, in all its modalities. The modifications introduced during RNA metabolism are considered by many to be a whole new level of gene expression regulation. By deciphering the regulatory code of RNA modifications, we will more completely understand the full potential of this macromolecule, unlock new functionalities for RNA, and likely provide novel therapeutic ingress points to the tremendous benefit of humanity. It is an undertaking worthy of continued research effort and investment.

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