SnapShot: RNA Structure Probing Technologies

Cell

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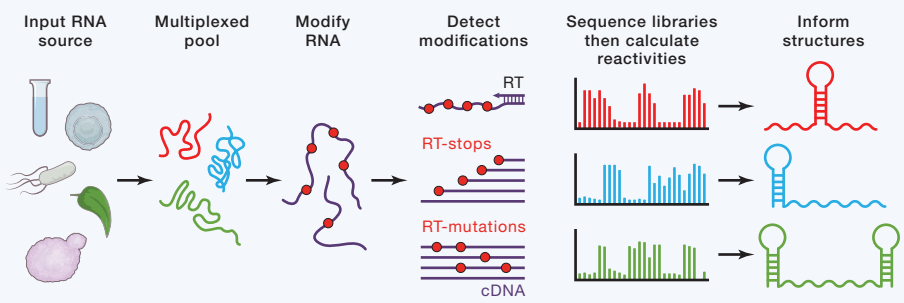
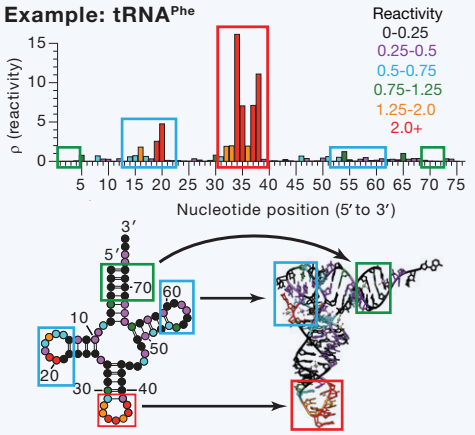
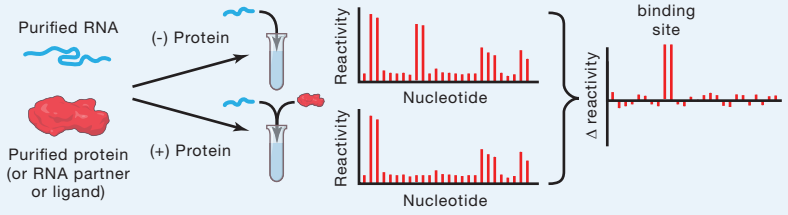
Overview of chemical probing workflow



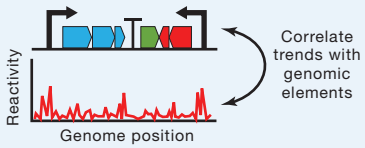
Example: tRNA^{Phe}



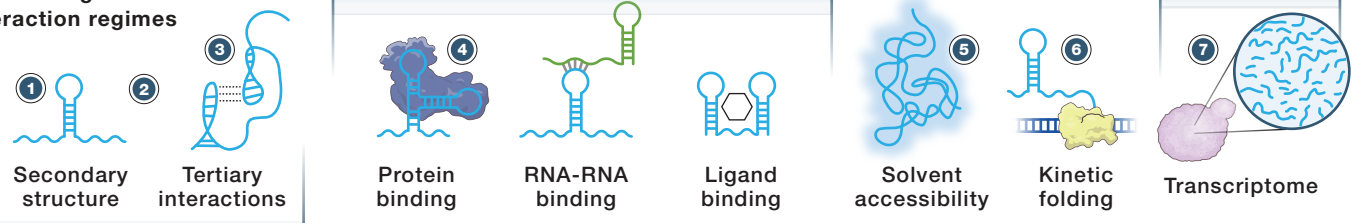
Comparison experiments



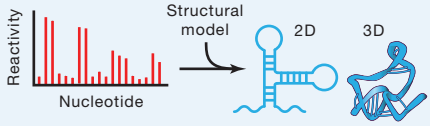
Genome-wide structure analysis



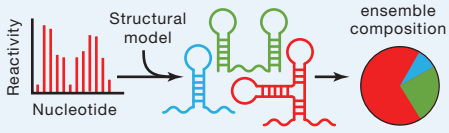
RNA folding and interaction regimes



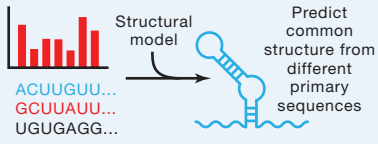
2D/3D structure prediction



Predicting ensembles of structures



Comparative structure prediction



Biological question	Comparison experiments	Structural mechanism questions
1. What is the secondary structure of the RNA? Does it contain long-range interactions or subdomains?	*In vitro*: full-length RNA vs. subdomains probed individually	Are there subdomains and, do they have defined/conserved structures?
2. How does the structure change as the RNA is thermally unfolded?	*In vitro* or *in vivo*: probe over a range of temperatures	What is the structural order of melting? Does melting expose functional sequences?
3. Does the RNA form tertiary structures?	*In vitro*: probe over a range of [Mg++]	Are the tertiary structures long range? How do they contribute to the overall RNA fold?
4. Where does a protein, ligand, or *trans*-acting RNA bind?	*In vitro* or *in vivo*: probing with and without protein/ligand/RNA	What is the structure of the binding site?
5. How does the RNA present interaction regions within the cell?	*In vitro* or *in vivo*: solvent accessibility probing	What does the surface of the RNA fold look like?
6. Do functional RNA structures form during transcription?	*In vitro*: cotranscriptional vs. equilibrium refolded probing	What is the cotranscriptional folding pathway of this RNA?
7. How does RNA structure vary across the transcriptome?	*In vivo* or *ex vivo*: genome-wide probing to compare reactivity across mRNAs	Do common features of RNAs, such as translation start sites, share common structural patterns?

See online version for legends and references

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Chemical probing coupled to high-throughput sequencing offers a flexible approach to uncover many aspects of RNA structure relevant to its cellular function and interactions. Chemical probes preferentially react with RNA in regions that are unconstrained—i.e., flexible, unstructured, unpaired, and unbound by other RNAs, proteins, or ligands. These covalent modifications can then be mapped by high-throughput sequencing to obtain structural information across a complex pool of RNA molecules simultaneously. A variety of inputs can be used in these experiments, ranging from *in vitro* purified RNA to RNA from whole cells and tissues. Following the probing reaction, RNA is reverse transcribed into cDNA and sequenced to detect modifications either as truncated cDNA products (RT-stops) or modification-induced mutations (RT-mutations). The distribution of modifications across a molecule is then used to calculate a "reactivity" value for each nucleotide in the RNA, with higher reactivities typically corresponding to more unconstrained positions (Strobel et al., 2018).

Chemical probing reactivities can be used to uncover many layers of RNA structure. Since the probes are sensitive to the structural environment of each nucleotide (McGinnis et al., 2012), reactivities can reflect base-pairing interactions, tertiary interactions, and other environmental effects such as ion-mediated interactions, protein/RNA/ligand interactions, and temperature-dependent changes in RNA flexibility. Moreover, different probes access different structural information (Strobel et al., 2018): some modify the backbone of the RNA, while others preferentially modify specific bases (Ehresmann et al., 1987). The choice of probe is an important consideration; for example, probes with long half-lives are better suited to probing RNA structures inside cells (Spitale et al., 2015). A table listing common probes and their properties is below, with additional probes discussed in Strobel et al, 2018.

Chemical probing can be used to address many biological questions about RNA structure and function. For example, a protein-RNA interaction can be characterized by comparing probing experiments performed on purified RNA and RNA folded in the presence of the RNA-binding protein (RBP). By looking for changes in reactivities between the two conditions, RBP interaction sites and resulting RNA structural changes can be uncovered (Smola et al., 2015). Similar comparisons can reveal where ligands bind RNAs, how RNA folds change in the complex cellular environment (Spitale et al., 2015), how RNA folds change during transcription (Watters et al., 2016), and many other findings (see table on front page).

Chemical probing data can also be leveraged alongside computational methods to yield higher-resolution RNA structural models. Single-structure methods can use reactivity information from experiments or an existing database (Yesselman et al., 2018) to increase the accuracy of 2D or 3D structural predictions (Lorenz et al., 2016). Since RNAs often fold into an ensemble of different structures in solution, multistate methods have recently been developed to extract this population-level information from bulk probing data in order to predict the ensemble of distinct folds of an RNA molecule, as well as their relative distributions within the population (Li and Aviran, 2018). Comparative methods have also been developed to ask questions about the conservation of structural elements between different sequences, and genome-wide tools are useful for linking reactivity patterns to genomic elements (Spitale et al., 2015, Mustoe et al., 2018, Strobel et al., 2018).

High-throughput chemical probing offers a powerful and growing suite of experiments to uncover the RNA structure-function relationship. However, in some cases, it can be difficult to unambiguously assign structural changes from experimental reactivity changes, as many different changes in structural context can lead to similar observed changes in reactivity. Low amounts of input RNA can result in low signal and therefore inaccurate reactivity estimates. Another existing limitation is that different steps in probing experiments can introduce bias into the data (Strobel et al., 2018). Protocols are being continuously developed to remove these biases. General considerations of sequencing depth should always be taken into account; studying genome-wide (Mustoe et al., 2018), cotranscriptionally folded (Watters et al., 2016), and long RNAs in particular requires increased sequencing depth to produce robust reactivity estimates. Additionally, the use of multiple probes to study the same RNA is often advantageous to leverage the complementary information offered by different probes.

Chemical probing of RNA structure continues to increase in power and resolution. There are also exciting opportunities to continue to merge these techniques with other high-throughput methods, such as cross-linking immunoprecipitation, to study the structural basis of RNA-protein interactions, the impact of RNA structure on translation, and many other features of the RNA structure-function relationship. Overall, we anticipate that these techniques will help uncover new RNA functional roles and the structural aspects of these mechanisms across the cell.

Probe	Structural feature probed	Molecular weight (g/mol)	Quenching half-life (37 °C)	Modifies	Used with in-cell probing?
1-methyl-7-nitroisatoic anhydride (1M7)	Nucleotide dynamics	222.2	14 s	2′ OH (all nts)	Y
Benzoyl cyanide (BzCN)	Nucleotide dynamics	131.1	0.25 s	2′ OH (all nts)	
2-methylnicotinic acid imidazolide (NAI)	Nucleotide dynamics	187.2	33 min	2′ OH (all nts)	Y
2-(azidomethyl)nicotinic acid imidazolide (NAI-N3)	Nucleotide dynamics	228.2	33 min	2′ OH (all nts)	Y
Dimethyl sulfate (DMS)	Base pairing context	126.1	User defined quenching	G, A, and C	Y
N-cyclohexyl-*N*′-(2-morpholinoethyl) carbodiimide metho-p-toluenesulfonate (CMCT)	Base pairing context	423.6	User defined quenching	G and U	
Kethoxal	Base pairing context	148.2	User defined quenching	G and C	
Hydroxyl radical	Solvent Accessibility	17.01	User defined quenching	Backbone	Y
Nicotinoyl Azide (NAz)	Solvent Accessibility	148.1	Solvent quenched, ps timescale	G and A	Y

ACKNOWLEDGMENTS

This work was supported by grants from the Arnold and Mabel Beckman Foundation, the NIH (T32GM083937, 1DP2GM110838, and R01GM120582), and Searle Funds at the Chicago Community Trust. M.E.E. and A.M Y. contributed equally to this work. We thank Kyle Watters for example data and plots for tRNA.

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  DOI https://doi.org/10.1016/j.cell.2018.09.024