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Measuring RNA Structure Transcriptome-Wide with icSHAPE

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Abstract

RNA molecules can be found at the heart of nearly every aspect of gene regulation: from gene expression to protein translation. The ability of RNA molecules to fold into intricate structures guides their function. Chemical methods to measure RNA structure have been part of the RNA biologists toolkit for several decades. These methods, although often cumbersome and difficult to perform on large RNAs, are notable for their accuracy and precision of structural measurements. Recent extension of these methods to transcriptome-wide analyses has opened the door to interrogating the structure of complete RNA molecules inside cells. Within this manuscript we describe the biochemical basis for the methodology behind a novel technology, icSHAPE, which measures RNA flexibility and single-strandedness in RNA. Novel methods such as icSHAPE have greatly expanded our understanding of RNA function and have paved the way to expansive analyses of large groups of RNA structures as they function inside the native environment of the cell.

Keywords

RNA structure; icSHAPE; Chemical Probing; RNA sequencing

1. Introduction

For several decades it was thought that RNA molecules act as messengers between the genetic code and protein translation[1–5]. However, recent sequencing of complex transcriptomes has revealed that almost the entirety of genomes is transcribed into RNA molecules that do not encode for proteins[6, 7]. These results have challenged the aforementioned notion and opened the door to the possibility that many RNA molecules perform function outside of encoding for proteins.

Recent investigations into the functions of RNA have revealed they play important roles in almost every facet of gene regulation. For example, long non-coding RNAs can control gene

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expression by interacting with chromatin remodeling proteins and DNA medication proteins to regulate gene expression[3]. Also, long non-coding RNA molecules can directly control RNA steady state levels[4]. Small non-coding RNAs can interact with messenger RNAs and control protein synthesis or effect RNA decay[8]. Non-coding portions of messenger RNAs can interact with and bind to small molecules to control protein expression through feedback loops[9, 10]. These careful analyses of RNA function has greatly expanded our understanding of RNA biology and underscored the need to gain a more mechanistic understanding of how RNA can perform such a myriad of biological functions.

RNA molecules can fold into intricate secondary and tertiary structures, which guide their ability to interact with themselves and other trans-acting biomolecules[11]. Identifying and accurately measuring the structural elements used by RNA to control their function has been an area of active research for some time. Early emphasis was placed on utilizing RNase enzymes, which can recognize and cleave at either single-stranded or double stranded regions within an RNA; sites of RNA cleavage can be identified using denaturing gel electrophoresis[12, 13].

Chemical mapping can be used to read out RNA structure. Direct methylation of the RNA through reaction with dimethylsulfate, hydroxyl radical cleavage of solvent exposed residues, hydroxyl acylation by solution electrophiles, or by metal catalyzed cleavage are currently the most rigorous methods for determining the secondary and tertiary structure of RNAs.[14] While these methods are quite useful for traditional probing techniques, many of these chemical methods are not generalizable and often have biases in their reactivities; this limits the ability to obtain structural information for every nucleotide.

A general chemical functionality that all RNAs have is a 2'-hydroxyl group in the ribose ring. 2'-hydroxyl is more chemically reactive at single-stranded positions than at nucleotides constrained by base pairing.[15, 16] Reagents that modify RNA as a function of RNA 2' hydroxyl reactivity can be used to read out RNA structure by reverse-transcription primer extension in experiments termed RNA SHAPE (Selective 2' Hydroxyl Acylation and Primer Extension, Figure 1, A).[15, 16] SHAPE has been proven to be highly accurate at identifying single stranded residues and can also be merged with structure prediction programs to obtain highly accurate secondary structure models.[17] SHAPE is widely used and has been proven to be a very powerful at measuring RNA structure *in vitro*

2. A transition of SHAPE from *in vitro* into living cells

To transform SHAPE to be performed in vivo the first challenge was to develop reagents that could actually be used inside cells. Traditional SHAPE reagents provide robust signal *in vitro*; however, they have recently been found to have limited utility in complex cellular environments[18].

To overcome the limitations of SHAPE probing *in vivo* we instead developed SHAPE reagents eventually settling on an acylimdazole core (Figure 1, B) that provides both increased solubility and slower reaction kinetics of acylation; both of these parameters are very useful for the design of in-cell reagents to measure RNA structure with SHAPE[19].

New acylimidazole reagents were benchmarked against the 5S ribosomal RNA (5SrRNA). 5SrRNA was used for the first iteration because it is highly expressed and its structure had been studied at great length. *In vitro* comparison of NAI (Figure 1, B outlines the synthesis of NAI), an acylimidazole reagent with a nicotinoyl core demonstrated its ability to measure 2'-OH flexibility with high accuracy, similar to canonical SHAPE anhydride reagents used previously. It is worth noting that in the first iteration of these probes a less-reactive acylimidazole with a furoic acid scaffold was much less-reactive. This difference is likely due to nicotinoyl ring being more electron withdrawing, increasing the electrophilicity of the carbonyl carbon. In contrast the furoyl ring is not as electron withdrawing and may contain a resonance form that donates electron density into the carbonyl carbon, rendering it less reactive to 2'-OH acylation. Thee lessons are important for understanding the limitations of SHAPE reagent design.

Testing in mammalian cells demonstrated that NAI can robustly modify 5SrRNA inside cells and revealed differences in modification patterns suggesting that NAI was measuring protein binding or changes in structure due to the RNA-RNA interactions in the cellular environment (Figure 1, C-E). Closer inspection revealed that nearly all sites of differential reactivity were due to protein binding or interactions with 18SrRNA. Furthermore, the *in vivo* SHAPE reactivity pattern closely resembled the b-factor values (thermal motion) from published crystal structures, further demonstrating the power of *in vivo* SHAPE to accurately measure the idiosyncrasies associated with RNA structures as they would exist inside living cells.

NAI was shown to be able to measure RNA structure in many different cell types and organisms, including yeast, bacteria, and fly cells. Additionally, NAI is able to penetrate into the nucleus of mammalian cells, as reverse transcription on nuclear associated RNAs demonstrated RNA modification consistent with the known structures of some spliceosomal RNAs. Overall, these data demonstrate that NAI is a robust reagent for measuring RNA structure inside many different cellular environments and suggest NAI will be proven to be very powerful for measuring RNA structure in native environments.

3. Transcriptome-wide measurements of RNA structure with icSHAPE

The merger of footprinting with deep sequencing is progressing at a very fast rate. In similar fashion to the traditional methods many of the first transitions were using nuclease footprinting. First performed on the yeast transcriptome, nuclease footprinting was the first to demonstrate the feasibility of the approach[20]. Nuclease digestion of RNA has been extended to plant transcriptomes and other organisms. These results have revealed that there are unique structural signatures at translation start sites and sites of posttranscriptional regulation, such as RNA-binding protein binding sites[21–23]. Although useful, the same limitations described above apply in such experiments, and the inability to use nuclease digestion *in vivo* further limits the utility of in-cell measurements of RNA structure.

DMS probing was the first to be extended into a transcriptome-wide method of measuring structure inside cells [24, 25]. These studies revealed the power of single-nucleotide resolution measurements. DMS profiling in Arabidopsis seedlings revealed RNA structure

signatures associated with translation start sites and also demonstrated that stress-response genes may have pre-programed structure states to regulation translation[26]. DMS probing in yeast and mammalian cells demonstrated that many parts of RNA structure can be dynamic and are under constant remodeling with the help of helicase proteins that can mold RNA structures[24]. The key limitation with DMS is that in both cases nearly 70% of the structural information comes from a single residue, adenosine. This result suggested at the time that if a more un-biased method, such as SHAPE, would be extended in the same way, it would be very powerful.

Recent efforts have been focused on extending this technique to transcriptome-wide measurements. The first reported use of SHAPE with deep sequencing reported that SHAPE reactivities, derived from deep sequencing, could be directly compared with those from traditional footprinting assays[27]. In this case the sites of RT-stop were cloned and identified by deep sequencing (Figure 2, A). SHAPE-Seq accurately inferred secondary and tertiary structural information, detect subtle conformational changes due to single nucleotide point mutations, and simultaneously measure the structures of a complex pool of different RNA molecules.

A modified SHAPE protocol, termed SHAPE-MaP was also developed[28, 29]. SHAPE-MaP works by taking advantage of the ability of reverse transcription enzymes to incorporate nucleotide mutations at sites of acylation. In this case the modifications can be used to map the reactivity, instead of RT-stops (Figure 2, B). Random priming can be used for RT. This is an important point because it negates the need of adapter ligation, which can introduce biases into the data, due to enzyme-mediated ligation[30]. SHAPE-MaP–guided modeling identified greater than 90% of accepted base pairs in complex RNAs of known structures, and was used to derive a new structural model for the HIV-1 RNA genome. Overall, these exciting results demonstrated new multiple ways to transition SHAPE from a traditional chemical probing method to sequencing.

Each of these early SHAPE efforts was done on a relatively small and simple RNA pool and was performed *in vitro* on a set of transcribed RNA. The major hurdle we predicted for successful transcriptome-wide analysis of SHAPE was to find a way to get over low signal-to-noise ratios from the data. SHAPE experiments traditionally rely on reverse transcription of modified RNA to generate cDNAs for analysis. One major caveat to this approach is that under single-hit kinetics many of the RNA molecules are in fact not modified. This can introduce false positives during the RT step, which will result in alterations in the conclusions from the data[31, 32]. To overcome this obstacle we hypothesized that enrichment for modification sites would decrease the noise and enrich for sites of SHAPE modification. Toward that end, we designed a dual reagent (NAI-N_3), which can acylate RNA just like prior acyl imidazole reagents, but could also be enriched through strain-promoted alkyne-azide cycloaddition (SPAAC). We termed this approach *in vivo* click selective 2′-hydroxyl acylation and profiling experiment (icSHAPE, Figure 2, C)[31]. As such, modified sites can be appended with a biotin molecule and then enriched through streptavidin binding. We demonstrated that this approach can not only enrich for sites of modification, but also deplete for sites of spurious RT stops. In parallel to our efforts another group demonstrated that enrichment of SHAPE stops can greatly increase the signal-to-noise

level in RT experiments[32]. Overall, the design of new SHAPE reagents and the accompanying protocol for library generation (Figure 2, D) were critical to the success of the development of icSHAPE.

The icSHAPE approach proved very robust at identifying unique structural aspects of translation start sites as well as ribosome pause sites, revealing novel structural insights into how translation start site selection may be controlled through RNA structure. Further, icSHAPE analysis when merged with orthogonal experiments identifying RNA-protein interactions or RNA modifications could be utilized to predict these interactions on a transcriptome-wide scale. icSHAPE may find wide-spread utility in large scale analysis of the mechanisms controlling RNA biology and function inside the cellular environment. The complementarity of different chemical methods (DMS and SHAPE) for measuring RNA structure is sure to find synergy in the different analyses for an overall view of how RNA structure controls biology.

4. Important notes on the data generated from icSHAPE

icSHAPE data is generated from sequencing a large amont of RT stops (cDNAs) and mapping them back to a referece genome or transcriptome. As with every sequencing experiment there are a few parameters that must be taken into consideration when deigning the experiment and also when interpreting the quality of the data. For human transcriptomes we utilize approximately 500ng of poly(A)$^+$ or ribosome-depleted RNA for the input. This amount of RNA is useful for generating icSHAPE scores for >13,000 transcripts. To estimate the sequencing depth needed, users should be mindful of the complexity of their RNA pool. For example, a smaller trancriptome (bacteria), we estimate ~50million reads should be useful for a reasonable representation of the total RNA. For human transcriptomes, we recommend ~150–200million reads to have high and full coverage of the majority of enriched RNAs.

Each icSHAPE library has a built in positive control: ribosomal RNA. Although we traditionally perform polyA enrichment, still there is a significant amount of rRNA in the samples (~5–8%). The users can use the reads from rRNA and compare to manual footprinting to test how well their structure profile matches that from traditional RNA structure probing methods. As an alternative, users can utilize positive control, well folded RNAs, in the library preparation.

For reproducibility measurements two criteria can be evaluated: (1) the expression level of the RNA between replicates and (2) the RT-stop reproducibility between replicates. The former should be an estimate of the coverage of the samples and how well different types of RNAs are represented in the total pool of RNAs. Agreement of RT stops should also be very informative as to the accuracy of icSHAPE. The DMSO (input) samples should be more randomly distributed and have lower agreement for RT stops. In contrast, the icSHAPE samples should have a higher level of agreement because there is a selection for RT stops by the enrichment of acylated hydroxyls.

5. Merger of icSHAPE with RNA points of interest

A major goal of large scale icSHAPE analysis is the merger of such data with orthogonal datasets, such as RNA modification or protein binding sites. The initial analyses of icSHAPE data proved to be very fruitful in this regard as we were able to identify sites of protein binding and RNA modification.

As an example data analysis can be performed with RNA binding proteins. RNA binding proteins are critical for the regulation of every RNA from transcription to decay. For our example data we utilized the RNA binding protein Rbfox2, which is an RBP that can bind to regions in the 3'-UTRs of RNAs and regulate their decay. For such analyses we relied on publically available cross-linking immunoprecipitation (CLIP data) to compare to the icSHAPE data (Figure 3, A)[31]. We calculated metagene structure profile around different functional sits by averaging all valid reactivity score 25 nucleotides upstream and downstream of the binding sites of RNA-binding proteins Rbfox2. As shown in Figure 3, B, this revealed that the binding sites of Rbfox2 have unique structural features when comparing the icSHAPE profiles of RNA inside and outside cells. This data demonstrates that icSHAPE cen be used to footpring RNA binding proteins, transcriptome wide. For further analysis of icSHAPE datasets, additional CLIP datasets for over 300 RNA binding proteins, is now publically available at https://www.encodeproject.org/.

To go a step further, icSHAPE data can also be used to elucidate the mechanistic basis of RNA structure in controlling posttranscriptional interactions and RNA biology. icSHAPE data can also be compared to RNA methylation profiling and we used this data to illustrate the power of icSHAPE profiling in understanding the structural basis of control for RNA methylation (Figure 3, C). For such a comparison we generated our own data for m6A with antibody m6A profiling[31]. 10 nucleotides upstream and downstream of the RNA methylation m^6A site were interrogated. Our initial data (Represented in Figure 3, D in red) revealed an icSHAPE profile suggesting single strandedness. However, this posed two possible models: 1) the single stranded profile was the result of modification or 2) the single stranded profile is necessary for recognition by the methyltransferase for modification. To distinguish between these possibilities the key methyltransferase METTL3 was knocked out using CRISPR-Cas9. icSHAPE profiling at the same sites revealed that the structure profile in the knockout cells was double stranded, suggesting a model in which double stranded RNA is recognized by the methyltransferase. At about the same time a more focused, single gene structure profiling, also revealed that methylation occurs on double stranded RNA molecules[33]. As such icSHAPE can be used, with additional datasets, to gain structural insight into the mechanisms performed on RNA.

6. Future directions on datasets and further comparisons to be done with the method

The initial RNA structure profiling performed with icSHAPE focused on developing a robust method for interrogation of RNA structure using the SHAPE chemistry. Additional analyses will need to be done in a more comprehensive way to better understand the transition from chemical modification to sequencing signal. For sure all the manipulation

and enrichment steps introduce a level of bias that blurs the signal from the raw RNA modification step to sequencing analyses.

Additional work should be done to better understand how icSHAPE can be merged with other datasets to understand the role of RNA structure in controlling posttranscriptional pathways. Additional protein CLIP datasets and RNA modification datasets continue to come online. Other modifications beyond m6A also continue to be profiled. As such, icSHAPE is poised to continue as a powerful method for interrogating RNA structure on a transcriptome-wide level.

Acknowledgments

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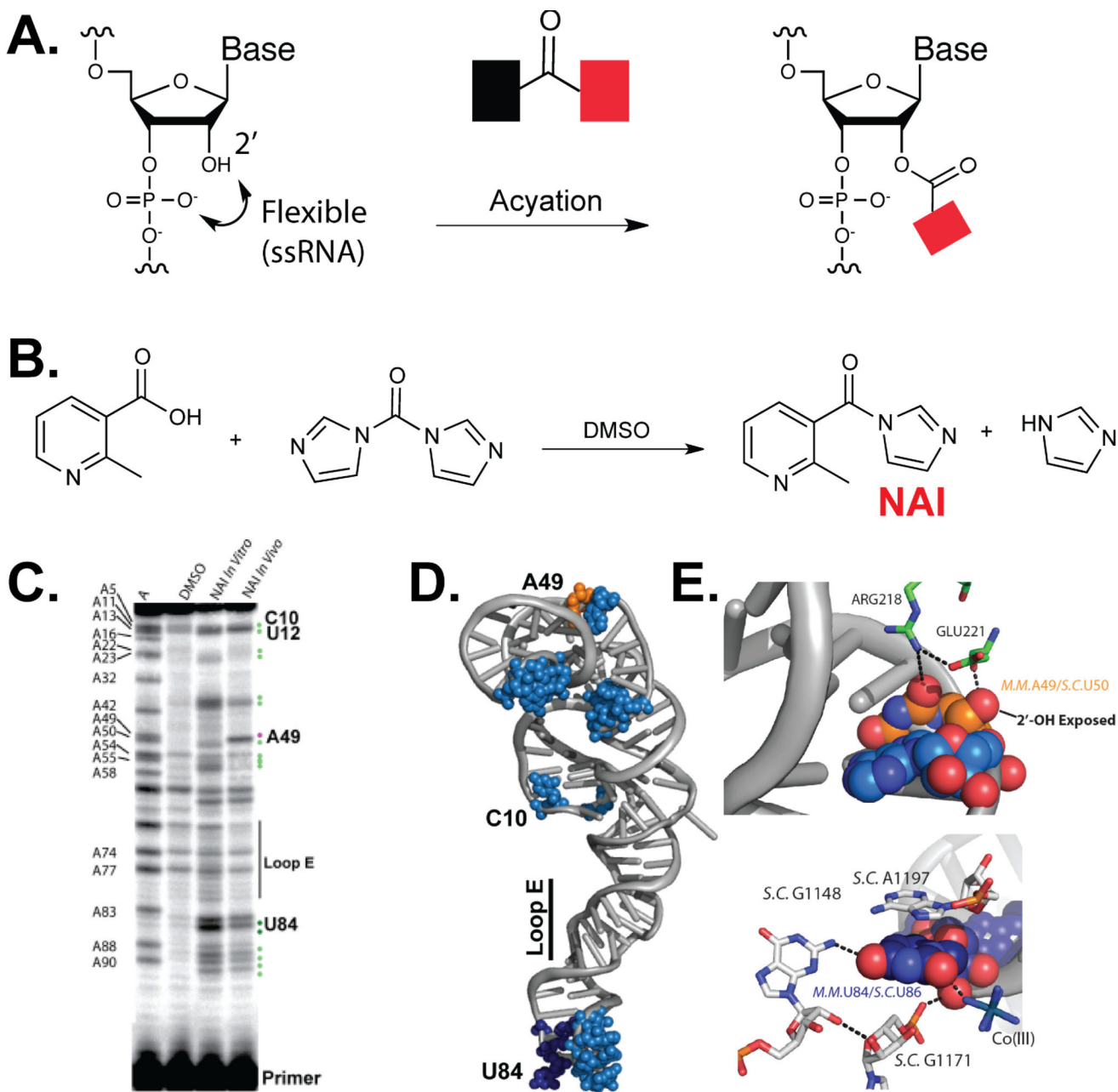
Figure 1. SHAPE and reagents to extend SHAPE to inside cells

A. Schematic of the SHAPE acylation reaction at single stranded residues. B. Chemical reaction to prepare NAI. C. Typical results for 5SrRNA SHAPE probing. D. Difference SHAPE map for 5SrRNA SHAPE probing. Residues in blue are less reactive in vivo. Orange is more reactive *in vivo*. E. Close up structural analysis of 5SrRNA difference SHAPE reactivity.
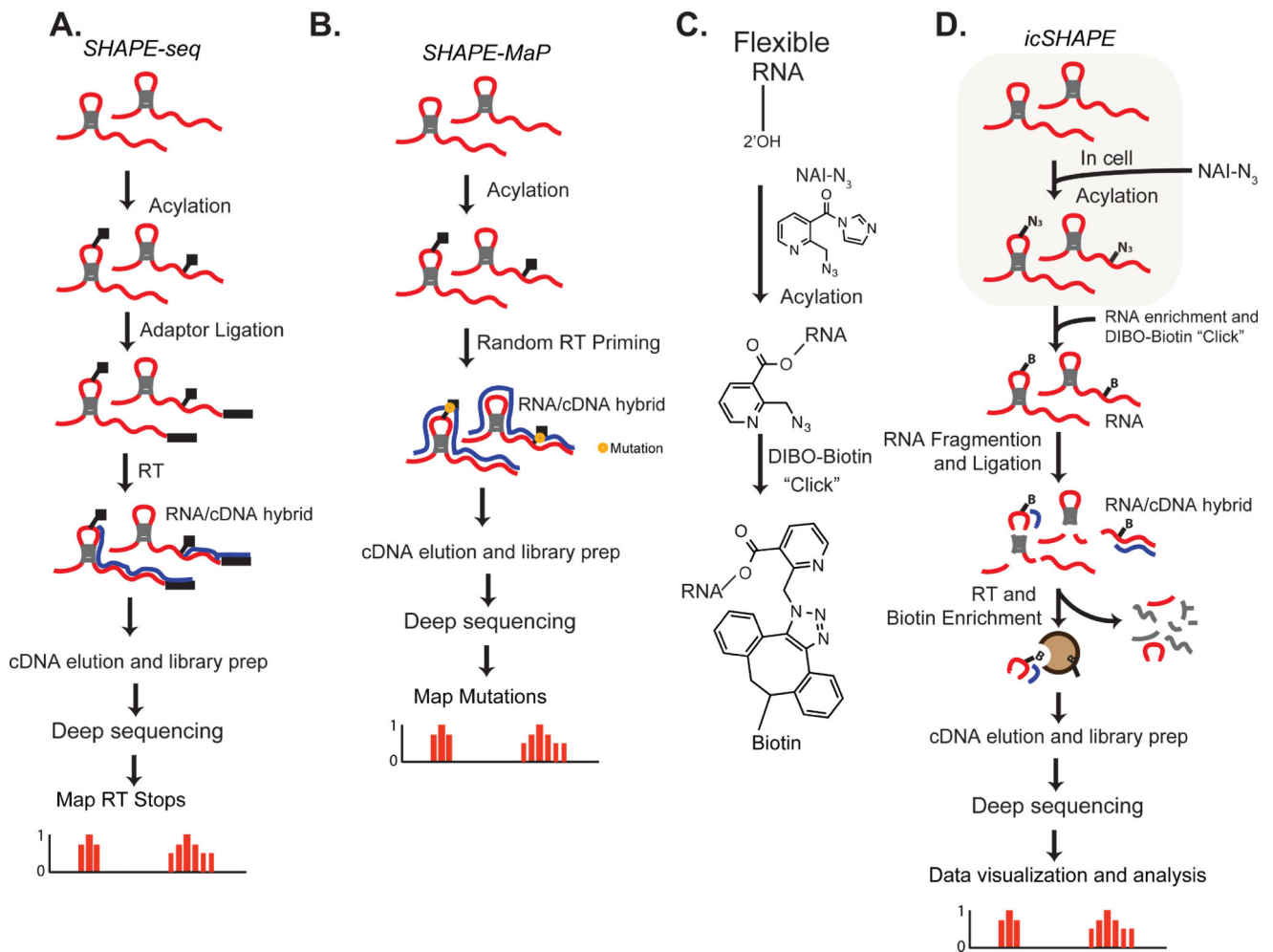
Figure 2. icSHAPE protocol

A. Schematic for SHAPE-Seq. B. Schematic for SHAPE-MaP C. Schematic of the chemical reactions in icSHAPE protocol. D. Overview of the protocol to generate sequencing libraries using icSHAPE.
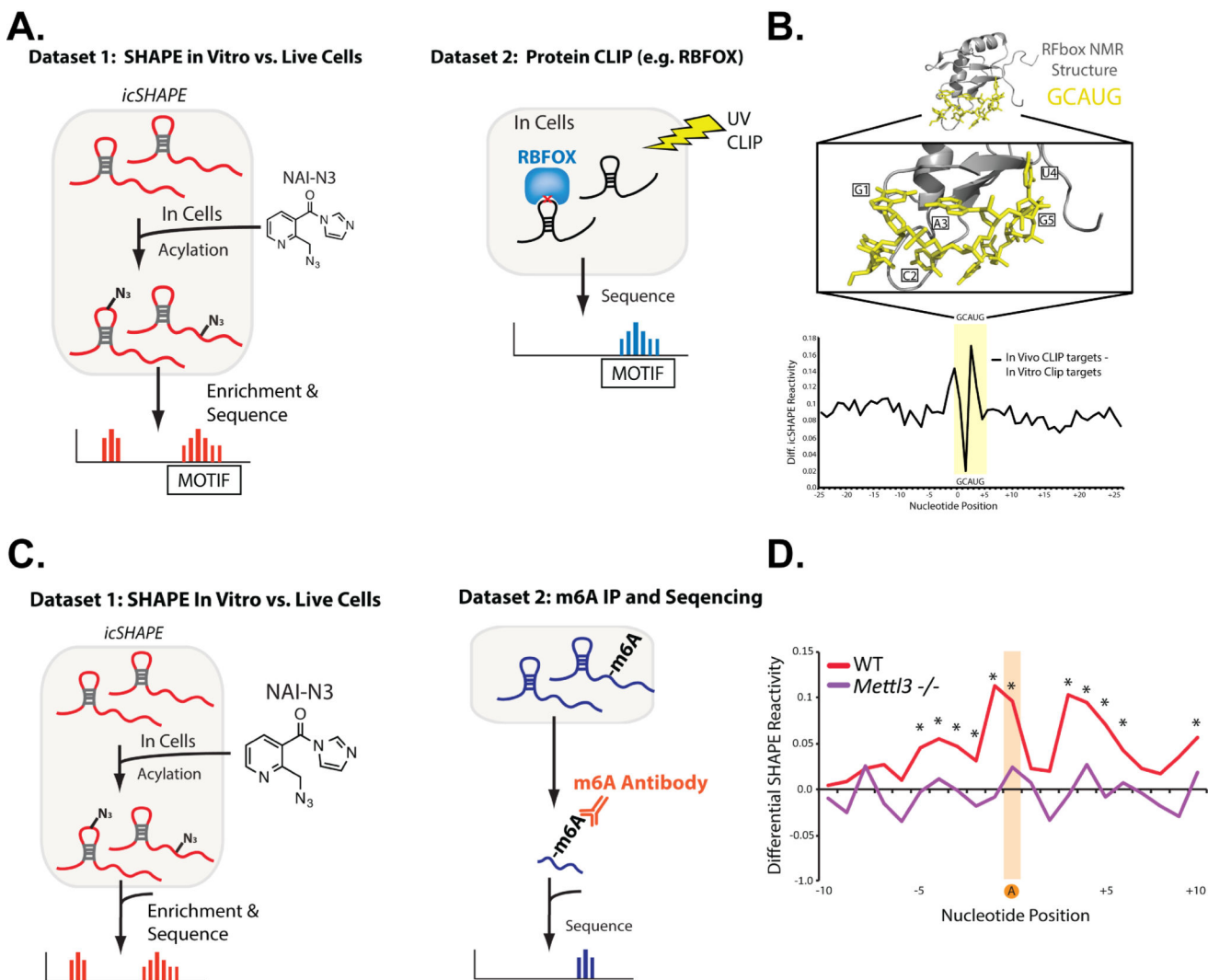
Figure 3. Representative data from compared datasets for icSHAPE and RNA-protein interactions and RNA modification
(A) Cartoon demonstrating the comparison of icSHAPE data and RBFOX CLIP data. (B) Data representation of the icSHAPE structure profile and its match to the RBFOX-RNA structure (C) Cartoon demonstrating the comparison of icSHAPE data and m6A methylation profiling. (D) Data representation of the icSHAPE structure profile at m6A sites in WT and Mettl3 KO cells.