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General Principles for the Detection of Modified Nucleotides in RNA by Specific Reagents

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Epitranscriptomics heavily rely on chemical reagents for the detection, quantification, and localization of modified nucleotides in transcriptomes. Recent years have seen a surge in mapping methods that use innovative and rediscovered organic chemistry in high throughput approaches. While this has brought about a leap of progress in this young field, it has also become clear that the different chemistries feature variegated specificity and selectivity. The associated error rates, e.g., in terms of false positives and false negatives, are in large part inherent to the chemistry employed. This means that even assuming technically perfect execution, the interpretation of mapping results issuing from the application of such chemistries are limited by intrinsic features of chemical reactivity. An important but often ignored fact is that the huge stochiometric excess of unmodified over-modified nucleotides is not inert to any of the reagents employed. Consequently, any reaction aimed at chemical discrimination of modified versus unmodified nucleotides has optimal conditions for selectivity that are ultimately anchored in relative reaction rates, whose ratio imposes intrinsic limits to selectivity. Here chemical reactivities of canonical and modified ribonucleosides are revisited as a basis for an understanding of the limits of selectivity achievable with chemical methods.

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1. Introduction

Detection and reproducible mapping of modified nucleotides defining the RNA epitranscriptome is an essential step in understanding their physiological functions.[1] Since such nucleotides are found in a wide spectrum of RNA types and living species, mapping and identification approaches need to be adapted to RNA length, diversity, and abundance. Ideally, such methods would be sensitive, selective, robust with respect to reproducibility[2] and provide a quantitative readout of the RNA modification stoichiometry throughout an entire transcriptome. Despite impressive progress toward this goal, there is still significant room for improvement.

In practice, detection of RNA modifications in different RNA species have evolved from classical physicochemical approaches (e.g., specific radioactive labeling followed by 2D thin layer chromatography,[3] RNA oligonucleotide fingerprinting,[4] digestion to nucleosides followed by reverse phase high-pressure (performance) liquid chromatography (HPLC) or reverse transcription (RT)-arrest profiles upon primer extension,[3,5] to modern combinations of HPLC with tandem mass-spectrometry, (LC-MS/MS).[6] Most recently, various deep-sequencing-based techniques of RNA modification mapping were established,[7] many of which use chemical treatments that differentially affect modified versus unmodified nucleotides. From a basic perspective, it is the particular chemical properties of modified nucleotides that allow to distinguish them from parental unmodified residues by approaches rooted in biophysics, biochemistry, or organic chemistry. Biophysical parameters used for such distinction include, e.g., differential mobility in chromatography, electrophoresis, and mass-spectrometry-based protocols. Biochemical parameters concern the recognition by proteins, e.g., specific antibodies, or base-pairing properties during complementary DNA (cDNA) synthesis by a reverse transcriptase.

Each of these properties can, in principle, be modulated by specific in vitro derivatization of either only modified nucleotides (positive detection) or only unmodified ones (negative detection), thus potentially enhancing distinction by any of the above principles. Such chemical reaction products are precious for improved reliability of mapping protocols, since they provide opportunities for intrinsic specificity controls (mock vs treated sample), and thus angle for reduction of background noise and false-positive peak calling.[8]

The use of chemical reagents for RNA analysis stems from the early discoveries of chemical reactivity of the ribose and canonical nucleobases/nucleosides/nucleotides.[9] Certain combinations of chemicals and derivatization techniques were developed to induce site-specific cleavages in the RNA chain, and thus constitute the historic foundations of direct RNA sequencing[10] and for structural probing.[11] Given that some of the chemical derivatives generated in such experiments are structurally identical to natural modifications, these protocols also provided a molecular basis for the detection of certain modifications. Cases in point are m^3C (3-methylcytidine) and m^7G (7-methylguanosine) (see **Table 1** for nomenclature), generated upon treatment of RNA with dimethyl sulfate (DMS). Further early "chemistries" used for the detection of native modifications are listed in our previous reviews.[6g,12]

Growing interest to the RNA epitranscriptomics field stimulated by the seminal rediscovery of N^6-methyladenosine (m^6A) mapping in mRNAs[13] greatly stimulated research that focused on coupling specific chemical treatments to deep sequencing protocols.[14] As mentioned above, such methods are generally more specific compared to antibody-driven enrichment (Me-RIP and similar protocols), since mock (untreated) sample provided important internal control. Thus, a number of chemical-based deep sequencing approaches were developed and successfully applied to RNA modification mapping.[7,15] With the progress in the mapping field came a rediscovery of early nucleic acid chemistry. Some of it has been included in deep sequencing methods, and some more is certain to come. There are, however, fundamental differences between detecting a modified nucleotide in a short RNA on a sequencing gel, and extracting valid positive signals from big data sequencing approaches in the order of 10^7 nucleotides. One such fundamental difference is the impact of background noise. Unnoticed by many, the signal-to-noise ratio has become a crucial but often underestimated parameter in transcriptome mapping. Just because finding a modification in a short RNA can succeed even with a low signal-to-noise (S/N) ratio, this does not at all guarantee that the same method will give helpful results when applied to an entire transcriptome, because the S/N will start producing false positives at a rate that no bioinformatic treatment can filter out any more. As opposed to antibody-based methods, which rely on differential binding constants and thus mostly on thermodynamic parameters, the S/N of chemical treatments is mostly dependent on kinetic parameters, i.e., on relative reaction rates of modified versus unmodified nucleotides with the reaction under investigation. Although this erects intrinsic and thus insurmountable limits for the selectivity of any given reagent, the S/N ratio can be optimized with respect to, e.g., temperature, solvent, salt, and pH conditions—within said limits. Identification of which condition is most worth optimizing requires some inspiration from basic considerations of the organic chemistry of nucleosides, which we will discuss in the following section.

2. Inherent Reactivity of Nucleosides

A variety of methods have been explored for the detection of RNA modifications, some of which rely on, or are improved by, compounds that react more or less specifically with modified nucleosides. For a better understanding of the use of specific reagents, we will first briefly outline the reactivity of ribonucleotides toward chemical reagents as a basis for a discussion of selectivity. The nucleobases in RNA are aromatic heterocycles, whose nitrogen atoms dispose of a lone electron pair that invariably makes them nucleophiles, albeit of variegated reactivity. Exocyclic amino groups are anilinic and relatively electron poor, making them clearly less reactive than aliphatic amines. Nitrogens in purines and pyrimidines, as part of aromatic rings, are sp^2 hybridized. Both types of heterocycles are electron poor, and thus susceptible to nucleophilic attacks. Their electron withdrawing effect further decreases the nucleophilic character of the exocyclic nitrogens.

Figure 1 shows a selection of reagents that have been employed in the study of modified as well as unmodified RNA nucleosides, roughly segregated into carbon electrophiles in red, nucleophiles in blue, and oxidizing agents in green. Note that some reagents may have dual character, e.g., NO^+ is both oxidizing and electrophilic. Also, reactivities shown in Figure 1 are incomplete in the sense that several of the reagents are known to attack more than one position. As an example, it was shown early on that all heteroatoms in nucleobases are reactive toward alkylation, albeit with a very strong carbon electrophile generated from ethylnitrosourea.[16] Indeed, proper control of reaction conditions is exceedingly important for tuning selectivity. For milder electrophiles like bromomethylcoumarins, strong influence of pH and solvent on the selectivity of alkylation have been demonstrated.[17]

DMS, a popular reagent in RNA structural probing,[11b,18] and methyl methanesulfonate are known to alkylate several nitrogens to different degrees. This also includes, e.g., N^6-A, a reaction which rarely appears in literature because detection for the purpose of structural probing is not straightforward.[19]

The four canonical nucleobases show patterns of alternating nucleophilic and electrophilic centers that conform to intuitive reactions of organic chemistry. Electrophilic sites of particular interest are the six positions in both pyrimidines, which are part of a Michael system. In vivo, a thiolate attack on this position initiates the catalytic cycle used by several methyltransferases to methylate the C5 position in uridines (U) and cytidines (C).[20]

The 5–6 double bond is also, to a degree, vulnerable to oxidation. This reaction is enhanced by the presence of a methyl group at the 5-position in 5-methyluridine (m^5U) and 5-methylcytidine (m^5C). For DNA, the use of permanganate, osmium tetroxide, and related osmium complexes were described for the discrimination of the 5mC nucleobase from C, and for footprinting experiments with thymidines (T).[21] However, similar osmate compounds, when tested for differential reactivity toward m^5C and T versus C and U in RNA, showed insufficient selectivity for epitranscriptomic applications.[22]

Modifications of the ribose are surprisingly limited in terms of chemical diversity. In addition to DNA being an extensively modified RNA, methylation and ribosylation at the 2′-OH are the only known ribose modifications. While ribosylation appears to be restricted to specific tRNAs,[23] ribose methylation is widespread and of significant current interest. Chemistry either targets the vicinal diol structure common to riboses or exploit the protection against degradation offered by these modifications, which is highlighted in **Figure 2**. Electron-poor boronate reagents profit from chelating effect of lone electron pairs of the alcoholic oxygen atoms to form relatively stable adducts with applications

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Table 1. Short forms for modifications used in this review. (Note: Please be aware of differences in nomenclature when it comes to modifications. In the context of RNA, nucleoside and short forms like m^{5}C (5-methylcytidine) are used. Whereas DNA professionals usually refer to the nucleobase and change order to 5mC (5-methylcytosine). To indicate a deoxyribonucleoside an extra "d" is added as in m^{5}dC (5-methyldeoxycytidine). In case of modifications on exocyclic nitrogens, *N* is added before the full name (see m^{1}A vs m^{6}A)).

Abbreviation	Structure	Common name	Abbreviation	Structure	Common name
m^{7}G		7-Methylguanosine	m^{3}C		3-Methylcytidine
D		Dihydrouridine	m^{1}A		1-Methyladenosine
m^{6}A		*N*6-methyladenosine	m^{1}G		1-Methylguanosine
m^{2,2}G		*N*2,*N*2-dimethylguanosine	hm^{5}C		5-Hydroxymethylcytidine
m^{5}U		5-Methyluridine	f^{5}C		5-Formylcytidine
m^{5}C		5-Methylcytidine	Gm		2′-*O*-methylguanosine
ac^{4}C		*N*4-acetylcytidine	mcm^{5}s^{2}U		5-Methoxycarbonylmethyl-2-thiouridine

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Table 1. Continued.

Abbreviation	Structure	Common name	Abbreviation	Structure	Common name
I		Inosine	ho^5C		5-Hydroxycytidine
Ψ		Pseudouridine	Q		Queuosine
s^2U		2-Thiouridine	acp^3U		3-(3-Amino-3-carboxypropyl) uridine
m^6t^6A		N^6-methyl-N^6-threonylcarbamoyl-adenosine	yW		Wybutosine
s^4U		4-Thiouridine			

in chromatography and electrophoresis. The Malaprade reaction, a rather specific oxidation of vicinal diols by periodate, has been exploited for an RNAseq-based mapping approach.[18a] Addressing the free singular 2′-OH function within the RNA chain, the RiboMethSeq method profits from the susceptibility of conventional RNA phosphodiester backbones to alkaline hydrolysis. Methylated and ribosylated nucleotides stand out, because their "capping" of the 2′-OH function protects the local phosphodiester bond against cleavage.[24] Also of interest are SHAPE (selective 2′-hydroxyl acylation analyzed by primer extension) reagents,[25] which specifically react with free (i.e., nonconstrained) 2′-OH groups, although this property has not yet been exploited in the detection of ribose modifications.

Table 2 is a substantial, yet nonexhaustive list of reagents that have either been evaluated for, or applied to, modification mapping, or represent yet unexploited options. It also contains several more reagents, which are missing from Figure 1, because their reaction is documented only with modified nucleotides. Examples are hydride reagents: $NaBH_4$ is used to selectively reduce the 7–8 double bond in m^7G,[41–44] and $NaCNBH_3$ to reduce the 5–6 double bond in N^4-acetylcytidine (ac^4C) as is shown in **Figure 3**.[46,47,76]

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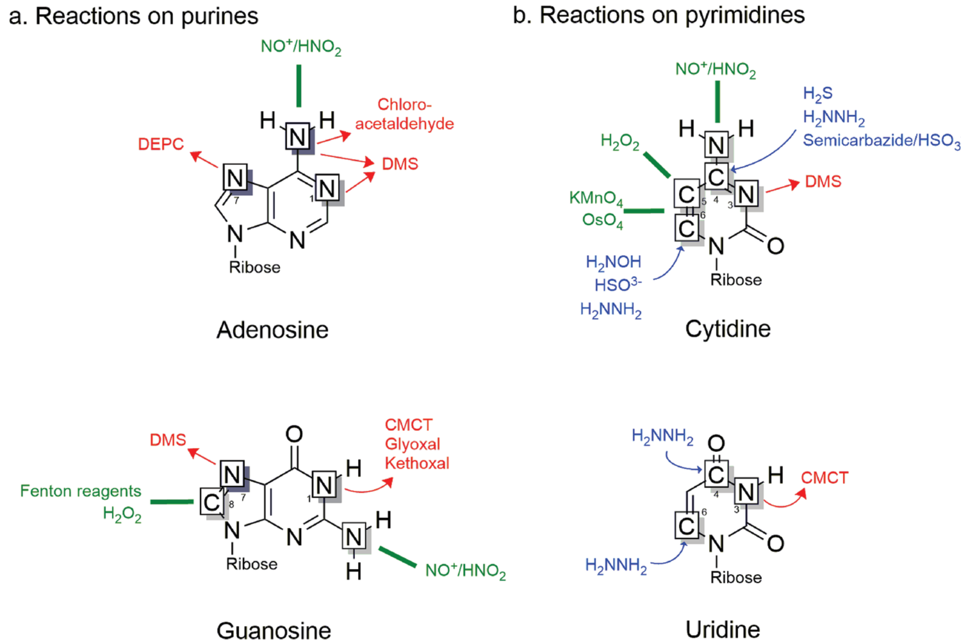

Figure 1. Reactivity of the four major ribonucleobases. Electrophiles are in red, nucleophiles are in blue, and oxidizing agents are in green. For nucleophilic and electrophilic attacks, arrows delineate the move of electron lone pairs. DMS: dimethyl sulfate; CMCT: 1-cyclohexyl-3-(2-(4- morpholinyl)ethyl) carbodiimide tosylate; DEPC: diethyl pyrocarbonate.

3. Detection Principles

In vitro, modified nucleotides can be discriminated from the overwhelming stoichiometry of the four canonical nucleotides by several properties. These may be roughly divided into biophysical and biochemical, although the border between these is becoming increasingly blurred. Chemical reagents affect and potentially improve both of the former, since they alter yet again the relevant biophysical and biochemical properties of the analyzed RNA. In what follows, we will first briefly review the biophysical approaches, which include altered behavior of modified RNA in chromatography or electrophoresis, and in mass spectrometry of either modified oligonucleotides or nucleosides. Subsequently, we will discuss biochemical approaches, which include differential recognition by RNA binding proteins, e.g., antibodies, and differential turnover by enzymes, especially by polymerases during reverse transcription into cDNA. The uncontested front runner in terms of impact in

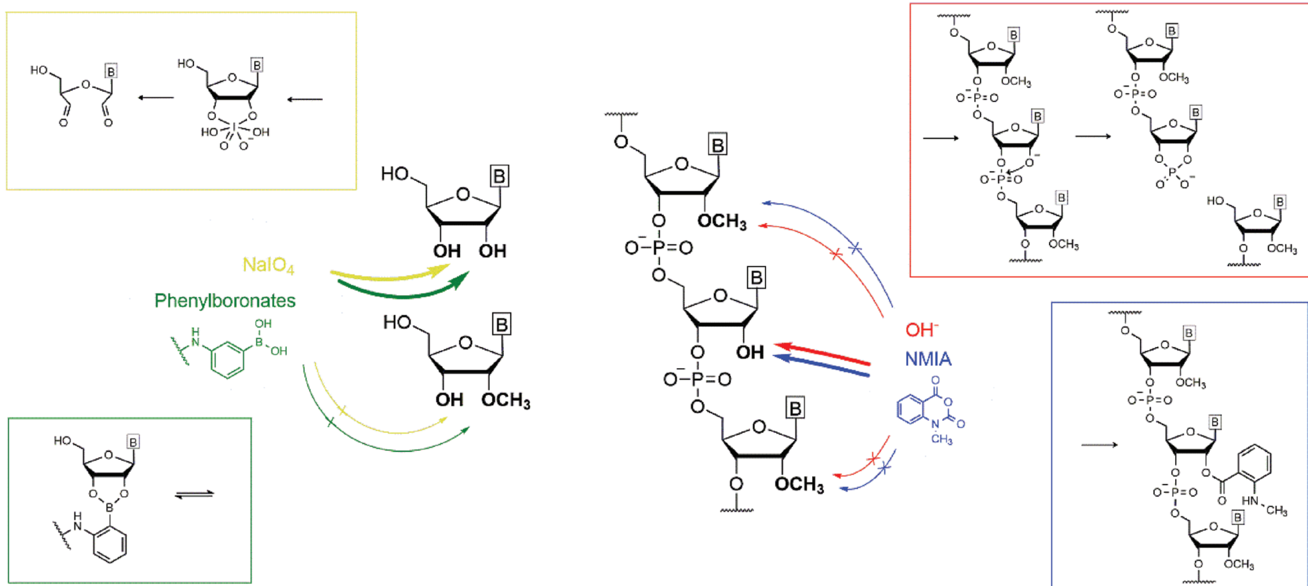

Figure 2. Chemical reagents acting on the ribose moiety of RNA particularly at the unmethylated 2′-OH position. Shown are sodium periodate ($NaIO_4$), phenylboronates, alkaline conditions (OH^-), and the SHAPE reagent NMIA (*N*-methylisatoic anhydride).

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Table 2. List of reagents displaying differential chemical reactivities of modified nucleotides.

Reagent	Target nucleotides	Reaction type	Detection	References
Dimethyl sulfate (DMS)	Unpaired nucleobases: *N*[1]-A, *N*[3]-C, *N*[7]-G	Methylation	Primer extension, RT arrest	[11b]
			High throughput-sequencing	[18,26]
Bromomethyl-coumarins	Ψ, thiouridines	*N*-alkylation, *S*-alkylation	LC fluorescence	[17,27]
	s^4U	*S*-alkylation	LC-MS/MS, gel electrophoresis	[28]
Diethyl pyrocarbonate (DEPC)	Unpaired adenosines (*N*[7]-A)	Carbethoxylation	Primer extension, RT arrest	[29]
Glyoxal + Borat	Guanosine (Adduct with Inosine is not stable)		RNase T_1 cleavage, primer extension	[30]
Osmate complex	m^5C, m^5U	Stable adduct	Oligo LC-MS	[22,31]
	s^4U	Conversion into C	High throughput-sequencing (TUC-seq)	[32]
CMCT 1-cyclohexyl-3-(2-(4-morpholinyl) ethyl)carbodiimide tosylate	Ψ	*N*-acylation	Primer extension, RT arrest	[33]
			MALDI-MS	[34]
			High throughput-sequencing	[35]
	Thiouridines	*N*-Acylation	MS	[36]
N_3-CMC	Ψ	*N*-acylation + biotin conjugation (click-reaction)	High throughput-sequencing (CeU-Seq)	[37]
Hydrazine	m^3C	See Figure 7 Nucleophilic addition, depurination	Aniline-induced chain scission	[10]
			High throughput-sequencing (HAC-seq)	[38]
	Unmodified uridines (Ψ resistant)	See Figure 7 Hydrazinolysis, ring opening	Aniline-induced chain scission	[33a,39]
			High throughput-sequencing (HydraPsiSeq)	[40]
Sodium borohydride $NaBH_4$	m^7G yW	See Figure 3 Reduction, depurination	Aniline-induced chain scission	[10,41]
	m^7G		High throughput-sequencing	[42]
		Reduction, hydrolysis, ARP pulldown	BoRed-Seq	[43]
		Reduction	Immunoprecipitation (m^7G-MeRIP-seq) and m^7G-seq	[44]
	D	Reduction, ring-opening	[^{3}H]$NaBH_4$, determination of radioactivity	[9b,45]
	ac^4C	See Figure 3 Reduction	Misincorporation signals in cDNA	[46]
Sodium cyanoborohydride $NaCNBH_3$	ac^4C	Reduction to *N*[4]-acetyltetrahydrocytidine	C > T misincorporation	[47]
Sodium periodate	Ribose 2′-OH (2′-OCH_3 is inert)	See Figure 2 Oxidation to dialdehyde	*β*-elimination resistance	[48]
			Electrophoresis	[49]
			High throughput-sequencing	[50]
NMIA (*N*-methylisatoic anhydride)	Ribose 2′-OH (2′-OCH_3 is inert)	See Figure 2 Acylation	Primer extension (SHAPE)	[25]
Phenyl boronates (see APB)	Ribose 2′-OH (2′-OCH_3 is inert)	See Figure 2 Formation of anionic complexes	Chromatography	[51]
Alkaline treatment	Ribose 2′-OH (2′-OCH_3 is inert)	See Figure 2 Alkaline hydrolysis	Primer extension, gel electrophoresis	[4,52]
			High throughput-sequencing (RiboMethSeq)	[24,53]
	m^7G, m^3C, ho^5C, D	See Figure 6 Alkaline hydrolysis	Aniline-induced chain scission, high throughput-sequencing (AlkAniline-Seq)	[54]
Methylvinylsulfone	Ψ	*N*-alkylation	MS	[33b,55]

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Table 2. Continued.

Reagent	Target nucleotides	Reaction type	Detection	References
APB ((*N*-acryloylamino)-phenyl -3-boronic acid)	Q	Chelation by *cis*-diols	Gel electrophoresis	[56]
			Affinity chromatography	[57]
APM-gels ((*N*-acryloylamino)-phenyl-4-mercuric chloride)	Sulphur-containing nucleotides, e.g., s^2U, s^4U	Mercury–sulfur interaction	Gel electrophoresis	[58]
Methylthiosulfonate-biotin	s^4U	Disulfide formation	Affinity enrichment	[59]
Acrylonitrile	Ψ	*N*-alkylation	MS	[60]
	I	*N*-alkylation	High throughput-sequencing (ICE-seq)	[61]
Acrylamido-fluorescein	I	Inosine labeling	Immunoprecipitation, fluorescence spectroscopy	[62]
Bisulfite	Cytosine, m^5C is inert	See Figure 4 Conversion to uridine	Sequencing of nonconverted m^5C	[63]
	Ψ	Ribose ring opening + reorientation	High throughput-sequencing (bisulfite sequencing)	[64]
	m^1A	Dimroth rearrangement to m^6A	High throughput-sequencing (bisulfite sequencing)	[64]
Development bisulfite sequencing	hm^5C	Conversion to cytosine-5-methylsulfonate	High throughput-sequencing (bisulfite sequencing)	[65]
Pretreatment with $NaBH_4$	f^5C	Reduction to hm^5C (protection from bisulfite-mediated deamination)	High throughput-sequencing (bisulfite sequencing)	[65,66]
Pretreatment with *O*-Ethylhydroxylamine	f^5C	Condensation to an oxim (protection from bisulfite-mediated deamination)	High throughput-sequencing (bisulfite sequencing)	[67]
Hydrochloric acid (HCl)	yW	Depurination	Aniline-induced chain scission, gel electrophoresis	[68]
Nitrous acid (HNO_2)	m^6A	See Figure 5 A-to-I deamination	High-throughput sequencing (NOseq)	[69]
NHS-derivates, Isothiocyanate	Modification with $-NH_2$	*N*-alkylation	Various, e.g., fluorescent labeling	[70]
Dansyl chloride	Modifications with $-NH_2$	Condensation	Fluorescent labeling	[71]
Amines (in carbodiimide)	Modifications with $-COOH$ (e.g., m^6t^6A, acp^3U)	Conjugation	Various, e.g., gel electrophoresis	[72]
Iodoacetamide derivates	Thiolated nucleotides (s^2U, s^4U)	*S*-alkylation	Various, e.g., fluorescent labeling	[73]
	s^4U	*S*-alkylation	High throughput-sequencing (SLAM seq)	[74]
Peroxotungstate	hm^5C	Oxidation to trihydroxylated-thymine (^{th}T)	High throughput-sequencing (WO-seq)	[75]
+ Naeglaria Tet-like oxygenase (NgTET1)	m^5C	Oxidation to hm^5C	High throughput-sequencing (TAWO-seq)	[75]

current literature and biological applications are RNAseq-derived methods, and the corresponding section is consequently the largest in this review.

3.1. Biophysical Approaches

The common denominator of biophysical approaches is separation of modified RNA, or its constituent monomers, for subsequent detection based on their separation characteristics. Separation is typically based either on differential affinity for a solid phase in chromatography, or on differential migration in an electric field, as embodied in mass spectrometry and electrophoresis.

3.1.1. Mass Spectrometry

Mass spectrometry is an intrinsically "modification aware" approach that can detect and quantify modified RNA components by their inferred molecular mass after ionization. In

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Figure 3. Reduction of N^7-methylguanosine and N^4-acetylcytidine and downstream reactions. Reduced modifications can be detected in RT signatures and additionally the resulting abasic site from 2H-m^{7}G can react with a biotin-coupled aldehyde reacting probe (ARP).[43]

combination with specific fragmentation analyses, this property allows specific detection of any modification in principle, but current methods suffer from low ionization efficiency and therefore low sensitivity for RNA oligonucleotides containing sequence information. Among the few reagents in use here is vinyl sulfonate for selective derivatization of pseudouridine (Ψ) and inosine (I), which create nucleotide derivatives whose molecular mass is distinctly set apart from all others, thereby facilitating data workup.[33b,55,60]

3.1.2. Gel and Nanopore Electrophoresis

Gel and nanopore electrophoresis are based on differential migration behavior of RNA in an electric field. In contrast to mass spectrometry, where such migration is unmitigated in vacuum, friction by a liquid medium is an important factor in electrophoresis. Affinity electrophoresis employs strong interactions of functional groups in modified RNA with affinity mediated compounds integrated into the gel matrix. The most renowned variants in the RNA field are the boronate (APB) and mercury (APM) gels, which allow strong retardation of RNA with modifications containing vicinal diols and sulfur, respectively.[56,58] Further factors affecting migration behavior are gel matrices, which retard RNA migration dependent on their mesh size. Of note, RNA containing nucleotide modifications do show electrophoretic mobility different from an unmodified analogous sequence in denaturing gels, and alterations of RNA structural features brought about by RNA modification are liable to altering migration behavior in native gels.[77] While these features have never been systematically exploited, they greatly matter in electrophoresis experiments with nanopore devices. In nanopore sequencing, the "mesh" of conventional electrophoresis gels is reduced to a single pore, and rather than watching the progress of an RNA molecule through all the pores of a mesh, this technology observes biophysical parameter of the RNA molecule as it is being dragged through a proteinaceous pore by electrostatic forces. During the translocation of a single RNA molecule, the electric resistance of the pore/RNA complex shows significant alterations as a function of the composition of a pentanucleotide section of the RNA inside the pore at a given timepoint. This parameter is affected by the chemical structure of each of the five nucleotides in the tunnel and can thus be exploited for detection of noncanonical structures.[78] Different approaches were put forward to tease it signals corresponding to modifications. Most basic by comparing sequencing runs between modified and unmodified sequences, or more sophisticated, by retraining machine learning algorithms used to deconvolute the raw signal. While applications to RNA modifications are scarce and in their infancy,[79] detection of 5mC in DNA by nanopore is about to become commonplace.[80] Clearly, changes of the chemical structure of modified nucleotides by specific reagents offer further possibilities for efficient discrimination of the corresponding signals from the canonical nucleotides.

3.1.3. Fractionation and Enrichment Techniques

Fractionation and enrichment techniques rely on differential interaction of modified RNA with a matrix. In conventional stationary phase applications like reverse phase (RP) and hydrophilic interaction liquid chromatography, nucleoside, nucleotides, or RNA fragments are separated based on individual lipophilicity and hydrogen bond capacity.[81] Because these parameters are typically altered by modifications, separation can often, but not always, be achieved using conventional matrices without implementation of particular affinity tags. An interesting application of affinity chromatography was the addition of boronate groups to create specific retention of *cis*-diol groups in RP chromatography,[51] much like in APB gel electrophoresis discussed above.

While conventional chromatography relies on a maximum of theoretical plates, each of moderate separation efficacy, many fractionation techniques in modern life sciences use a single separation step. In such cases, the stationary phase is typically

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conjugated to an affinity mediating molecular entity such as an antibody. The corresponding pulldown methods are variations of what is commonly called MeRIP (methylated RNA immunoprecipitation).[82] Extensive development of MeRIP techniques involved transition to covalent interactions between RNA and stationary phase,[83] but the ultimately limiting factors appears to be the quality of the antibodies used, in terms of affinity and specificity for specific modifications.[84] Since this review focuses on chemical reagents, we will not further discuss protein-mediated affinity pulldown techniques in detail.[85] However, emulations of these approaches using chemical reagents that conjugate an affinity tag to a modification site have raised some attention, e.g., an azide derivative of CMCT (N_3-CMC) for selective transfer of a clickable azide moiety to Ψ.[37] Affinity enrichment, followed by RNAseq, was then achieved after conjugating a biotin to the azide via click chemistry. A similar scheme used persulfide chemistry to transfer a biotin moiety onto 4-thiouridines (s^4U) residues.[59] An advancement of this concept used a derivative of the methyl-group donor *S*-adenosyl-L-methionine to enzymatically transfer a clickable propargyl group onto RNA methylation sites.[86]

3.2. Biochemical Approaches

The use of antibodies for the separation, as discussed above, could arguably be listed as a biochemical method as well. Other proteins that can achieve biochemical discrimination of modified and unmodified nucleotides are polymerases that reverse transcribe modified templates, i.e., RT enzymes. Here, the detection of modification events is not straightforward, because of the particular properties of sequencing technologies. RNAseq-based methods are still dominated by Illumina sequencing, although other technologies are emerging. By design, Illumina generates only signals for four deoxynucleotides, requiring cDNA synthesis by reverse transcription of RNA. While this workflow does not provide direct recognition of modified RNA, the reverse transcription process transports cryptic pieces of information that must then be revealed by bioinformatics means. Here, a large variety of small molecule reagents have been applied to what the current jargon calls epitranscriptome mapping, i.e., determination of modified nucleotides in the sequences of cellular RNA.

3.2.1. Mapping by Reverse Transcription Signature

A very basic type of information transmission on modifications in an RNA template involves misincorporation during reverse transcription into cDNA. Alterations of the molecular structure of modifications, either native or enhanced chemical treatments, can lead to the incorporation of noncomplementary deoxynucleotides. The oldest and most straightforward example is inosine. This modification, despite being derived from an adenosine, is faithfully reverse transcribed by all known reverse transcriptases into a deoxycytidine, meaning it behaves like a riboguanosine in the template.[87] Misincorporation induced by other modifications are less complete, and less clearly defined, but early studies suggest that a majority of modifications, possibly all, give rise to at least spurious misincorporations.[88] Importantly, misincorporations need not be homogenous, i.e., more than a single mismatched deoxynucleotide can be incorporated into cDNA at variegated ratios.[89]

On the mechanistic molecular level, a plausible model considers the catalytic center of the reverse transcriptase enzyme. This site was optimized by evolution to handle the four canonical nucleotides according to Watson–Crick base pairing principles but is known to stall when modifications impede Watson–Crick recognition. In such cases, four general resolutions are conceivable on the molecular level, namely, i) continued reverse transcription with normal dNTP or ii) by misincorporation, iii) skipping of the modified nucleotide in the template, or iv) abortion of cDNA synthesis.[89,90]

Many modifications, in particular those with alterations on the Watson–Crick face, display a mixture of (i)–(iv). The relative contributions of (i)–(iv) form a reverse transcription signature that is somewhat characteristic for a given modification but is sequence specific and may also vary according to the enzyme and reaction conditions employed for reverse transcription.[89–91]

A number of reagents with selectivity for modifications have been employed to trace modifications by comparing RNAseq data from treated and untreated samples. The alkylation of inosine[61b] or the acylation of Ψ with CMCT[33a,39b,92] was among the first to be carried to a transcriptome wide stage. More recent developments include specific erasure of RT signatures, e.g., by enzymatic demethylation of m^3C, 1-methyladenosine (m^1A) 1-methylguanosine (m^1G), and N^2,N^2-dimethylguanosine ($m^{2,2}G$) by AlkB-type enzymes, which cause partial or complete disappearance of the corresponding reverse transcription signatures.[93] Of note, AlkB acts on a very limited number of methylated nucleotides, ignoring essentially all of the bulky hypermodifications at positions 34 and 37 in tRNA. The latter can therefore still impede RT after AlkB treatment.[94]

A specific case of erasure of the signature of m^1A is implemented via its Dimroth rearrangement to m^6A, with the latter having no RT-signature. This rearrangement as shown in **Figure** 4A also occurs under alkaline conditions, which are as well encountered in certain bisulfite treatment protocols that typically include an alkaline incubation step (vide infra). Disappearance of m^1A signatures after treatment under Dimroth rearrangement conditions has been used for validation of otherwise identified m^1A sites.[64,95]

3.2.2. Chemical Editing of Nucleotide Decoding Properties

The biological information of a nucleotide as part of the genetic code principally resides in its pattern of hydrogen bond donors and acceptors on the Watson–Crick face. Biological alterations of such a pattern include in particular enzymatic A-to-I conversion,[87] commonly termed "editing" because of associated changes of the genetic information content, based on the similarity of inosine to guanosine (vide infra). Similarly, C-to-U editing involves enzymatic deamination, which can easily be traced in RNAseq data.[96] Bisulfite treatment similarly converts cytidines to uridines but leaves m^5C residues intact. Originally developed for mapping 5mC in DNA, and later adapted to RNA by Schaefer & Lyko,[63a] numerous applications to full epitranscriptomes in the past decades have incited debates as to the

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Figure 4. A) Demonstration of the Dimroth rearrangement of m^1A into m^6A occurring under alkaline conditions. B) Bisulfite treatment of cytidine contrary to m^5C and Ψ.

borders of specificity, resulting in publication of numerous improved protocols.[97] As mentioned above, one of the latter was adapted to also detect m^1A and Ψ via chemically enhanced signature profiles.[64] As displayed in Figure 4B bisulfite is a nucleophilic reagent that, by reversible Michael addition to the 5–6 double bond, enables hydrolysis of the 4-aminofunction in cytidines. Several variations build on the basic concept of bisulfite sequencing for the mapping of oxidative derivatives of m^5C, such as 5-formylcytidine (f^5C).[67,98]

As illustrated in **Figure 5**, the deamination of adenosines by nitric acid in NOseq is based on the generation of an NO^+ electrophile from nitrite under acidic conditions, which converts exocyclic amino groups into leaving groups.[99] Because this reagent leads to the deamination of all exocyclic amino groups, there is comprehensive chemical A-to-I and C-to-U editing in RNA, which makes data treatment after Illumina sequencing more complicated than for bisulfite seq. Modifications, e.g., m^6A stall the deamination reaction on the level of the respective

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Figure 5. Molecular basis of NOseq. A) The generation of the reacting nitrosyl cation (NO^+). Deamination reactions are illustrated on B) adenosine residues and C) guanosines.

nitroso compound which inhibits A-to-I editing. Therefore, the cDNA differs in corresponding residues making them accessible for detection in bioinformatic data analysis.

3.2.3. Cleavage of the RNA Ribose-Phosphate Backbone

In several deep sequencing-based approaches differential chemical reactivity of modified/unmodified residues is used to compromise the integrity of the RNA ribose-phosphate backbone in a close proximity of the modified nucleotide. This can be ensured either by stimulation or preventing the RNA phosphodiester bond cleavage under alkaline conditions or by RNA nucleases. Alternatively, specific chemical treatment leads to the destruction of the nucleobase, followed by decomposition of the resulting RNA abasic site and subsequent cleavage of the ribose-phosphate backbone. Both approaches are now extensively used for RNA modification mapping.

Modulation of the RNA Cleavage under Alkaline Conditions or by Nucleases: As described above, the presence of 2′-OH moiety in ribose greatly destabilizes 3′-adjacent phosphodiester bonds, since deprotonation (or activation) of 2′-OH under alkaline conditions leads to phosphodiester bond cleavage, with concomitant formation of 3′-cyclophosphate and 5′-OH extremities. However, ribose methylation (Nm, 2′-O-methylation) strongly protects RNA from cleavage, since the 2′-OMe group shows only limited reactivity. This method was used in the past for 2′-OMe mapping,[52,100] but only coupling to deep sequencing allowed

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multiple high-throughput applications. The original protocol for alkaline cleavage-based RiboMethSeq using Ion Torrent sequencing was proposed by Nielsen lab[24a] and further developed to simplify library preparation steps and increase sensitivity, as well as to accommodate Illumina high throughput-sequencing approach.[24b,53b,101] Actual variants of RiboMethSeq use random fragmentation of RNA chains at high temperature and mild alkaline pH (≈9.4), followed by extensive 3′-end dephosphorylation to remove residual 2′,3′-cyclophosphate and/or 2′/3′-phosphates. Ligation-competent RNA fragments are cheated by 5′-phosphorylation by PNK/ATP. Adapter ligation to 3′-ends and to 5′-ends creates RNA templates for primer extension by RT. Since 2′-OMe protects the 3′-adjacent phosphodiester bond, fragments starting at N+1 nucleotide and ending at the Nm residue are underrepresented in the resulting library. This underrepresentation is seen as a gap in cumulated 5′/3′-end profile and can be used for quantitative evaluation of the modification level. RiboMethSeq is robust, requires only minute amounts of input RNA and thus extensively used to map and quantify 2′-OMe in rRNAs, tRNAs, and other, less abundant, RNA species.[53b,101b,102] Ribose methylation also prevents enzymatic (nuclease) cleavage of the RNA chain, but this is mostly seen as complication in periodate oxidation-based RibOxiSeq and Nm-Seq approaches, imposing two (or more) oxidation/removal cycles to reach the terminal 2′-OMe.[50]

Activity of RNA nucleases can be modulated (mostly inhibited) by RNA modifications, examples of such behavior include RNase T2 which is inhibited by 2′-OMe, RNase T1, sensitive to 2′-*O*-methylguanosine (Gm) and m^7G,[103] gamma-subunit of *Kluyveromyces lactis zymocin*,[104] specific to 5-methoxycarbonylmethyl-2-thiouridine (mcm^5s^2U) and *Escherichia coli* MazF toxin, an ACA-sequence-specific endoribonuclease, which is sensitive to m^6A.[105]

Modulation of the nuclease cleavage by chemical modification of guanosine residues was initially described for detection of inosine residues in RNA[30] and later coupled to deep sequencing-based protocol.[106] RNase T1 normally cleaves after both G and inosine residues in RNA, but protection of Gs by borate/glyoxal treatment directs RNase T1 cleavage to residual unprotected inosines, allowing detection of cleaved positions by deep sequencing.

RNA Backbone Cleavage Mediated by Decomposition of the RNA Abasic Site: The second group of methods does not use direct nucleolytic cleavage of the phosphodiester bond, but proceed via destruction or loss of the nucleobase, followed by the cleavage of RNA abasic site or its derivative by aniline. Several well-known chemical reactions converge to formation of the RNA abasic site, like the loss of purine (A/G) RNA bases and modified wybutosine (yW)[45b,107] under acidic conditions, the cleavage of the dihydrouridine ring upon alkaline treatment, the reduction of m^7G to dihydro-m^7G and ring opening of uridine and m^3C residues upon reaction with hydrazine.

Many of these detection principles are now successively coupled to library preparation and deep sequencing protocols called AlkAnilineSeq, TRAC-Seq, HAC-Seq, and HydraPsi-Seq.[38,40,54,108]

The AlkAnilineSeq protocol,[54] initially designed for m^7G detection, is based on the observation that mild alkaline hydrolysis at high temperatures (pH 9.2–9.3, 94–96 °C) is sufficient for efficient opening of the m^7G pyrrole cycle, making it sensitive for subsequent replacement by aniline and RNA abasic site decomposition as represented in **Figure 6**. Thus, previously described m^7G-specific $NaBH_4$ reduction becomes only optional. In addition, other RNA modified residues (m^3C, dihydrouridine (D) and 5-hydroxycytidine (ho^5C)) were shown

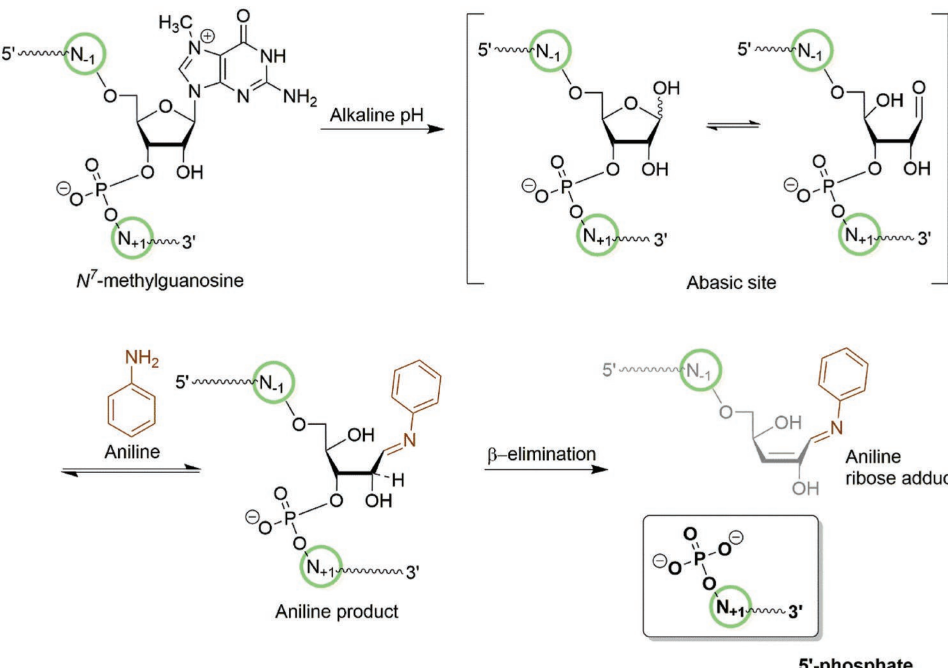

Figure 6. AlkAniline-sequencing reaction scheme showing abasic site generation after alkaline treatment and reaction with aniline. Aniline treatment catalyzes downstream *β*-elimination which results in the pivotal 5′-phosphate.

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to be sensitive to the treatment under the conditions of alkaline hydrolysis employed in the protocol. Decomposition of the RNA abasic site by aniline creates ligation-competent 5′-phosphate at the N+1 nucleotide, and preliminary removal of all potential phosphate residues (both 5′- and 3′-end) leads to high sensitivity and selectivity. The only limitation of AlkAnilineSeq resides in nonlinear response curves between the modification level and the observed signal, making the protocol extremely sensitive, but not truly quantitative, particularly at higher modification stoichiometries.

TRAC-Seq, BoRed-Seq, and MAP-Seq protocols[43,108,109] use m^7G-specific $NaBH_4$ reduction for m^7G detection in RNA, MAP-Seq and BoRed-Seq omit aniline cleavage steps and detect RNA abasic sites either by RT-misincorporation signature or as RT stop after Schiff-base formation with biotinylated aldehyde reactive probe (ARP) (Figure 3), respectively. TRAC-Seq proceeds with subsequent aniline cleavage and insures the detection of m^7G as an RT-stop.

Two last, recently described protocols use the same highly nucleophilic reagent (hydrazine, $H_2N\text{-}NH_2$) for detection of m^3C (HAC-Seq)[38] and Ψ (HydraPsiSeq),[40] which can be seen in **Figure 7**. Both protocols stem from direct RNA sequencing methods proposed in late 1970, which used hydrazine cleavage for direct cleavage at U and C residues in RNA.[10] In application of DMS for structural probing[11a] it was noticed that m^3C is particularly sensitive to hydrazine cleavage, while U and C cleavages require more harsh treatment conditions. Thus, in HAC-Seq, sensitive m^3C residues are cleaved by hydrazine/aniline treatment and comparative analysis of cleavage profiles allows to conclude on the presence of m^3C methylation. In HydraPsiSeq, hydrazine is used to produce random cleavages at all U residues in RNA, and, since Ψ is not sensitive to hydrazine, reveal "protected" U positions, which corresponds, in a great proportion, to Ψ. Since alkaline hydrolysis is used as a preliminary fragmentation step in the HydraPsiSeq protocol, "sensitive" G (m^7G) and C (m^3C) residues are also detected. HydraPsiSeq and HAC-seq protocols use the same ligation principle, described above to AlkAnilineSeq, and HydraPsiSeq is somehow conceptually similar to RiboMethSeq since "protected" U residues are detected and quantitative readout is insured by measurement of the protection level.

4. An Opinionated Outlook

In this review, we have outlined how and on what basis a large number of small molecules have been used for modification detection, with an emphasis on mapping of larger epitranscriptomes. We cannot neglect to add some critical remarks. While during the early phase of the resurge of the RNA modification field, every method was greeted with high attention and confidence, many of the corresponding mapping methods were later alleged to have overstated the number of modifications. Reasons for this might include political considerations, given that higher numbers of modification sites imply higher biological relevance. However, we also perceive an underrepresentation of concepts and arguments from organic chemistry in the field. The use of small molecule reagents in epitranscriptomics is, for the most part, rooted in vintage organic chemistry. Current controversies on the validity of modification mapping approaches develop arguments that are strongly focused on bioinformatic parameters which are arbitrarily set, i.e., human-guessed thresholds. By contrast, the intrinsic limits set by kinetic reaction rates are frequently being ignored, and basic characterizations of fundamental parameters such as pH are often being neglected. The pH is of particular importance, since we are typically looking at reactivity in aqueous solution, where the pH governs the protonation state of both nucleophiles and electrophiles, and thus their respective reactivity. The pH buffer system therefore affects all those reagents listed in Table 2 that act as either nucleophile or electrophile. It similarly activates or deactivates the reactive RNA counterpart, e.g., the nucleobase. The optimum is often a compromise trying to balance selectivity, specificity, and degradation characteristics. As an example

Figure 7. Reaction of hydrazine with 3-methylcytidine and uridine.

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lowering the pH has allowed Schaefer & Lyko,[63a] to adapt bisulfite sequencing from DNA to RNA, where other groups before them have tried and failed. More recently, tardive pH optimization has caused significant confusion as to the occurrence of ac^4C in different RNA species.[46,47,76] Beyond the pH value, factors known to affect reactivity and selectivity of reactions organic chemistry include organic solvent content and polarity, temperature, and pressure, to name just a few.

While we are certain that several brands of vintage organic chemistry still await their application to deep sequencing, one must be aware that some reagents might simply not be selective enough for application to full-fledged epitranscriptomes, despite being able to discriminate short modified versus unmodified RNAs.[17,22] Rather, it might be helpful to the community to determine the size of a "limited transcriptome,"[99] such as, e.g., the collective of tRNAs and rRNAs of $\approx 10^4$ nucleotides, that can be meaningfully investigated with a given combination of reagent and reaction conditions. For example, at false positive rate of 1 in 1000, we expect 10 false positives in transcriptome of that size, which is a manageable number for validation by orthogonal methods. An application of the same method in a typical mammalian mapping exercise, however, will cause 10 000 false positives out of a transcriptome of 10^7 in size, a number that is far from trivial to handle. Although such considerations are, in principle, affected by the number of modifications actually present in the epitranscriptome under investigation, their scarcity renders this effect negligible. In the case at hand, natural abundance of modifications would only "save" the day, if it were in the order of 10^6, while any native abundance of 10^5 or lower would lead to at least 50% of predicted signals being false positives. Incidentally, many first-ever reports on a new mapping method come up with modification numbers in this order of magnitude, i.e., 10^5. A simple rule-of-thumb recommendation would therefore be to choose a transcriptome for mapping, whose size roughly matches the inverse of the false positive rate.

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Conflict of Interest

The authors declare no conflict of interest.

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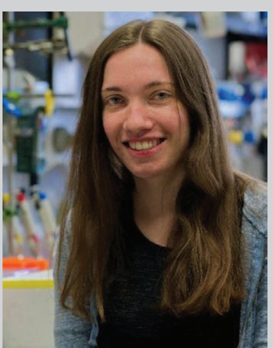

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