



HAL
open science

Instrumental analysis of RNA modifications

Yasemin Yoluç, Gregor Ammann, Pierre Barraud, Manasses Jora, Patrick A Limbach, Yuri Motorin, Virginie Marchand, Carine Tisné, Kayla Borland, Stefanie Kellner

► **To cite this version:**

Yasemin Yoluç, Gregor Ammann, Pierre Barraud, Manasses Jora, Patrick A Limbach, et al.. Instrumental analysis of RNA modifications. *Critical Reviews in Biochemistry and Molecular Biology*, 2021, 56 (2), pp.178-204. 10.1080/10409238.2021.1887807 . hal-03402194

HAL Id: hal-03402194

<https://hal.science/hal-03402194v1>

Submitted on 25 Oct 2021

HAL is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.



Distributed under a Creative Commons Attribution - NonCommercial - NoDerivatives 4.0 International License

Instrumental analysis of RNA modifications

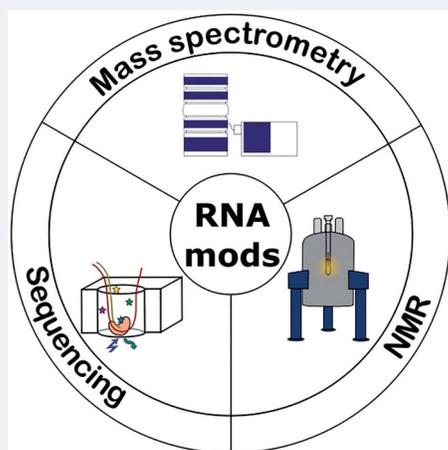
Yasemin Yoluç^a , Gregor Ammann^a , Pierre Barraud^b , Manasses Jora^c , Patrick A. Limbach^c , Yuri Motorin^d , Virginie Marchand^e , Carine Tisné^b , Kayla Borland^a and Stefanie Kellner^{a,f} 

^aDepartment of Chemistry, Ludwig Maximilians University, Munich, Germany; ^bExpression génétique microbienne, UMR 8261, CNRS, Institut de biologie physico-chimique, IBPC, Université de Paris, Paris, France; ^cDepartment of Chemistry, University of Cincinnati, Cincinnati, OH, USA; ^dUniversité de Lorraine, CNRS, UMR7365 IMoPA, Nancy, France; ^eUniversité de Lorraine, CNRS, INSERM, Epitranscriptomics and RNA Sequencing Core facility, UM S2008, IBSLor, Nancy, France; ^fInstitute of Pharmaceutical Chemistry, Goethe-University, Frankfurt, Germany

ABSTRACT

Organisms from all domains of life invest a substantial amount of energy for the introduction of RNA modifications into nearly all transcripts studied to date. Instrumental analysis of RNA can focus on the modified residues and reveal the function of these epitranscriptomic marks. Here, we will review recent advances and breakthroughs achieved by NMR spectroscopy, sequencing, and mass spectrometry of the epitranscriptome.

GRAPHICAL ABSTRACT



ARTICLE HISTORY

Received 4 November 2020

Revised 18 January 2021

Accepted 5 February 2021

KEYWORDS

Epitranscriptome; sequencing; NMR; mass spectrometry; RNA modification

Overview

RNA is a dominant macromolecule in all organisms. Its monomeric units are the canonical nucleosides cytidine, uridine, guanosine, and adenosine which are interconnected by a 3'-5'-phosphate backbone. After transcription, enzymes target the RNA and introduce a variety of modifications onto nucleobases or the ribose 2'-OH. Overall, more than 150 unique RNA modifications have been identified in many RNA types and throughout all domains of life.

The detection, localization, and quantification of these RNA modifications are crucial for understanding the elusive function of RNA modifications. Here, we

review the most common techniques for instrumental analysis of modified nucleosides and discuss recent advances in the field as well as the strengths and weaknesses of every approach. As shown in Figure 1, NMR spectroscopy is mostly suited to study the impact of RNA modifications on RNA structure and stability in addition to recent progress made in RNA modification dynamics. One of the fastest developing fields is RNA sequencing, which allows localization of modifications at nucleotide resolution. Many RNA modifications, such as thiolation, amino acid addition, and even methylations lead to a change in the chemical reactivity of the

CONTACT Stefanie Kellner  stefanie.kellner@cup.uni-muenchen.de  Department of Chemistry, Ludwig Maximilians University, Butenandtstr., 5-13, Munich 81377, Germany,  Institute of Pharmaceutical Chemistry, Goethe-University, Max-von-Laue-Str., 9, Frankfurt 60438, Germany

© 2021 The Author(s). Published by Informa UK Limited, trading as Taylor & Francis Group.

This is an Open Access article distributed under the terms of the Creative Commons Attribution-NonCommercial-NoDerivatives License (<http://creativecommons.org/licenses/by-nc-nd/4.0/>), which permits non-commercial re-use, distribution, and reproduction in any medium, provided the original work is properly cited, and is not altered, transformed, or built upon in any way.

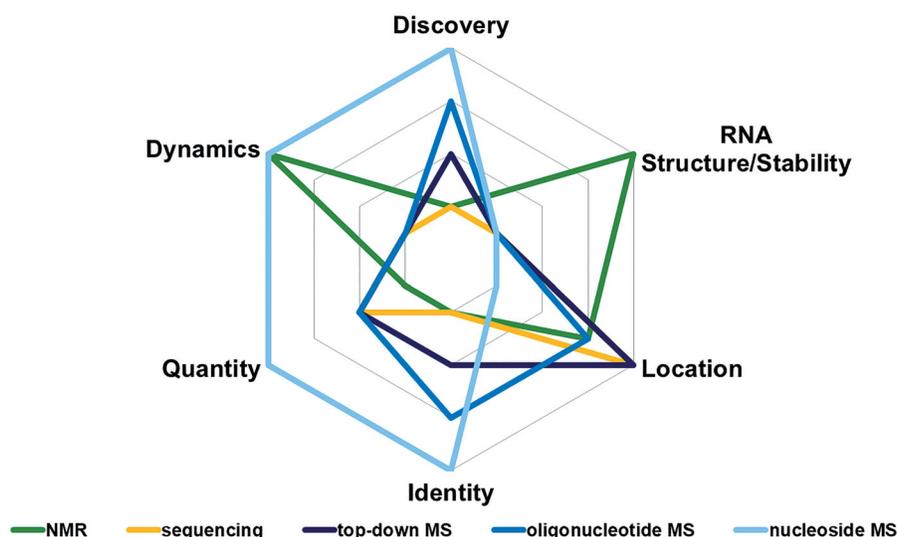


Figure 1. Strengths (outside) and weaknesses (inside) of current methods for RNA modification analysis. Legend: Discovery (and chemical characterization of novel RNA modifications), (impact of RNA modifications on) RNA Structure/Stability, Location (of modification within the sequence), Identity (chemical structure of modification, especially isomer discrimination), Quantity (of modification), Dynamics (of RNA modifications). See colour version of this figure at www.tandfonline.com/ibmg.

RNA. The resulting differential reactivity of modified nucleosides is exploited for their detection by sequencing but also by mass spectrometry, the last field discussed in this review. Mass spectrometry (MS) of RNA modifications can be performed on full-length RNA (top-down MS), partial RNA hydrolysates (oligonucleotide MS), or complete hydrolysates (nucleoside MS).

Interestingly, NMR, MS, and sequencing analyses complement each other as they annihilate the major pitfalls of the other. For example, mass spectrometry is ideally suited to unambiguously identify the chemical nature of the modified nucleoside even with minute amounts of sample material. Unfortunately, nucleoside MS depends on the complete enzymatic digestion of the RNA and thus all sequence information and the location of the modified nucleoside remains unknown. Sequencing on the other hand is, if carefully conducted, perfectly able to pinpoint the location of a modified nucleoside within the RNA but it does not deliver any information about the chemical nature of it. Thus, MS and sequencing are orthogonal techniques that benefit from each other while NMR analysis adds information on the structural impact of an RNA modification. In this work, we discuss the basic principles of NMR, sequencing, and MS analyses, with the main focus on the most recent advances in these fields.

NMR spectroscopy in the analysis of RNA modifications

The use of NMR in the study of RNA modifications goes back to the origins of the study of biological

macromolecules by NMR (Crawford et al. 1971; Koehler and Schmidt 1973; Kan et al. 1974). The large chemical diversity contained in modified nucleotides, as compared with the homogeneous chemistry of the canonical RNA nucleotides, is associated in NMR with a large variety of signals, which often are located in regions of the spectra that are empty of canonical RNA signals. These signals, appearing at particular positions, and most of the time isolated from each other, are thereby relatively easy to recognize. Pioneering studies have used the isolated signals arising from nucleotide modifications in tRNAs as molecular probes to explore the three-dimensional folding and the conformational stability of these molecules (Kan et al. 1974; Kastrop and Schmidt 1978). In addition, signals from tRNA modifications have played a leading role in the chemical shift assignment process for this family of RNAs. Some isolated signals in the upfield part of the spectra, mostly methyl groups, were indeed used as independent and unambiguous signals for the assignment of the imino groups in the downfield part of the spectra (Hilbers et al. 1983; Roy and Redfield 1983; Hare et al. 1985; Heerschap et al. 1985; Choi and Redfield 1986). These signals include the methyl group of 5-methyluridine (m^5U54) (~ 1.00 ppm), 5-methylcytidine (m^5C49) (~ 1.55 ppm), 7-methylguanosine (m^7G46) (~ 3.75 ppm), 1-methyladenosine (m^1A58) (~ 3.00 ppm), and 2-methylguanosine (m^2G10) (~ 2.75 ppm) that are found in many tRNAs including the yeast tRNA^{Phe} (Agris et al. 1986).

After these original studies, the investigation of RNA modifications with NMR has expanded beyond the study of tRNA structure and stability. Occasionally, NMR

has been used for the identification of modifications contained in a given RNA (Sakamoto et al. 1993; Tisné et al. 2000; Gaudin et al. 2003; Wurm et al. 2010; Ranaei-Siadat et al. 2013). But NMR has been and is still mostly used in detailed inspections dealing with the characterization of the specific effects caused by RNA modifications. For instance, NMR combined with UV-melting curves has been used to decipher the contribution of specific modifications in RNA stability (Kumar and Davis 1997; Strebitzer et al. 2018). Similarly, the ability of NMR to assess the dynamic behavior of macromolecules at atomic levels was used to demonstrate that the presence of m⁶A perturbs the hybridization kinetics of RNAs, and slows down the annealing of m⁶A-containing RNAs (Shi et al. 2019). In addition, several structural studies have analyzed the role and the effect of modifications in the conformation of tRNA anticodon stem-loops (Stuart et al. 2003; Durant et al. 2005; Vendeix et al. 2012), overall showing that modifications stabilize and restrict the conformations of the anticodon domain in tRNAs (Agris 2008). In another example, deuterium exchange NMR experiments were used to show that the dynamics and flexibility of *Escherichia coli* tRNA^{Val} are significantly affected by post-transcriptional modifications, with some base pairs being 20 times more stable in the native tRNA than in the unmodified one, although the local and global structure of this tRNA seems rather unchanged in the presence or absence of modifications (Vermeulen et al. 2005). Finally, several NMR studies have also investigated in detail the structural effects produced by specific modifications, such as Ψ (Davis and Poulter 1991), m⁶A (Zhou, Parisien, et al. 2016), D (Dyubankova et al. 2015), m¹A and m¹G (Zhou, Kimsey, et al. 2016), further expanding the repertoire of NMR applications associated with the study of RNA modifications.

Recently, a novel approach using NMR has been introduced to analyze RNA modifications. Following this methodology, NMR offers a way to monitor tRNA maturation in a non-disruptive and continuous manner thereby providing information on the temporality of tRNA modification events (Barraud et al. 2019). The strategy relies on the introduction of ¹⁵N isotope-labeled tRNAs into unlabeled cell extracts containing the cellular enzymatic activities and on the use of isotope-editing in NMR experiments to only detect the tRNA of interest within the complex cell extract environment. With this experimental setup, RNA modification events are directly monitored in a time-resolved fashion, by measuring successive NMR experiments on

a single sample directly incubated in the NMR spectrometer (Figure 2). This methodology consists of three main steps. In the first step, a ¹⁵N-labeled tRNA sample and an unlabeled cell extract are produced. The ¹⁵N-labeled tRNA sample is produced by *in vitro* transcription with ¹⁵N-labeled nucleotides and purified by ion-exchange chromatography (Catala et al. 2020b). This tRNA contains no post-transcriptional modifications. The unlabeled cellular extract is produced from the lysis of cell culture. To preserve as far as possible the cellular enzymatic activities in the extract, cell lysis is performed under gentle conditions and in the presence of anti-proteases (Gato et al. 2021). In the second step, the NMR signature of each individual modification is obtained from the NMR chemical shift assignments of different tRNA samples with different modification content. The comparison of the NMR spectra of the unmodified tRNA (Figure 2(B)) with the NMR spectra of the modified tRNA provides the means to identify the NMR signature of each post-transcriptional modification (Catala et al. 2020a). In the third step, the NMR monitoring of tRNA maturation is performed. The ¹⁵N-labeled tRNA sample is mixed with the unlabeled cell extract to yield the *in extract* NMR sample (Figure 2(A)). This sample is incubated directly in the NMR spectrometer and series of NMR experiments are measured in order to monitor tRNA maturation events in a time-resolved fashion (Figure 2(C)). In the NMR spectra, the progressive appearance of new signals and the correlated disappearance of signals from the unmodified tRNA sample are the signature of chemical modifications being introduced in the initial transcript. These changes in the NMR spectra can be analyzed in the light of the NMR signature of the individual modifications previously identified. Since NMR spectra are measured as a time course series, this methodology enables the identification of early and latter modifications events. For instance, in the yeast tRNA^{Phe}, this methodology demonstrated that in the T-arm, modifications Ψ55, T54 and m¹A58 are introduced in a defined sequential order (Figure 2(C)), which is controlled by a strong circuit of modifications (Ψ55 → T54 → m¹A58) (Barraud et al. 2019). Modifications circuits can be complicated to identify (Han and Phizicky 2018; Barraud and Tisné 2019), and this NMR approach adds to the available tools for the analysis of potential cross-talks between modification events. Overall, NMR spectroscopy provides the means to observe sequential orders in the introduction of modifications along the tRNA maturation pathway (Figure 2(C)) and to identify modification circuits.

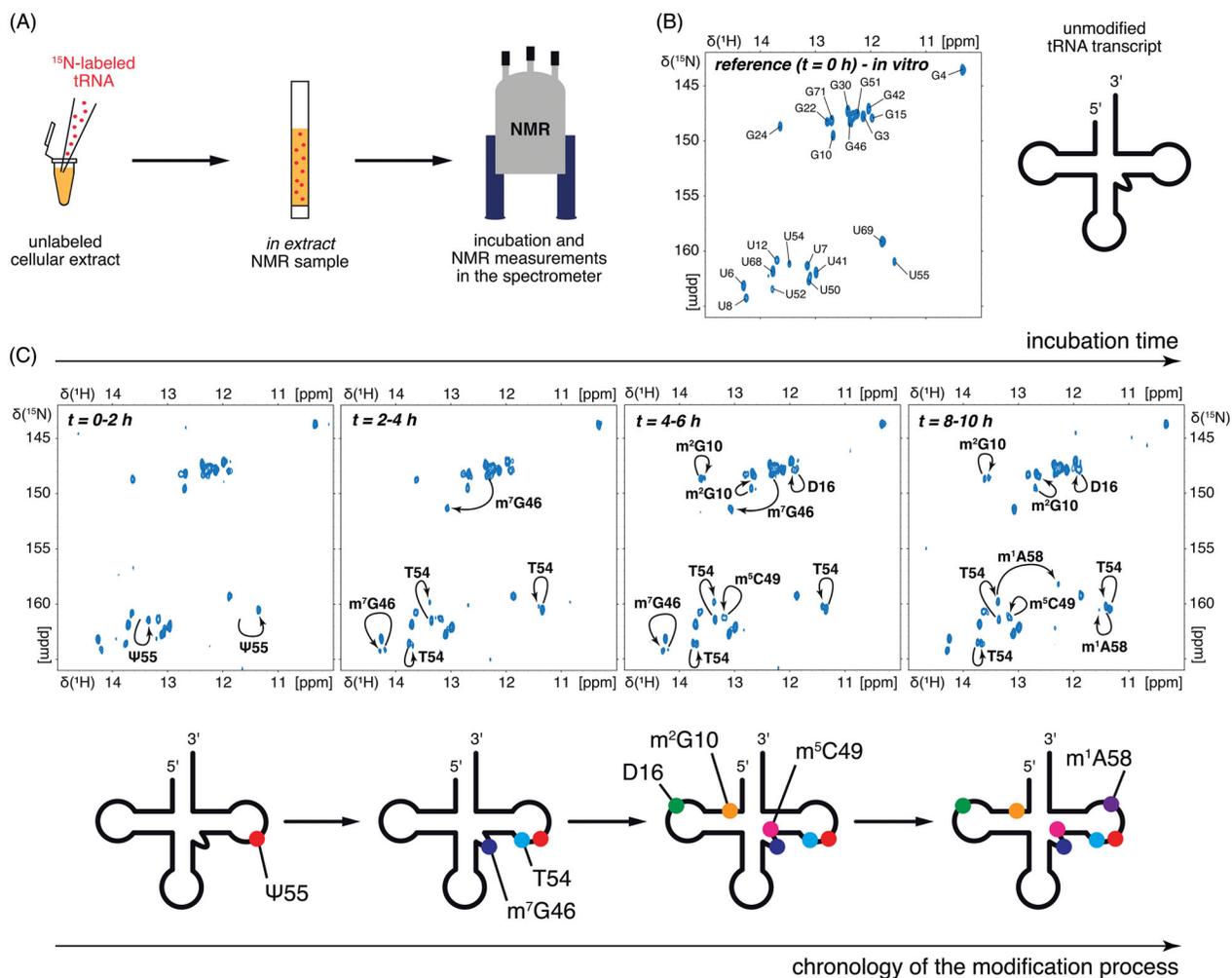


Figure 2. Time-resolved NMR monitoring of tRNA modifications. (A) A ^{15}N -labeled tRNA transcript (represented as red dots) is introduced in an unlabeled cellular extract (in orange). This mix is transferred to an NMR tube to yield an in extract NMR sample. The tRNA maturation sample is then directly incubated in the NMR spectrometer. (B) Imino (^1H , ^{15}N) correlation spectrum of a ^{15}N -labeled yeast tRNA^{Phe} measured *in vitro* to provide a reference spectrum (left part) and schematic representation of this unmodified tRNA transcript (right part). (C) Successive NMR measurements provide time-resolved information on the tRNA maturation process. Top part: Imino (^1H , ^{15}N) correlation spectra of ^{15}N -labeled yeast tRNA^{Phe} measured during a continuous incubation at 30 °C in yeast extract. Each NMR spectrum measurement spreads over 2 h (incubation time indicated on each spectrum). NMR chemical shift changes (indicated with arrows on the NMR spectra) are identified and linked to specific modification events. Bottom part: Schematic view of the sequential order of the introduction of modifications in yeast tRNA^{Phe} corresponding to the NMR spectra depicted above. The acquisition of NMR spectra in a continuous and time-resolved fashion enables the identification of a sequential order in the introduction of post-transcriptional modifications and thereby gives access to the chronology of the modification process. See colour version of this figure at www.tandfonline.com/ibmg.

Advances in chemical labeling of modified RNA in the context of instrumental analysis

Many RNA modifications, such as thiolation, amino acid addition, and even methylations lead to a change in the chemical reactivity of the RNA. Thus, early analyses focused on the differential reactivity of modified nucleosides for their detection. With the advent of instrumental analysis, the benefit of these chemical reactions was more and more forgotten and they have

been rarely used (Kellner et al. 2010; Heiss and Kellner 2017). Only those compatible with modern detection techniques such as mass spectrometry (Durairaj and Limbach 2008) or sequencing (Motorin and Helm 2019) are now commonly used as they aid in the detection of certain RNA modifications. For example, pseudouridine (Ψ) and inosine (I) are similar to their canonical precursor (U and A, respectively) in their mass and base-pairing abilities. Due to isomerization, Ψ gains additional functionalities which react with acrylonitrile (Figure

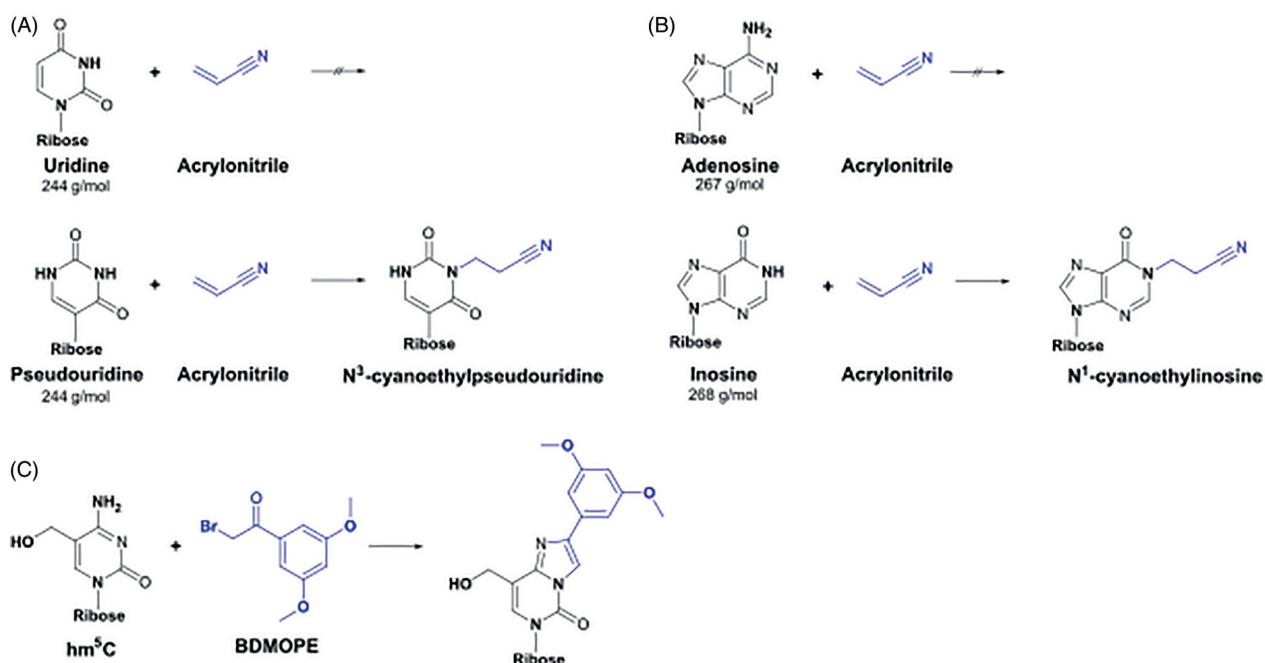


Figure 3. Selected chemical labeling strategies for detection of modified nucleosides. (A, B) Reaction of uridine and adenosine with acrylonitrile is not observed (upper panels), whereas pseudouridine and inosine are successfully cyanoethylated (lower panels). Modified nucleotides are afterwards detected through mass spectrometry and sequencing. (C) 5-hydroxymethylcytosine (hm^5C) reacts with 2-bromo-1-(3,4-dimethoxyphenyl)-ethanone (BDMOPE) which allows highly sensitive mass spectrometric detection of hm^5C (Feng et al. 2020). See colour version of this figure at www.tandfonline.com/ibmg.

3(A) (Mengel-Jørgensen and Kirpekar 2002) and thus facilitate detection by, for example, mass spectrometry (Emmrechts et al. 2005; Hossain and Limbach 2007; Durairaj and Limbach 2008) and even sequencing (Bakin and Ofengand 1993; 1998) becomes possible. Similarly, A-to-I editing can be visualized by cyanoethylation of inosine (Figure 3(B)) (Sakurai et al. 2010). Further developments of acrylonitrile-based reagents have led to the detection of inosine through fluorescence and bioaffinity enrichment (Knutson et al. 2018) and through inosine chemical erasing sequencing, ICE-Seq (Okada et al. 2019). The most trusted methods for RNA modification analysis through sequencing are based on the use of specific chemical reagents. This allows one to compare an untreated (mock) sample with a treated one and exclude (or at least reduce) random noise in peak calling. Such derivatization methods were developed for numerous naturally modified nucleotides in RNA, like inosine (Suzuki et al. 2015), 5-methylcytosine (Tuorto et al. 2012; Schaefer et al. 2017; Khoddami et al. 2019), pseudouridine (Carlile et al. 2014; Lovejoy et al. 2014; Schwartz et al. 2014; Li et al. 2015), 1-methyladenosine (Dominissini et al. 2016; Safra et al. 2017), 4-acetylcytidine (Thomas et al. 2018; Sas-Chen et al. 2020), and 7-methylguanosine (Enroth et al. 2019; Pandolfini et al. 2019).

Another progress was recently achieved for the sensitive detection of modified cytidines. So far, the

quantification of these modifications appeared to be challenging due to their low ionizability by mass spectrometry. Despite several attempts by chemical derivatization (Tang et al. 2015; Huang et al. 2016; Guo et al. 2017) and selective enrichment, it was not possible to quantify these nucleosides easily. In 2020, the Feng lab reported the use of bromoacetylated benzene reagents for efficient labeling of cytidines and many of their naturally occurring modifications (Feng et al. 2020). After the addition of the reagent to the N3 and N4 of cytidines (Figure 3(C)), the new chemical properties of the product are exploited in the context of LC-MS detection. The hydrophobicity leads to better retention on a reverse-phase (RP) column and thus the analytes reach the mass spectrometer at a higher organic solvent ratio which increases detection sensitivity. Furthermore, the tertiary amines are easily protonated in, for example, electrospray ionization and thus Feng et al. could show a 40- to 460-fold increased detection sensitivity for modified cytidines after the reaction with their chemical label (lower limit of detection (LLOD) of 0.06 fmol for m^5Cm , 0.17 fmol for hm^5Cm , 0.22 fmol for f^5Cm and 0.06 fmol for ca^5Cm). In this work, the authors quantified the abundance of modified cytidines in several human cell lines and even carcinoma tissue without utilizing a stable

isotope-labeled standard (SILIS). Due to the chemical simplicity of the labeling reagent, its synthesis with stable isotopes should be an easily possible and thus simple access to synthetically produced cytidine modification SILISs. From our perspective, the method appears to have a great potential for sensitive and accurate quantification of modified cytidines.

Sequencing

Deep sequencing protocols for high-throughput analysis of DNA are now widespread and indispensable for comprehensive projects in modern biology. The output of current DNA deep sequencing machines is now sufficient for a complete analysis of one human genome (or even several genomes) in only few hours. Such extraordinary performance is also employed for RNA analysis, like whole-transcriptome sequencing, mRNA alternative splicing, or single-cell transcriptome studies.

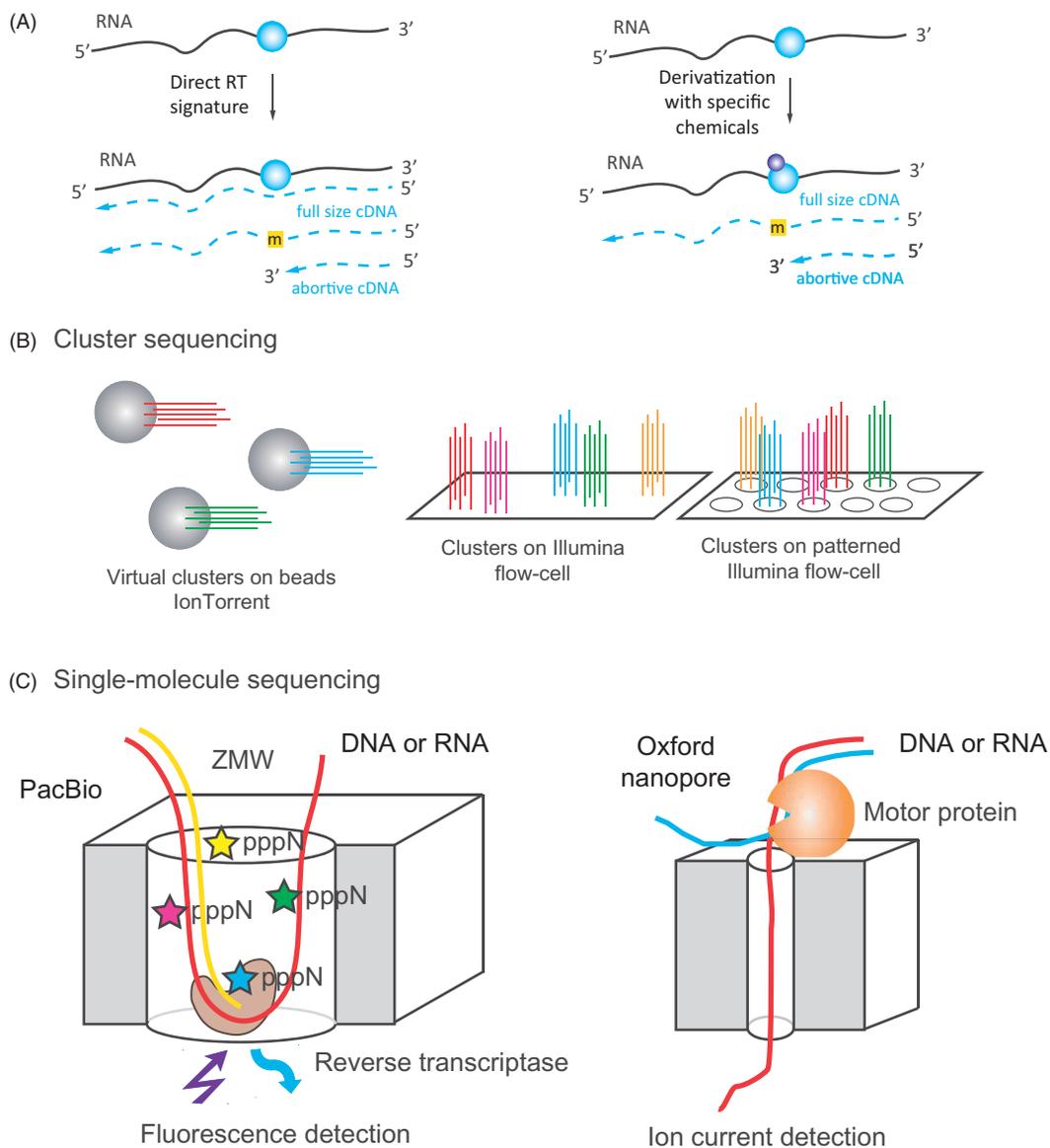
Post-transcriptional RNA modifications can be detected in RNA using various methods and approaches exploiting the chemical and physico-chemical properties of these non-canonical RNA nucleotides. In addition to classical RNA techniques like 5'/3' and specific internal labeling, methods based on next-generation sequencing of the second (NGS) and third (NNGS) generation become more and more popular. These approaches generally provide single-nucleotide resolution for identification of the modified RNA position but maybe less accurate in the identification of the exact nature of the modified residue, due to a rather generic treatment used during the library preparation step. The most popular and reliable methods using NGS analysis rely on various specific chemical treatments applied to alter particular modified RNA residues and thus to make them detectable either as RT-stop or mis-incorporation of nucleotides into cDNA. For the moment, NNGS approaches are mostly using ion-current profiles through a nanopore or kinetics of dNTP incorporation in a PacBio chip to deduce the presence of unusual modified nucleotide (Figure 4).

Except for very recent attempts of direct RNA sequencing by nanopores (see below) all other protocols for RNA modification analysis use the conversion of RNA into cDNA sequence by primer extension with reverse transcriptase (RT). This RT step is almost inevitably followed by PCR amplification, to obtain a sufficient amount of amplicons. Current technologies for DNA (direct RNA) deep sequencing belong to two distinct generations: Next Generation Sequencing (second generation, NGS), also called massive parallel

sequencing which uses amplified real or virtual DNA clusters (Illumina/Ion Torrent), and the third generation technologies (NNGS), based on single-molecule sequencing analysis (PacBio/Oxford Nanopore) (Figure 4).

Current sequencing technology for RNA modification analysis

The first protocols developed for the analysis of RNA modifications used a traditional RT-based RNA sequencing for detection of RT-stops due to impeded primer extension (Maden 2001; Motorin et al. 2007; Behm-Ansmant et al. 2011). With the development of deep-sequencing technologies, such low-throughput approaches were progressively replaced by next-generation sequencing, the most popular technology remains Solexa (now Illumina) sequencing-by-synthesis (SBS) protocol using fluorophores. The very first commercial Solexa/Illumina devices (like Genome Analyzer Ix released in 2009) were able to provide very short reads (with a maximum of 36 nt in length) and thus had limited applications for RNA modification analysis, which generally requires longer sequences to analyze. In addition, the sequencing output was extremely modest. When the HiSeq series of sequencers (HiSeq2000 and 1000) were released in 2011, their performance became fully compatible with the analysis of RNA modifications, first by analysis of RT signatures (Ryvkin et al. 2013). Other competitor technologies that emerged in the DNA deep sequencing field had very limited use in the analysis of RNA modifications, mostly due to high levels of intrinsic sequencing errors. Only one application of Ion Torrent sequencing was described for the original RiboMethSeq protocol developed by H. Nielsen's lab (Birkedal et al. 2015), but the use of Illumina sequencing is recommended. The current evolution of the NGS field left the space only for various Illumina devices employing SBS technologies differing by the number of fluorophores, mostly four (for older HiSeq/MiSeq series) and two (for newer NextSeq/NovaSeq devices). The currently available low-end Illumina sequencer (iSeq100) is not truly compatible with RNA modification analysis due to its very low throughput (4 mln reads/run), and more productive machines, at least MiniSeq or MiSeq (25 mln reads/run) are required. Optimal performance is achieved with NextSeq series (NextSeq550 and NextSeq2000) which are able to generate 400–1000 millions reads/run since RNA modification analysis routinely requires 15–25 mln of raw reads per sample, or more (up to 100–200 mln) in case of whole-transcriptome RNA modification analysis. Even if the sequencing



quality of the newest two colors NextSeq/NovaSeq machines is somehow lower, compared to the four colors HiSeq, the use of patterned flow cell limits overloading issues and improves the quality of the raw reads. The only current competitor technology is not second-generation sequencing, but single-molecule nanopore sequencing, as discussed below.

Analysis of RNA modifications by NGS

Different principles are currently used for the detection of RNA modifications in the epitranscriptome using NGS and NNGS. We classify them in:

1. analysis of RNA-signatures (natural/enhanced or chemically induced) visible in sequencing profiles

2. treatment-induced cleavage of the RNA phosphodiester chain followed by selective ligation of sequencing adapters
3. affinity-based enrichment protocols exploiting specificity of polyclonal or monoclonal antibodies (ab) and specific enzymes installing modifications in RNA.

In many instances, the developed protocols use a combination of these different principles (like ab-driven enrichment followed by specific chemical treatment). Several recent reviews provide more detailed information on these subjects (Helm and Motorin 2017; Hartstock and Rentmeister 2019; Krogh and Nielsen 2019; Linder and Jaffrey 2019; Motorin and Helm 2019; Zhao et al. 2020).

Naturally existing RT signatures of modified nucleotides

A natural RT signature consists of an altered reading of the modified nucleotide during primer extension by RNA-dependent DNA polymerase (reverse transcriptase, RT). Depending on the nature of the nucleotide such a signature may represent a “mutation” compared to the expected reference sequence, or constitute a more complex profile composed of mis-incorporations at different proportions in addition to the abortive RT products ending at the modified nucleotide (Ryvkin et al. 2013; Hauenschild et al. 2015; Tserovski et al. 2016). Such RT signatures can be altered or manipulated by pretreatment of the RNA template (e.g. by demodification/removal of modified residues) (Cozen et al. 2015; Zheng et al. 2015), by the choice of particular conditions of the primer extension reaction (Incarnato et al. 2017; Kristen et al. 2020), or by the use of non-natural dNTP substrate(s) (Hong et al. 2018) and RT active site mutants (Aschenbrenner and Marx 2016; Aschenbrenner et al. 2018). In vivo metabolic labeling with SAM analogs also allowed the incorporation of reactive chemical groups at positions of certain modifications (instead of methyl groups) and thus also helps to alter RT signature of the RNA template (Hartstock et al. 2018; Shu et al. 2020).

Chemically induced RT signatures or RT stops

The most trusted methods for RNA modification analysis are based on the use of specific chemical reagents able to distinguish modified RNA residues from unmodified counterparts (Behm-Ansmant et al. 2011; Heiss and Kellner 2017) as discussed in the Section “Advances in chemical labeling of modified RNA in the context of instrumental analysis”.

Chemically induced cleavage of the ribose-phosphate backbone and selective ligation

Another application consists of a specific chemical cleavage of the RNA ribose-phosphate backbone at an RNA modification by one specific reagent or combination of reagents. In some instances, enhanced cleavage (positive signal) is in fact replaced by increased protection against cleavage (negative signal), thus allowing to distinguish modified and unmodified residue. The most popular applications of this approach are:

1. detection of 2’O-methylations (Nm) by RiboMethSeq (Birkedal et al. 2015; Marchand et al. 2016) and Nm-Seq/RibOxiSeq (Dai et al. 2017; Zhu et al. 2017)
2. detection of 7-methylguanosine (m⁷G) (Lin, Liu, et al. 2018; 2019), 3-methylcytosine (m³C)/dihydrouridine (D)/5-hydroxycytosine (ho⁵C) by AlkAnilineSeq (Marchand et al. 2018)
3. mapping and quantification of pseudouridines (Ψ) by HydraPsiSeq (Marchand et al. 2020). A selective RNA protection against enzymatic cleavage was implemented for m⁶A detection by MazF endonuclease (Garcia-Campos et al. 2019).

Antibody-based enrichment methods (MeRIP-Seq, i/miCLIP)

The use of the specific antibodies for the detection of RNA modifications was already proposed and successfully implemented in the late 70s (reviewed in (Feederle and Schepers 2017)). This development is still ongoing and, in few instances, highly specific antibodies can be obtained (Matsuzawa et al. 2019). However, the majority of antibodies against RNA modified nucleotides/nucleosides have poor affinity and specificity (Mishima et al. 2015) and enrichment factors for modified RNA are only very modest (Slama et al. 2019). Taking these considerations into account, it is not surprising that multiple artifacts in RNA modification mapping result from antibody cross-reactivity, uncertain specificity, and low enrichment (discussed in (Grozhhik et al. 2019; McIntyre et al. 2020)). Despite these limitations, RNA modification-specific antibodies are widely used to RIP and CLIP protocols applied to RNA modification mapping.

Analysis of RNA modifications by NNGS (single molecule sequencing)

The use of single-molecule sequencing approaches (NNGS or third-generation deep sequencing) is an

attractive alternative to the classical cluster sequencing protocols. Indeed, cluster sequencing involves amplification steps, providing only an average picture of modifications in a population of RNA molecules. To get information about the exact combination of modifications in a given RNA molecule (individual modification pattern), a single molecule analysis should be performed (Xu and Seki 2020).

The proof of principle for the analysis of RNA modifications (namely m⁶A) by single-molecule sequencing was established already in 2013, by using PacBio SMRT technology. HIV-1 and AMV RT were loaded to zero-mode waveguides (ZMWs) arrays and extension of DNA primer on m⁶A-modified RNA template was monitored (Vilfan et al. 2013). Even if the precision of the RNA sequencing remains limited, the analysis of the reverse transcriptase kinetics can be used to discriminate RNA base modifications.

More recently, single-molecule direct RNA sequencing by nanopores (Oxford Nanopores) was used for the detection of RNA modifications. It was demonstrated that m⁶A RNA modifications can be detected with high accuracy, in the form of systematic errors and decreased base-calling qualities (Liu et al. 2019). Analysis of raw ion current profiles for direct MinION nanopore sequencing of full-length 16S rRNA revealed 7-methylguanosine (m⁷G) as well as pseudouridine modifications (Smith et al. 2019). The major challenge in the field of direct RNA sequencing and RNA modification mapping by nanopores consists of appropriate data analysis softwares and algorithms. Analysis can be either done by standard base-calling and identification of “sequencing signatures” (Lorenz et al. 2020), or by extremely laborious, but direct analysis of ion current traces (Cozzuto et al. 2020; Ding et al. 2020; Jenjaroenpun et al. 2021).

Mass spectrometry

Mass spectrometry is a valuable tool to analyze RNA modifications. Low-resolution instruments such as quadrupoles are usually very sensitive and can detect attomole amounts of low abundance modifications. High-resolution MS, such as time-of-flight (TOF) or orbitrap instruments, can detect the exact mass of a modification and allow for elemental composition prediction and subsequent fragmentation of the modification, which can provide further structural information. The analysis of RNA can be conducted in a variety of ways and when combined, a broad spectrum of information becomes available. Depending on the enzymatic

treatment of the RNA prior to MS, we discuss 3 major types of MS analyses:

1. Top-down analysis of non-hydrolyzed RNA allows for total mass analysis and modifications can be identified and their location can be determined specifically within the sequence context
2. Bottom-up MS of partially hydrolyzed RNA allows for mass mapping and provides some sequence context for modifications but requires a sequence to map it back to
3. Nucleoside MS of fully hydrolyzed RNA can provide information on the chemical identity of modifications even at incredibly low abundance

Top-down MS

Top-down MS features the injection of undigested RNA into the mass spectrometer. This idea first came up in the early 1990s (McLuckey et al. 1992; Limbach et al. 1995) when oligonucleotides were “sequenced” using mass spectrometers and when MS was used to determine the molecular mass of tRNA isoacceptors and 5S rRNA and to detect potential modifications (Limbach et al. 1995). Later, in the mid to late 2000s approximately 20 nt long oligonucleotides were analyzed (Kellersberger et al. 2004; Huang et al. 2008) and in 2010 the length was extended to 61 nt (Taucher and Breuker 2010). At around that time, the first studies analyzing full-length tRNA by QqTOF (Huang et al. 2010) and FT-ICR (Taucher and Breuker 2012) mass spectrometers appeared. The study using the FT-ICR mass spectrometer achieved full sequence coverage by combining Electron Detachment Dissociation (EDD) and Collision-Activated Dissociation (CAD) experiments. Using the nomenclature for nucleotide fragments (McLuckey et al. 1992), CAD results mainly in complementary (i.e. the sum of their masses equals the mass of the intact RNA) c- and y-type fragment ions along with undesired base-loss fragments and secondary fragments where neither the 3'- nor the 5'-terminus is present (Figure 5 (A–C)).

The EDD pathway produces non-complementary d- and w-type fragment ions, whose masses add up to the mass of the intact tRNA + 97.98 Da (+H₃PO₄) (Taucher and Breuker 2010, 2012). This information can be combined to sequence RNAs and to locate the mass-altering post-transcriptional modifications present in the sample. Other dissociation techniques for oligonucleotides that were investigated in the past include UV-Photo Dissociation (Smith and Brodbelt 2010, 2011), Infrared Multiphoton Dissociation (Smith and Brodbelt 2011),

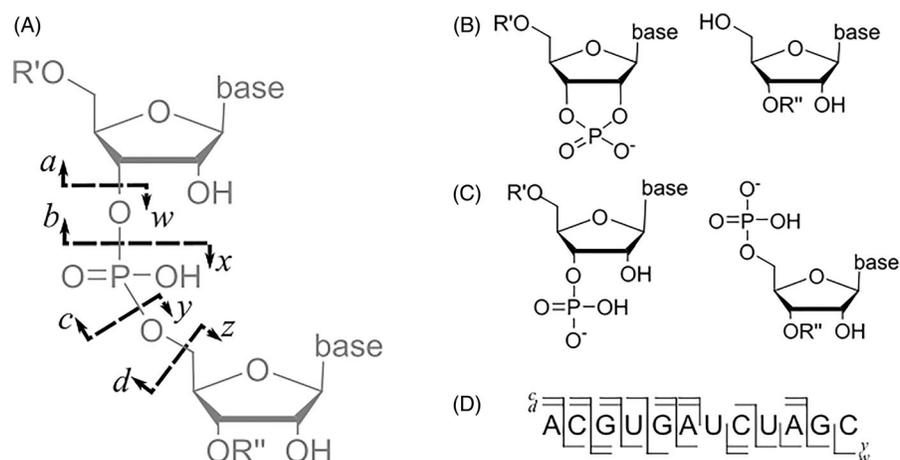


Figure 5. (A) Nomenclature of top-down fragment ions (McLucky et al. 1992). (B) Complementary c (left) and y (right) type fragment ions. (C) Non-complementary d (left) and w (right) type fragment ions. (D) Possible cleavage map of a 12 nt long RNA.

and Electron Photodetachment Dissociation (Yang and Håkansson 2009; Smith and Brodbelt 2010), Electron Capture Dissociation (Mo and Håkansson 2006) and Negative Electron Transfer (Huang and McLucky 2011; Gao and McLucky 2013). An important measure for assessing top-down techniques is called sequence coverage. It describes the number of cleavage sites where at least one resulting product ion could be detected and is typically reported as a sequence cleavage map (Figure 5(D)).

In 2020, the Bruker lab published a report of the novel dissociation technique Radical Transfer Dissociation (RTD) (Calderisi et al. 2020). In RTD, the reagent cobalt(III)hexamine is concomitantly introduced into the mass spectrometer to induce the formation of RNA radical ions, which dissociate into d- and w-type fragments upon collisional activation. At the same time, c- and y-type ions are produced through the well-known CAD phosphodiester bond cleavage. In their publication, they report full sequence coverage of up to 39 nt long RNAs. The proposed mechanism for radical dissociation neither involves the nucleobase nor the 2'-OH group, consequently, RTD might be especially useful for the analysis of modified RNA. In addition, base-loss and internal fragmentation are reduced under RTD. Therefore, it is especially recommended for mapping of labile RNA modifications such as 5-formylcytidine and wybutosine and its derivatives. Moreover, RTD has comparably low requirements for instrumentation: Measurements can be conducted on any mass spectrometer that is equipped with an ESI source and a collision cell (Calderisi et al. 2020).

More recently in 2020, the Coon lab used fluoranthene cations as a Negative Electron Transfer reagent and an infrared laser to excite precursor ions (activated-ion negative electron transfer dissociation, AI-NETD)

(Peters-Clarke et al. 2020). Similar to what has been demonstrated for other electron-based MS/MS approaches, d- and w-type ions predominate in the MS/MS spectra generated by AI-NETD and only minute levels of base-loss and internal fragment ions are observed. The application of this method leads to full sequence coverage of the 21 nt long luciferase antisense siRNA when all fragment ion types are considered. A major benefit of this approach is the independency of FT-ICR instrumentation and thus it is more accessible. Another advantage highlighted by the authors is the lower complexity of spectra generated by their approach in comparison to traditional collision-based dissociation methods, as this method produces fewer secondary and base loss fragments. However, while full sequence coverage of 21 nt luciferase antisense siRNA was obtained by AI-NETD, its performance on longer RNA and more complex mixtures remains to be investigated. RNA-protein complexes can also be investigated using top-down analysis as recently reviewed (Schneeberger and Breuker 2017).

To summarize, top-down analysis of RNA is a technology that is still being developed. Its major benefits comprise the ability to perform de-novo sequencing, identify mass-altering modifications of nucleobases as well as localize these modifications. Furthermore, laborious digestion steps are not necessary. However, the fact that equal-mass modifications (e.g. m^1A and m^6A) cannot be distinguished from each other is a major disadvantage. In addition, the analysis of mass-silent modifications (such as pseudouridine) is challenging. Moreover, pure RNA is required for the top-down analysis, as the analysis of mixtures is laborious or even impossible. Further, the analysis and interpretation of top-down MS spectra are less straightforward due to high spectral complexity, especially as currently there is

no software available to support top-down MS analysis of RNA. With the influx of oligonucleotide-based vaccines and therapeutics exiting clinical trials and entering the pharmaceutical market, we expect an increased interest in the analysis of full-length RNA and thus finally software tools for data processing will be developed.

Modification mapping of oligonucleotides by LC-MS/MS

In the bottom-up approach, a single RNA or a mixture of RNA sequences are digested to oligonucleotides amenable to separation and subsequent analysis by tandem mass spectrometry (MS/MS). The general bottom-up approach for RNA modification mapping has not changed since introduced by McCloskey and colleagues over 25 years ago (Kowalak et al. 1993). Moreover, the key experimental factors and considerations that impact the success of this approach are well known. These include the approach for generating oligonucleotides from the RNA sample; chromatographic methods for the separation of oligonucleotide mixtures; MS and MS/MS approaches to generate sequencing ladders from oligonucleotides; and the data analysis steps that can simplify both interpretation of MS/MS data, RNA sequence reconstruction and subsequent mapping of modifications onto those RNA sequences.

In general, a review of advances in each of these areas finds that there have been incremental improvements within each step that – when combined – have led to major enhancements in the overall analytical strategy. These enhancements now permit the complete mapping of RNA modifications onto messenger RNA (mRNA) sequences, total transfer RNA (tRNA) mixtures from organisms, and ribosomal RNAs (rRNAs) (Jiang et al. 2019; Jora et al. 2019; Yu et al. 2019; Thakur et al. 2020; Wein et al. 2020). While these are impressive achievements, the field could benefit from a radical rethinking of what a bottom-up approach to RNA modification mapping could entail and achieve. In particular, given the advances in NGS and NNGS methods including the goal of single copy RNA sequencing and modification placement, which are built off a completely different technology platform, the inherent advantages of MS for directly detecting an intrinsic property of the molecule (i.e. the m/z or mass of the molecule) argues for continued investments and advancements in this platform for RNA modification mapping.

Digestion of RNA sample into oligonucleotides

Traditionally, RNA is enzymatically digested to oligonucleotides using site-specific ribonucleases (RNases). The

most commonly used RNases are RNase T1 (guanosine-, N^2 -methylguanosine-, and inosine-specific), RNase A (pyrimidine-, and pseudouridine-specific), and RNase U2 (purine-selective). In general, RNases are selected based on the desirable generation of relatively longer (5-15 nucleotides long) and sequence-unique oligonucleotides. While the use of single RNases has been proven effective throughout the years, the inherent challenge has been that a single RNase limits the analyst's ability to accurately reconstruct the entire RNA sequence. Not surprisingly then, much of the recent work in this area has focused on identifying and implementing new RNases that can be used in combination to increase sequence coverage through the generation of overlapping digestion products (Figure 6).

In a study mapping *E. coli* total tRNA, Addepalli and coauthors (Thakur et al. 2020) demonstrated that the combination of RNases T1, MC1 (uridine-specific), and cusativin (cytidine-specific) led to an overall $\geq 75\%$ sequence coverage. They also showed that for *E. coli* 23S rRNA, sequence coverage was enhanced from $\sim 25\%$ (single RNases) to 85% when the same set of RNases were combined. In a similar fashion, Hua et al. have shown that similar sequence coverage is achieved when RNases T1, colicin E5 (cleaves between guanosine and uridine), and mazF (cleaves at ACA sequences) are used in parallel to map mRNAs (Jiang et al. 2019). An alternative approach was the development of a nonspecific RNase U2 mutant, which led to complete sequence coverage of modified RNAs through the generation of overlapping digestion products in a single-pot digestion reaction (Solivio et al. 2018).

While these technical achievements demonstrate new analytical approaches that can be taken, most of the RNases employed in these seminal studies (i.e. MC1, cusativin, colicin E5, and U2 mutant) were produced in-house. As a result, such approaches are commonly plagued by the lack of batch-to-batch or interlaboratorial reproducibility of in-house expressed and purified RNases. Thus, in addition to illustrating the efficacy of bottom-up MS-based approaches to locate modified nucleosides within different RNA species, these studies strongly advocate for the batch production and commercialization of reproducible complementary RNases.

If the analysis of multiple tRNAs is desired it needs to be taken into account that lots of tRNA isoacceptors do have similar sequences and therefore similar or even equal masses, which hampers sequencing by mass spectrometry, as isotopic pattern overlays may occur often. In order to overcome this obstacle, different isoacceptors can be separated by two-dimensional polyacrylamide gel (2D gel). This way tRNA_{GCU}^{Ser} and tRNA_{CAG}^{Leu}

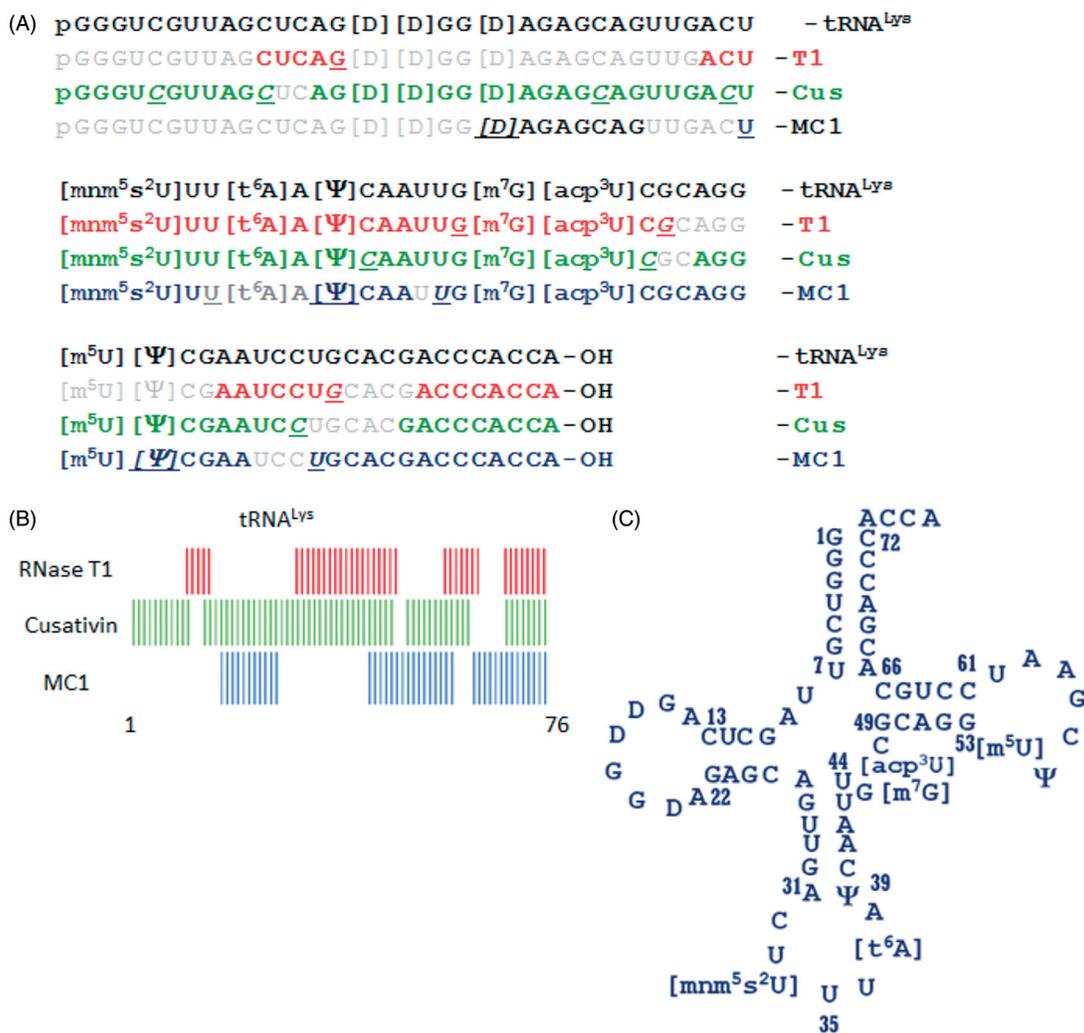


Figure 6. RNA modification and sequence mapping of *E. coli* tRNA^{Lys} from the digestion products of RNases T1, cusativin, and MC1. (A) The observed digestion products of each ribonuclease are matched against the known sequence of tRNA^{Lys}. The cleavage sites for each enzyme is underlined in an oligomer. Note the overlapping regions between the sequences of digestion products. (B) A schematic view of looking at the overlaps between the observed digestion products of the three ribonucleases. (C) The clover-leaf model of 2D structure of *E. coli* tRNA^{Lys} is depicted. Figure reproduced with permission from (Thakur et al. 2020). See colour version of this figure at www.tandfonline.com/ibmg.

were sequenced. However, a major weakness is a necessity for ethidium bromide staining – a hazardous substance, which renders the technique obsolete (Fradin et al. 1975; Gaston and Limbach 2014). To obtain specific short oligonucleotide fragments, the isoacceptors are digested with certain RNases in an “in gel digestion”. Subsequently, these oligonucleotides are separated on a nano-ion-pair reversed-phase high-performance liquid chromatography (nano-IP-RPHPLC) (Taoka et al. 2010) and are analyzed by MS/MS. This method enables the identification of tRNA isoacceptors by using genomic databases. Mass-silent modifications such as pseudouridine cannot be detected by this method, thus, CMCT derivatization is recommended prior to analysis. Moreover, methyl groups cannot be localized in modified nucleotides. However, if the tRNA

sequence is known, it is possible to predict the respective methyl group position.

While conventional mapping of modifications by MS is done through the use of RNase-based digestion, Zhang et al. have conceptually demonstrated the sequencing and modification mapping of RNA through sequence ladders generated by acidic digestion (50% v v⁻¹ formic acid) (Bjorkbom et al. 2015; Zhang, Shi, et al. 2019). While the efficacy of the approach on more labile modifications and more complex RNA mixtures has not been demonstrated yet, their 3'- and 5'-end labeling strategies (through the addition of tags such as biotin, cyanine3, and cyanine5) (Zhang, Shi, et al. 2019) may pave the way for a multiplexed qualitative and quantitative analysis of modified oligonucleotides by bottom-up MS approaches. Looking forward, alternative

approaches generating longer oligonucleotides that remain amenable to mass spectrometry sequencing and analysis would greatly benefit the field. As the read length increases, the analyst's confidence in modification mapping accuracy improves. Further, strategies such as end-labeling could lead to significant enhancements in throughput as compared to what is currently achievable by more conventional approaches.

HPLC separation of oligonucleotides

In addition to developments in sample preparation strategies, separation approaches employed for modification mapping have also been improved. In general, oligonucleotides are subject to chromatography prior to MS analysis. To date, the most common chromatography approach is the use of reversed-phase high-performance liquid chromatography (RP-HPLC). In general, the typical RP-HPLC mobile phases – both aqueous and organic – can be selected for compatibility with MS conditions. Moreover, standard RP-HPLC stationary phases and columns have been optimized for various flow rates amenable to electrospray ionization.

Due to the relatively high hydrophilicity conferred by the negatively charged phosphate backbone, the use of ion-pairing reagents as mobile phase modifiers is needed to achieve chromatographic retention and selectivity of oligonucleotides during MS analysis (Biba et al. 2017; Li et al. 2018). Such a chromatographic approach is named ion-pair reversed-phase liquid chromatography (IP-RP-LC). Different alkylamine modifiers have been used during IP-RP-LC of oligonucleotides, and broadly investigated by the Bartlett lab (McGinnis et al. 2013; Li et al. 2018). Some of their recent studies have led to models able to predict optimal ion-pairing reagents based on sample hydrophobicity (McGinnis et al. 2013), as well as a better understanding of the retention mechanism under ion-pairing conditions (Li et al. 2018). Nonetheless, the use of alkylamine triethylamine (TEA) and the counter ion hexafluoroisopropanol (HFIP), developed by Apffel et al. (Apffel et al. 1997), is still a method of choice for oligonucleotide analysis by LC-MS (Baldrige et al. 2018; Lin, Miyauchi, et al. 2018). While TEA contributes to excellent chromatographic performance, HFIP helps with the ionization of oligonucleotides, improving the sensitivity of oligonucleotide analysis (Biba et al. 2017). These chromatographic conditions under nanoflow-based LC-MS analysis have been used to map modifications in nanogram aliquots of complex RNA samples (Yamauchi et al. 2016; Taoka et al. 2018; Nakayama et al. 2019; Wein et al. 2020).

One drawback of IP-RP-LC is that the mobile phase modifiers (alkylamines/HFIP) are often linked to ion

suppression, observed when subsequent LC-MS analyses are conducted in positive ion mode (e.g. during proteomics analyses). Such contamination concerns have led to the common practice of having dedicated LC and MS systems for oligonucleotide analysis, limiting the number of groups interested in this field. Due to the costly burdens of dedicated instruments, alternatives to IP-RP-LC of oligonucleotide have been investigated. The Kellner lab has shown that modifications on tRNA transcripts may be mapped using similar conditions used for nucleoside analysis (Hagelskamp et al. 2020). In their approach, chromatographic separation of modified oligonucleotides is achieved using an octadecylsilane (C18)-derived RP-LC column (Fusion-RP), and mobile phases consisting of water, acetonitrile, and ammonium acetate. The mass spectrometric analysis is done in positive ion mode (as opposed to the traditionally used negative ion mode). While the efficacy of this method to chromatographically resolve complex oligonucleotide mixtures (e.g. total tRNA digests) is yet to be demonstrated, it is a suitable alternative for the study of synthetic or purified RNA species. Such an approach led to identifying that A34 of tRNA^{Val}_{AAC} is deaminated by the human adenosine deaminase tRNA specific enzyme 2/3 (ADAT2/3) (Hagelskamp et al. 2020). It also supported the discovery that *E. coli*'s alpha-ketoglutarate-dependent hydroxylase AlkB is an eraser with higher specificity toward m¹A1408 of the 16S rRNA (Hagelskamp et al. 2020).

Among the alternatives being explored to IP-RP methods, hydrophilic interaction liquid chromatography (HILIC) represents a promising alternative. The Limbach lab has shown that a performance comparable to IP-RP-LC in terms of chromatographic retention, selectivity, and resolution, as well as mass spectrometric sensitivity, is feasible through HILIC-MS (Lobue, Jora, et al. 2019). In this approach, water-, acetonitrile-, and ammonium acetate-based mobile phases are used. However, the column used in their study (HILICpak VN-50 2D) is only commercially available in relatively large particle size (5 μm) and limited column dimensions (2.0- or 4.6-mm internal diameter, by 150-mm length), thus limiting the chromatographic and mass spectrometric performance of the approach. Another recent study has demonstrated that similar chromatographic performance (under similar mobile phase conditions) may be achieved during oligonucleotide analysis using another commercially available HILIC column (BEH amide) (Demellenne et al. 2020). This column is available in a wider range of column and particle sizes, thus expanding possibilities in terms of method development and optimization. Continued efforts around HILIC-based

approaches, and other alternative LC methods, are needed to identify those LC-MS conditions that can achieve the performance found in current IP-RP methods while minimizing concerns about instrument carry-over, contamination, and sample suppression that are often shared anecdotally as reasons to avoid LC-MS analyses of oligonucleotides.

Independent of the LC method, modification mapping of complex RNA samples (such as total tRNA) by LC-MS is challenging. More often than not, oligonucleotides generated from RNase digestion of RNA mixtures are challenging to completely resolve chromatographically. Further, another challenge is the finite dynamic range (i.e. difference between the most abundant and least abundant species) of mass spectrometers (usually up to 10^4 – 10^5) (Zubarev 2013) that impact the ability to detect co-eluting oligonucleotides. To overcome such challenges, labor-intensive offline methods that minimize sample complexity are traditionally employed prior to RNase treatment and LC-MS analysis (Kumazawa et al. 1992; Suzuki and Suzuki 2007). In a more elegant fashion, online approaches that explore the orthogonality of the different LC modes employed during oligonucleotide analyses have been recently developed (Goyon and Zhang 2020; Li et al. 2020). More specifically, one-dimensional anion exchange chromatography (AEX) or IP-RP-LC methods have been coupled to HILIC, IP-RP-LC, or RP-LC as the second LC dimension to facilitate the characterization of synthetic oligonucleotide impurities (Goyon and Zhang 2020; Li et al. 2020). Considering the demonstrated potential of two-dimensional-LC (2D-LC) approaches to resolve synthetic oligonucleotides, we anticipate that 2D-LC approaches have the potential to facilitate modification mapping in complex biological samples. In this sense, a comprehensive investigation regarding the orthogonality of the different LC modes employed for modification mapping, as well as investigation of the retention mechanisms driving the separation of oligonucleotides in these different LC modes is advised.

MS/MS sequencing of oligonucleotides

The information generated through tandem mass spectrometry (MS/MS) plays a pivotal role in successful modification mapping. Among MS/MS tools, collision-induced dissociation (CID) is the most widespread fragmentation technique used. Under CID, precursor ions are accelerated by an electric field, and through collisions with a neutral gas (generally He, Ar, or N₂) bond cleavage is enabled, generating fragment ions. During oligonucleotide analysis, CID leads to fragmentation within the phosphodiester backbone of

oligonucleotides in a ladder-fashion, generating c-, y-, a-, and w-type fragment ions, with c and y being the most abundant ions (Figure 5). Characteristic sequence ladders and mass shifts observed in these fragment ions help locate modification(s) within a given oligonucleotide precursor ion.

Mapping positional isomers such as 1-methyladenosine (m¹A), and N⁶-methyladenosine (m⁶A) within an RNA sequence by LC/MS-MS is not a straightforward task. Moreover, while pseudouridine (Ψ) may be easily characterized during nucleoside analysis, it adds another layer of complexity during modification mapping experiments. Because it is isomeric with uridine (U), pseudouridine cannot be directly identified based on its mass (or *m/z*) during oligonucleotide analysis by LC-MS/MS. Derivatization strategies mentioned earlier have been implemented for differentiating pseudouridine from uridine (Patteson et al. 2001; Mengel-Jørgensen and Kirpekar 2002; Durairaj and Limbach 2008). Even though derivatization facilitates mapping, this strategy requires extra experimental steps and is targeted to one modification (or class) at a time. Pomerantz and McCloskey have explored the characteristic C-glycosidic bond present in Ψ and the absence of “a-base” ions at the Ψ site to facilitate mapping of Ψ-containing oligonucleotides through MS/MS (Pomerantz and McCloskey 2005). In a similar fashion, the Taoka lab developed a pseudo-MS³-based mapping strategy that allows direct identification and mapping of ribonucleoside isomers. In their approach, two rounds of LC-MS/MS analyses are performed. In the first round, traditional oligonucleotide analysis is carried out. As such, modifications may be mapped, but the nature of the isomer present in a given oligonucleotide may not be identified. In the second round, in-source CID (in which c-, y-, w-, a-type ions are generated) and higher-energy collisional dissociation (HCD, in which ribonucleoside fingerprints are generated) are performed. In this step, the nature of the isomers (mapped within a given oligonucleotide in the first step) is identified. Such an approach has been used to identify Ψ in the human spliceosomal snRNAs (Yamauchi et al. 2016) and in the rRNA of *Saccharomyces cerevisiae* (Taoka et al. 2016), as well as to map modifications present in the rRNAs of the human 80S ribosome (Taoka et al. 2018) and in the rRNAs of *Leishmania donovani* (Nakayama et al. 2019).

The nature of MS/MS spectra generated through collision-based dissociations (CID and HCD) are highly dependent on the charge state and length of the oligonucleotide. Moreover, they may be convoluted due to the occurrence of ions resulting from internal

fragmentation and neutral loss of labile nucleobases (Schurch 2016), thus impacting data processing and sequence coverage. Alternative MS/MS strategies employing electrons or photons have been investigated throughout the years (Smith and Brodbelt 2010; Taucher and Breuker 2010; Huang and McLuckey 2011). Recently the Coon lab proposed activated-ion negative electron transfer dissociation (AI-NETD) as an alternative tool for oligonucleotide mapping and the Breuker lab developed a fragmentation approach termed radical transfer dissociation (RTD) (Calderisi et al. 2020). These two approaches are discussed in detail in the chapter *Top-down MS*.

It is undeniable that these MS/MS strategies facilitate modification mapping. Nonetheless, challenges still exist when it comes to improving the acquisition of MS/MS spectra of low abundance modified oligonucleotides. MS/MS analysis of oligonucleotides is traditionally done using data-dependent acquisition (DDA) mode (Cao and Limbach 2015). Here, precursor ions detected in the first MS stage are sequentially selected (usually the top 3–10 most abundant ions) for MS/MS analysis. Consequently, each MS/MS acquired spectrum has a precursor ion m/z linked to it, thus facilitating data interpretation. Because ions are introduced to the mass spectrometer for a limited amount of time (i.e. chromatographic peak width), and the co-elution issues mentioned above, it is not always possible to acquire MS/MS spectra for all oligonucleotides present in a given sample. To enhance the acquisition of MS/MS spectra of modified oligonucleotides, the Limbach lab has implemented an exclusion list-based protocol (Cao and Limbach 2015). As such, m/z s corresponding to unmodified oligonucleotides are excluded from MS/MS analysis. This exclusion list-based approach improved RNA modification mapping of bacterial and archaeal total tRNAs by 10–25% when compared to standard DDA. What remains to be shown is whether this or other DDA-based strategies will enable modification mapping of more complex RNA mixtures, including those from mammalian samples.

In addition to DDA, MS/MS spectra may also be acquired using data-independent acquisition (DIA) mode (Fernández-Costa et al. 2020). As such, all precursor ions detected at a given time within a predefined m/z range are concomitantly fragmented, thus resulting in a multiplexed MS/MS spectrum. Despite the challenges associated with the deconvolution of DIA-based MS/MS spectra, this strategy has been largely employed for protein sequencing (including mapping of post-translational modification) by LC-MS/MS (Zhang et al. 2020). Nonetheless, it has yet to be explored for RNA

modification mapping. Considering the potential of the approach to facilitate modification mapping in complex samples (e.g. human total tRNA), developments in this area are advised.

Software to analyze LC-MS/MS data

A few significant software and bioinformatics resources have been developed to facilitate modification mapping (Figure 7) (Rozenski and McCloskey 2002; Matthiesen and Kirpekar 2009; Nakayama et al. 2009; Nyakas et al. 2013; Sample et al. 2015; Paulines et al. 2019). Improved performance has been achieved by two recently developed open-source tools, RNAModMapper (RAMM) (Yu et al. 2017; Lobue, Yu, et al. 2019) and NucleicAcidSearchEngine (NASE) (Wein et al. 2020). Both software platforms offer algorithms capable of interpreting and mapping modifications in large LC-MS/MS datasets obtained during analysis of complex oligonucleotide mixtures (Baldrige et al. 2018; Solivio et al. 2018; Yu et al. 2019; Thakur et al. 2020; Wein et al. 2020). RAMM is a user-friendly tool that maps modifications via two independent modes: fixed, for targeted modifications/positions; and variable, in which modifications are mapped in an untargeted fashion. Data interpretation is done based on RNA/DNA sequence inputs, through a two-component scoring function, with user-defined and instrument-based scoring thresholds. NASE is an OpenMS-based (Rost et al. 2016) search engine that presents extra functionalities such as high-quality data visualization and quantification capabilities. Because it supports decoy RNA sequences as input in addition to the target sequence, false discovery rates are minimized in such a tool. Moreover, the incorporation of m/z corrections due to salt adducts (traditionally observed during oligonucleotide analysis (Sutton and Bartlett 2020)), and mass defects resulting from instrumental selection of higher abundance isotopologue peaks, significantly enhances the ability of NASE to identify modified oligonucleotides. Although these (and earlier developed) tools have paved the way to facilitate LC-MS/MS spectral interpretation of oligonucleotide data, manual review is still a necessary step. Thus, the community has yet a lot to gain from further developments on software platforms dedicated to MS-based modified oligonucleotide mapping.

Nucleoside MS

Unlike the previously described MS methods, nucleoside MS relies on complete enzymatic hydrolysis of RNA into its nucleoside building block. The hydrolysis is

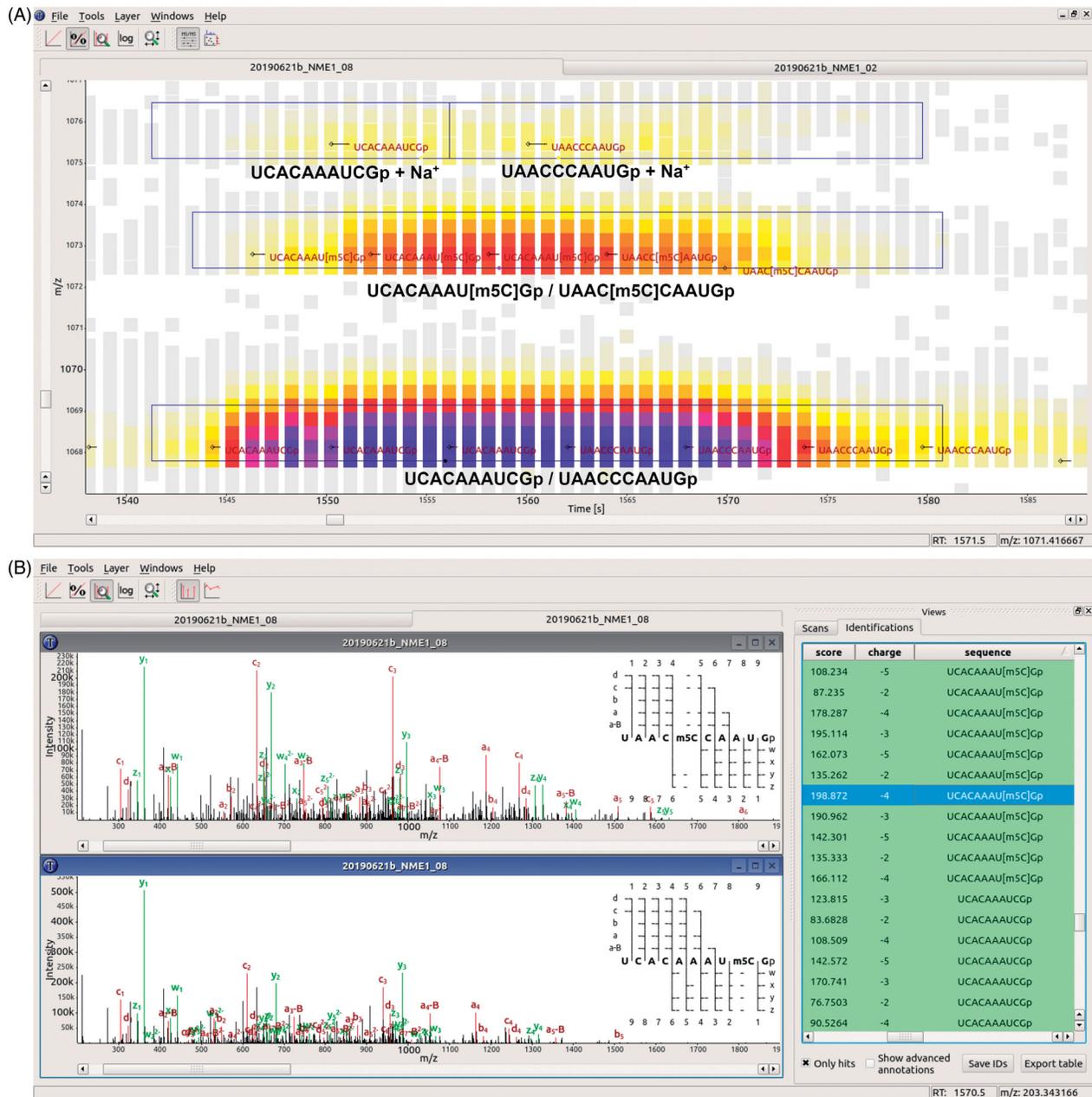


Figure 7. Interactive data visualization using TOPPView, showing data from the NCL1-treated NME1 sample. (a) MS1 view (RT-by- m/z) of a data section. LC-MS peaks are shown as small squares, colored according to their signal intensities. Small black diamonds and horizontal lines indicate MS/MS fragmentation events; oligonucleotide sequences identified by NASE from the MS/MS spectra are shown in dark red font. Black boxes outline features detected for label-free quantification, which have been annotated with the corresponding oligonucleotides. All oligonucleotides shown have a charge state of -3 . (b) "Identification view" comparing two MS/MS spectra, identified by NASE as the sequences "UAAC[m⁵C]CAAUGp" and "UCACAAAU[m⁵C]Gp". Matching peaks between the acquired and theoretical spectra are annotated and highlighted in red and green. On the right in each spectrum plot, an ion coverage diagram shows which of the theoretical fragment ions of the sequence were matched in the MS2 spectrum (in any charge state). Figure reproduced with permission from (Wein et al. 2020). See colour version of this figure at www.tandfonline.com/ibmg.

commonly achieved using endonucleases such as nuclease P1, nuclease S1 or benzonase and supported by the use of phosphodiesterase 1 (Crain 1990; Cai et al. 2015). The resulting 5'-phosphorylated nucleotides are dephosphorylated using alkaline phosphatase

and free nucleosides are released. The analysis of hydrolytic digests has several drawbacks. The first is the dependence of the result on the purity of the analyzed RNA. Contaminating RNA will obscure the quantitative result by either introducing unexpected RNA

modifications or by simply diluting the target RNA and thus the number of detectable RNA modifications. Thus, a quality control step, for example, (chip) gel electrophoresis or sequencing is crucial prior to hydrolysis to ensure comparable purity of samples. The second is the introduction of artifacts through the hydrolysis protocol. In the original protocol by Crain and colleagues (Crain 1990), the hydrolysis is performed in a two-step protocol first at pH 5 followed by a pH elevation to pH 8. These conditions may destroy labile RNA modifications such as cyclic N(6)-threonylcarbamoyladenosine (ct⁶A) which undergoes epimerization under mild alkaline conditions (Matuszewski et al. 2017). Other artifacts such as isocytidines emerge through amination/amination of carbonthiolated nucleosides during RNA hydrolysis (Jora et al. 2020). Furthermore, not all enzymes used for RNA hydrolysis are capable of cleaving modified nucleotides. For example, the He lab recently demonstrated that nuclease S1 alone is not capable of cleaving m⁷G from the mRNA cap. Only in the presence of phosphodiesterase 1, the complete m⁷G cap is cleaved and m⁷G is released (Zhang, Liu, et al. 2019).

In the past, nucleoside hydrolysate separation by thin-layer chromatography (TLC) of ³²P radioactively labeled nucleotides (Gupta et al. 1976; Stanley and Vassilenko 1978; Gupta and Randerath 1979; Keith 1995; Zhao and Yu 2004; Grosjean et al. 2007), or gas chromatography after nucleoside derivatization have been reported (Gehrke and Ruyle 1968; Lakings and Gehrke 1971; Gehrke and Patel 1976; Gehrke et al. 1980). Today, the resulting nucleoside mixture is most commonly separated using liquid chromatography (LC) before detection by UV absorption or MS (LC-UV or LC-MS). The field has recently seen substantial advances, especially in the context of separation techniques but also in terms of absolute quantification and analysis of RNA modification dynamics.

Advances in the separation of nucleosides

Most established separation systems rely on the use of reverse phase columns as described elsewhere (Pomerantz and McCloskey 1990). While these systems allow efficient separation and analysis of modified nucleosides from many native samples within less than 10 min (Heiss et al. 2017; Reichle, Kaiser, et al. 2018; Borland et al. 2019), recent advances to improve the separation efficiency have been made.

For example, new stationary phases, based on Hydrophilic interaction liquid chromatography (HILIC), have been reported. For native RNA hydrolysates, HILIC allows separation of nucleosides within 30 min (Lobue,

Jora, et al. 2019). Analysis of modified nucleosides from urine has a great potential as a diagnostic tool (Bryzgunova and Laktionov 2015) and here HILIC has also been used for efficient separation and sensitive detection of cytidine derivatives (Guo et al. 2018).

In 2018 Sarin et al. established a sensitive capillary nanoflow liquid chromatography mass spectrometry (nLC-MS) for absolute quantification of ribonucleosides. While the separation by liquid chromatography of some ribonucleosides remains challenging by the polar attributes of the analytes, the utilization of porous graphitic carbon (PGC) as column material can overcome this obstacle. This material is stable over broad temperature and pH ranges and possesses a polyaromatic scaffold which allows the separation of highly polar, charged, and structural similar analytes (Sarin et al. 2018).

Advances in absolute quantification

As described above, top-down and oligonucleotide MS are commonly done through negative ionization (e.g. deprotonation) of the phosphate backbone before analysis on high-resolution MS instruments. Due to the absence of phosphates after complete RNA hydrolysis to the nucleoside building block, ionization of nucleosides exploits the basic character of the nucleobases which are easily ionized through protonation. Thus, nucleoside MS is performed in positive ion mode. Quantification of small molecules is commonly done on low-resolution mass spectrometers consisting of two quadrupoles for ion selection interconnected through a collision cell that fragments the selected precursor ion (in the past another quadrupole). The principle is shown in Figure 8(A). Such triple quadrupole MS systems (QQQ-MS) are highly sensitive as the double selection of target analytes leads to substantial noise reduction and thus clear signals for quantification are received.

Even though a mass spectrometer might be a sensitive device for RNA modification analysis in the sub-femtogram range, its non-quantitative nature constitutes a drawback. To allow precise and accurate absolute quantification, stable isotope-labeled internal standards are pivotal for analysis. In the literature, the abbreviations SILIS (stable isotope-labeled internal standard) or ISTD/IS (internal standard) are commonly used, but the term SILIS is preferable as it is more clearly defined. In general, an IS is necessary to account for fluctuations in instrumental detection efficiency. In the case of mass spectrometry, fluctuations are caused by instrument runtime in between cleaning procedures, LC buffer composition, ion load of samples, and other mostly

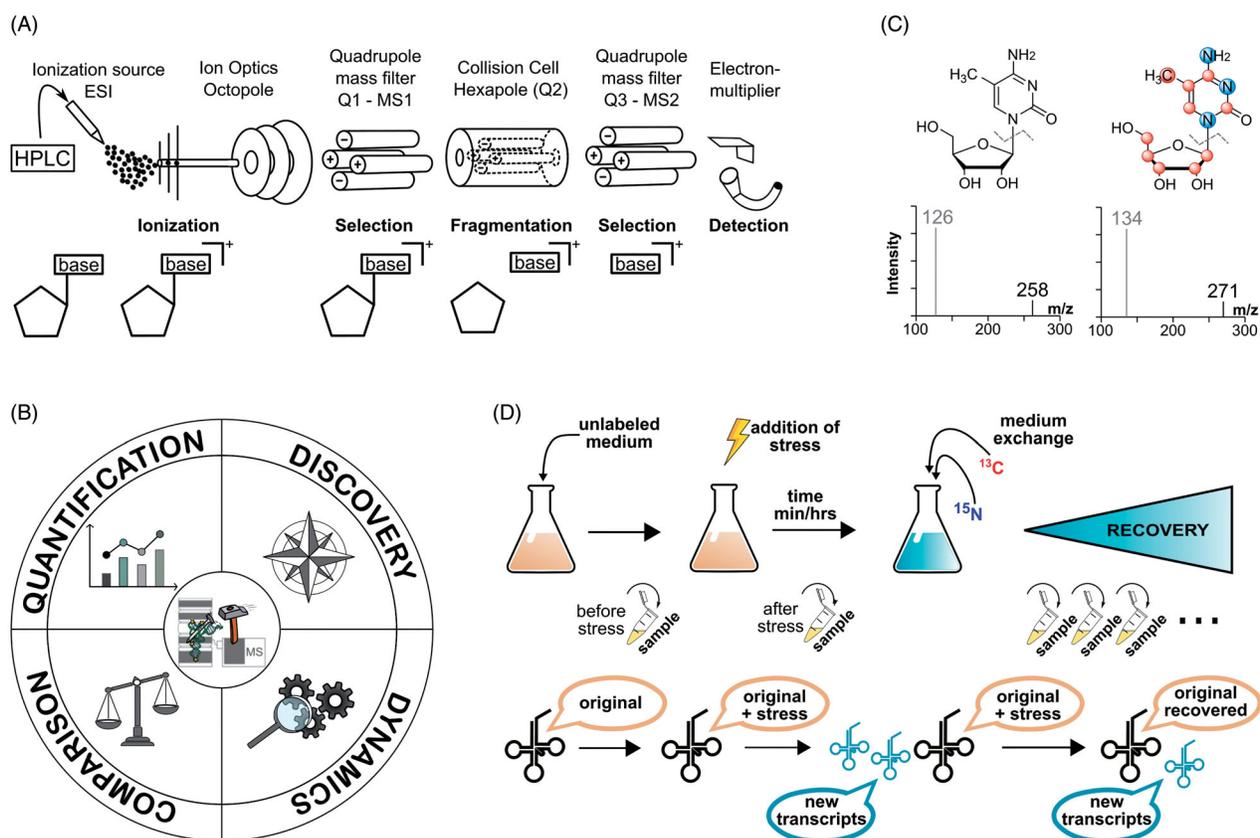


Figure 8. Principle and recent advances in nucleoside mass spectrometry (MS). (A) Instrumentation for sensitive detection of modified nucleosides by a triple quadrupole MS. After separation of the RNA hydrolysate via HPLC, the effluent is ionized in an electrospray ion source (ESI) and the nucleoside is ionized by protonation. The first quadrupole selects for the nucleoside ion. The collision cell fragments the nucleoside and the charged nucleobase remains. After another selection, the nucleobase enters the detector and a signal is recorded. (B) Chemical structure of 5-methylcytidine and its mass spectrum through targeted MS/MS analysis. The left structure is the natural isotopologue, the right structure an isotopologue where all carbon and nitrogen atoms have been exchanged to stable isotopes (here ¹³C and ¹⁵N). (C) Overview of different techniques utilizing stable isotope labeling for RNA modification analysis through mass spectrometry (NAIL-MS, nucleic acid isotope labeling coupled mass spectrometry). (D) Exemplary workflow of a dynamics NAIL-MS experiment. Cells are grown in, for example, unlabeled medium and stressed. After stress exposure the medium is exchanged to a labeled variant. Newly transcribed RNA and original RNA can be easily distinguished through their differential labeling. See colour version of this figure at www.tandfonline.com/ibmg.

uncontrollable factors. An IS is ideally identical to the analyte of interest in terms of detection efficiency but is yet sufficiently different to allow its discrimination from the actual analyte. Isotopologues are molecules that only differ in their isotope composition but retain identical physicochemical properties and thus behave identically on LC-MS systems. Many stable isotopes, especially ¹³C and ¹⁵N, fulfill this requirement and thus SILIS are now established aides in quantitative RNA modification analysis (Figure 8 (B)).

Here, synthetically produced SILIS (Brandmayr et al. 2012) and biosynthetically produced SILIS (Kellner, Ochel, et al. 2014; Borland et al. 2019) can be distinguished. Over the last decade, achievements have been made in terms of biosynthetically produced SILIS. While only 11 modifications could be used for quantification

in a SILIS in 2014 (Kellner, Ochel, et al. 2014) today up to 26 modifications produced in different organisms can be utilized as SILIS (Borland et al. 2019). In addition, there is now evidence that it is of no relevance in which organism the SILIS was produced as it can be used for quantification of RNA modifications in any other organism. With the help of SILIS whole modification profiles can be assessed (Borland et al. 2019).

Making RNA modification dynamics visible with NAIL-MS

With the rise of biosynthetic production of stable isotope-labeled RNA for mass spectrometry purposes, a new technique for tracing RNA modification dynamics emerged.

Nucleic acid isotope labeling coupled mass spectrometry (NAIL-MS) relies on the efficient and mono-isotopic labeling of RNA and is mainly used to follow changes of the epitranscriptome in a certain RNA population. However, NAIL-MS is also used in the context of RNA modification discovery (Dumelin et al. 2012; Kellner, Neumann, et al. 2014; Kellner, Ochel, et al. 2014; Thiaville et al. 2016; Dal Magro et al. 2018) quantification or multiplexing (Reichle, Kaiser, et al. 2018) (Figure 8(C)).

Dynamic NAIL-MS analyses are also referred to as pulse-chase experiments. Before experiment initiation, the cells of interest are grown in a medium containing controllable stable isotope sources. For example, in *E. coli* (Reichle, Kaiser, et al. 2018; Reichle, Weber, et al. 2018; 2019) and *S. cerevisiae* (Heiss et al. 2017) glucose can be used as either $^{13}\text{C}_6$ - or $^{12}\text{C}_6$ -glucose, while human cells require supplementation with stable isotope-labeled nucleosides or nucleobases for efficient labeling (Heiss et al. 2021). To assess the impact of a pulse on the epitranscriptome, the medium is exchanged to contain the other isotopologue of the nutrient. Through intelligent experiment design, it is possible to assess the impact of the pulse on the original transcripts alongside the modification kinetics of nascent RNA (concept shown in Figure 8(D)). Here, a maximal labeling efficiency of close to 100% is necessary. In some cases, the epitranscriptome of nascent RNA is not of interest and thus a lower starting labeling efficiency of around $\sim 50\%$ is acceptable.

NAIL-MS enables the differentiation between RNA degradation, RNA modification, or demodification. An extensively studied pulse for NAIL-MS studies is methyl methanesulfonate (MMS), which methylates nucleophilic positions in RNA. In the context of RNA methylation damage repair, NAIL-MS was used to discriminate the origin of methylation marks in damaged RNAs, and subsequently the repair of damage through enzymatic demethylation was visualized for m^1A , m^3C , and ms^2C in living *E. coli* cells (Reichle, Kaiser, et al. 2018; Reichle, Weber, et al. 2018; 2019). Interestingly, NAIL-MS revealed only low abundant methylation damage products in human tRNA and no repair was observable in living human cells (Heiss et al. 2021). This result highlights a major pitfall of NAIL-MS. The time resolution depends on the speed of labeled nutrient uptake upon medium exchange and afterward on the metabolic processing speed inside the cell. Thus, a delay from medium exchange to isotope-labeled RNA detection through LC-MS can be observed. If a biological process is faster than this delay, it is not possible to resolve the mechanisms of the epitranscriptome change through

NAIL-MS. Although there are limitations to NAIL-MS, it is currently the only available technique to allow a multi-layered analysis of the epitranscriptome and its dynamics.

Summary and outlook

To determine the structure, function, and impact of RNA modifications various techniques are available. Many require the use of expensive instrumentation dedicated to RNA modification analysis and often collaborative efforts of experts in these analyses are needed to study a given epitranscriptomic mark. Currently, N6-methyladenosine (m^6A), is one of the best-studied RNA modifications, as it regulates the fate and function of messenger RNA and thus impacts gene expression. Initial studies on m^6A writer and erasers (enzymes adding and removing m^6A from mRNA) strongly depended on nucleoside MS analyses and antibody-based sequencing techniques (Jia et al. 2011; Zheng et al. 2013; Liu et al. 2014). Many advances discussed in this review have been made in the context of m^6A detection. Nucleotide resolution mapping of m^6A is now possible without using antibodies of questionable specificity (Mishima et al. 2015; Grozhik et al. 2019; Slama et al. 2019; McIntyre et al. 2020) by using Oxford Nanopore sequencing (Li et al. 2020) or the endonuclease *mazF*, which shows an m^6A dependent cleavage ability (Garcia-Campos et al. 2019). In addition, top-down MS (Hoernes et al. 2016) and oligonucleotide MS (Jiang et al. 2019) are now capable to analyze full-length mRNA, and thus mapping m^6A modifications through MS is possible. However, software limitations for data analyses have to be overcome before broader applicability of MS becomes available. Our understanding of the m^6A modification has further deepened through NMR studies, where an impact of m^6A on RNA annealing was shown (Shi et al. 2019).

The power of combining sequencing and mass spectrometric analyses was recently presented by the Waldor lab. They predicted modified nucleosides in tRNAs of the uncharted organism *Vibrio cholerae* by comparing RT signature profiles with *E. coli* tRNA profiles. After sequencing, two RT signatures stood out. One of them was analyzed through nucleoside and oligonucleotide MS which led to the discovery of a novel modified nucleoside, namely acetylated 3-amino-3-carboxypropyl (acacp^3U) (Kimura et al. 2020). The discovery of novel modified nucleosides is currently supported through the continuously increasing sensitivity of modern mass spectrometers. Yet, this advance is connected to the risk of mis-interpretation of MS data

and reporting lowly abundant signals as novel epitranscriptomic marks. In fact, careful analysis of such native signals through high-resolution mass spectrometry and metabolic isotope labeling is necessary to exclude artifacts (Jora et al. 2020).

Recent advances in CryoEM analysis of large protein and RNA-protein complexes now allow “direct” observation of complex structures at almost atomic resolution. Therefore, it is tempting to interpret extra electronic density as post-transcriptional RNA modifications (Natchiar et al. 2017; Golubev et al. 2020; Sas-Chen et al. 2020; Stojković et al. 2020). While it is certain that CryoEM brings exceptional insights in structural analysis of RNA-protein complexes, its real value for “direct” observation of RNA modifications remains questionable. CryoEM data should be considered with extreme care since many of the newly identified nucleotides in human 80S ribosome (Natchiar et al. 2017) may be artifacts from tightly bound water molecules and/or Mg²⁺ atoms and were not confirmed by any other approaches (Taoka et al. 2018; Enroth et al. 2019).

The reversibility of RNA modifications through RNA erasers has introduced the dimension of time into the instrumental analysis of the epitranscriptome. The observation of RNA modification and demodification processes inside living cells is now possible by utilizing stable isotope labeling of RNA in combination with NMR and MS. Both techniques were introduced by demonstrating tRNA^{Phe} maturation processes. In yeast, sequential orders in the introduction of modifications along the tRNA maturation pathway were found alongside of modification circuits (Figure 2) (Barraud et al. 2019). By NMR, sequential incorporation of Ψ55 followed by m⁷G and m⁵U and finally m⁵C and m¹A was observed. In human HEK 293 cell tRNA^{Phe}, a slightly different sequential order was observed through NAIL-MS analysis. Ψ is quickly incorporated, potentially accompanied by m⁵U, followed by m⁵C and m¹A and finally, m⁷G occurs (Heiss et al. 2021). This study also showed that anticodon-loop modifications form rather slowly which implies that structure stabilization by modified nucleosides is a key necessity and must thus happen early on, while ac-loop modifications are not immediately needed and are potentially placed on-demand. Earlier studies on RNA maturation were done with radioactive labeling of RNA, as was the only study showing actual RNA demethylation *in vivo* (Ougland et al. 2004). NAIL-MS in *E. coli* confirmed the activity of the m¹A demethylase AlkB and furthermore its demethylation capacity and preference for 3-methylcytidine

(m³C) and 2-methylthiocytidine (ms²C) was revealed (Reichle, Weber, et al. 2018; Reichle et al. 2019). With NAIL-MS demethylation kinetics inside living cells observable a temporal view on the epitranscriptome becomes available.

Our way of life is currently dominated by the outbreak of the SARS-CoV-2 coronavirus. This virus is an RNA virus and according to one NNGS report, it is highly modified (Kim et al. 2020). So far Oxford Nanopore sequencing is the only resource to study the epitranscriptome of the SARS-CoV-2 virus as MS analysis depends on purified viral RNA free of contaminating host RNA. The development of more appropriate data analysis software will further increase the use of modern sequencing techniques for modification assignments. The importance of understanding the viral epitranscriptome was recently shown for HIV where RiboMethSeq revealed the presence 2'-O-ribose methylations and its role in evasion of innate immune sensing (Ringgaard et al. 2019).

To end the pandemic, a vaccine is urgently needed. mRNA-based vaccines against the SARS-CoV-2 spike protein are promising and are being intensively tested (Jackson et al. 2020; Mulligan et al. 2020). Vaccine RNA might also be modified, by, for example, 1-methylpseudouridine, which increases mRNA translation and dampens innate immune sensing (Karikó et al. 2008). If mRNA vaccines successfully clear all clinical trials, their production will start as billions of vaccine doses are required. Here, fast and reliable quality control of the produced mRNA and its epitranscriptome is needed. Thus, instrumental analysis of the epitranscriptome, especially top-down and oligonucleotide MS, is now more important than ever.

Disclosure statement

No potential conflict of interest was reported by the author(s).

Funding

This work was financially supported by the FRCREpiARN project from Grand Est Region (France) to Y.M and performed in the framework of EPITRAN COST initiative [CA16120]; P.B. and C.T. under Grant NMR-VitAmin [ANR-14-CE09-0012], CiMoDyMo [ANR-19-CE44-0013], and Labex DYNAMO [ANR-11-LABX-0011]; the US National Science Foundation under Grant [CHE150735] awarded to P.A.L. and J.M.; and Deutsche Forschungsgemeinschaft under Grant SFB1309 [Projektnummer 325871075], SPP 1784 [KE1943/4-1] and Emmy-Noether funding [KE1943/3-1].

ORCID

Yasemin Yoluç  <http://orcid.org/0000-0001-9535-5020>
 Gregor Ammann  <http://orcid.org/0000-0003-2135-058X>
 Pierre Barraud  <http://orcid.org/0000-0003-4460-8360>
 Manasses Jora  <http://orcid.org/0000-0002-1999-1508>
 Patrick A. Limbach  <http://orcid.org/0000-0003-1526-4546>
 Yuri Motorin  <http://orcid.org/0000-0002-8018-334X>
 Virginie Marchand  <http://orcid.org/0000-0002-8537-1139>
 Carine Tisné  <http://orcid.org/0000-0001-5534-4650>
 Stefanie Kellner  <http://orcid.org/0000-0003-3224-7502>

References

- Agris PF. 2008. Bringing order to translation: the contributions of transfer RNA anticodon-domain modifications. *EMBO Rep.* 9(7):629–635.
- Agris PF, Sierzputowska-Gracz H, Smith C. 1986. Transfer RNA contains sites of localized positive charge: carbon NMR studies of [¹³C]methyl-enriched *Escherichia coli* and yeast tRNAPhe. *Biochemistry.* 25(18):5126–5131.
- Apffel A, Chakel JA, Fischer S, Lichtenwalter K, Hancock WS. 1997. Analysis of oligonucleotides by HPLC-electrospray ionization mass spectrometry. *Anal Chem.* 69(7):1320–1325.
- Aschenbrenner J, Marx A. 2016. Direct and site-specific quantification of RNA 2'-O-methylation by PCR with an engineered DNA polymerase. *Nucleic Acids Res.* 44(8):3495–3502.
- Aschenbrenner J, Werner S, Marchand V, Adam M, Motorin Y, Helm M, Marx A. 2018. Engineering of a DNA polymerase for direct m6A sequencing. *Angew Chem Int Ed Engl.* 57(2):417–421.
- Bakin A, Ofengand J. 1993. Four newly located pseudouridylate residues in *Escherichia coli* 23S ribosomal RNA are all at the peptidyltransferase center: analysis by the application of a new sequencing technique. *Biochemistry.* 32(37):9754–9762.
- Bakin AV, Ofengand J. 1998. Mapping of pseudouridine residues in RNA to nucleotide resolution. *Methods Mol Biol.* 77:297–309.
- Baldrige KC, Jora M, Maranhao AC, Quick MM, Addepalli B, Brodbelt JS, Ellington AD, Limbach PA, Contreras LM. 2018. Directed evolution of heterologous tRNAs leads to reduced dependence on post-transcriptional modifications. *ACS Synth Biol.* 7(5):1315–1327.
- Barraud P, Gato A, Heiss M, Catala M, Kellner S, Tisne C. 2019. Time-resolved NMR monitoring of tRNA maturation. *Nat Commun.* 10(1):3373.
- Barraud P, Tisné C. 2019. To be or not to be modified: miscellaneous aspects influencing nucleotide modifications in tRNAs. *IUBMB Life.* 71(8):1126–1140.
- Behm-Ansmant I, Helm M, Motorin Y. 2011. Use of specific chemical reagents for detection of modified nucleotides in RNA. *J Nucleic Acids.* 2011:408053.
- Biba M, Foley JP, Welch CJ. 2017. Liquid chromatographic separation of oligonucleotides. In: Fanali S, Haddad PR, Poole CF, editors. *Liquid chromatography*, 2nd ed. Amsterdam (The Netherlands): Elsevier; p. 159–182.
- Birkedal U, Christensen-Dalsgaard M, Krogh N, Sabarinathan R, Gorodkin J, Nielsen H. 2015. Profiling of ribose methylations in RNA by high-throughput sequencing. *Angew Chem Int Ed Engl.* 54(2):451–455.
- Bjorkbom A, Lelyveld VS, Zhang S, Zhang W, Tam CP, Blain JC, Szostak JW. 2015. Bidirectional direct sequencing of noncanonical RNA by two-dimensional analysis of mass chromatograms. *J Am Chem Soc.* 137(45):14430–14438.
- Borland K, Diesend J, Ito-Kureha T, Heissmeyer V, Hammann C, Buck AH, Michalakos S, Kellner S. 2019. Production and application of stable isotope-labeled internal standards for RNA modification analysis. *Genes.* 10(1):26.
- Brandmayr C, Wagner M, Brückl T, Globisch D, Pearson D, Kneuttinger AC, Reiter V, Hienzsch A, Koch S, Thoma I, et al. 2012. Isotope-based analysis of modified tRNA nucleosides correlates modification density with translational efficiency. *Angew Chem Int Ed Engl.* 51(44):11162–11165.
- Bryzgunova OE, Laktionov PP. 2015. Extracellular nucleic acids in urine: sources, structure, diagnostic potential. *Acta Naturae.* 7(3):48–54.
- Cai WM, Chionh YH, Hia F, Gu C, Kellner S, McBee ME, Ng CS, Pang YLJ, Prestwich EG, Lim KS, et al. 2015. A platform for discovery and quantification of modified ribonucleosides in RNA: application to stress-induced reprogramming of tRNA modifications. *Methods Enzymol.* 560:29–71.
- Calderisi G, Glasner H, Breuker K. 2020. Radical transfer dissociation for de novo characterization of modified ribonucleic acids by mass spectrometry. *Angew Chem Int Ed Engl.* 59(11):4309–4313.
- Cao X, Limbach PA. 2015. Enhanced detection of post-transcriptional modifications using a mass-exclusion list strategy for RNA modification mapping by LC-MS/MS. *Anal Chem.* 87(16):8433–8440.
- Carlile TM, Rojas-Duran MF, Zinshteyn B, Shin H, Bartoli KM, Gilbert WV. 2014. Pseudouridine profiling reveals regulated mRNA pseudouridylation in yeast and human cells. *Nature.* 515(7525):143–146.
- Catala M, Gato A, Tisné C, Barraud P. 2020a. 1H, 15N chemical shift assignments of the imino groups of yeast tRNAPhe: influence of the post-transcriptional modifications. *Biomol NMR Assign.* 14(2):169–174.
- Catala M, Gato A, Tisné C, Barraud P. 2020b. Preparation of yeast tRNA sample for NMR spectroscopy. *Bio-protocol.* 10(12):e3646.
- Choi BS, Redfield AG. 1986. NMR study of isoleucine transfer RNA from *Thermus thermophilus*. *Biochemistry.* 25(7):1529–1534.
- Cozen AE, Quartley E, Holmes AD, Hrabeta-Robinson E, Phizicky EM, Lowe TM. 2015. ARM-seq: AlkB-facilitated RNA methylation sequencing reveals a complex landscape of modified tRNA fragments. *Nat Methods.* 12(9):879–884.
- Cozzuto L, Liu H, Pryszyk LP, Pulido TH, Delgado-Tejedor A, Ponomarenko J, Novoa EM. 2020. MasterOfPores: a workflow for the analysis of Oxford Nanopore Direct RNA Sequencing Datasets. *Front Genet.* 11:211.
- Crain PF. 1990. Preparation and enzymatic hydrolysis of DNA and RNA for mass spectrometry. *Methods Enzymol.* 193:782–790.
- Crawford JE, Chan SI, Schweizer MP. 1971. NMR studies of organic solvent denatured yeast phenylalanyl transfer RNA at 220 MHz. *Biochem Biophys Res Commun.* 44(1):1–7.
- Dai Q, Moshitch-Moshkovitz S, Han D, Kol N, Amariglio N, Rechavi G, Domissini D, He C. 2017. Nm-seq maps 2'-O-

- methylation sites in human mRNA with base precision. *Nat Methods*. 14(7):695–698.
- Dal Magro C, Keller P, Kotter A, Werner S, Duarte V, Marchand V, Ignarski M, Freiwald A, Müller RU, Dieterich C, et al. 2018. A vastly increased chemical variety of RNA modifications containing a thioacetal structure. *Angew Chem Int Ed Engl*. 57(26):7893–7897.
- Davis DR, Poulter CD. 1991. 1H-15N NMR studies of *Escherichia coli* tRNA(Phe) from hisT mutants: a structural role for pseudouridine. *Biochemistry*. 30(17):4223–4231.
- Demelonne A, Gou MJ, Nys G, Parulski C, Crommen J, Servais AC, Fillet M. 2020. Evaluation of hydrophilic interaction liquid chromatography, capillary zone electrophoresis and drift tube ion-mobility quadrupole time of flight mass spectrometry for the characterization of phosphodiester and phosphorothioate oligonucleotides. *J Chromatogr A*. 1614:460716.
- Ding H, Bailey AD, Jain M, Olsen H, Paten B. 2020. Gaussian mixture model-based unsupervised nucleotide modification number detection using nanopore sequencing readouts. *Bioinformatics*. 36(19):4928–4934.
- Dominissini D, Nachtergaele S, Moshitch-Moshkovitz S, Peer E, Kol N, Ben-Haim MS, Dai Q, Di Segni A, Salmon-Divon M, Clark WC, et al. 2016. The dynamic N(1)-methyladenosine methylome in eukaryotic messenger RNA. *Nature*. 530(7591):441–446.
- Dumelin CE, Chen Y, Leconte AM, Chen YG, Liu DR. 2012. Discovery and biological characterization of geranylated RNA in bacteria. *Nat Chem Biol*. 8(11):913–919.
- Durairaj A, Limbach PA. 2008. Improving CMC-derivatization of pseudouridine in RNA for mass spectrometric detection. *Anal Chim Acta*. 612(2):173–181.
- Durant PC, Bajji AC, Sundaram M, Kumar RK, Davis DR. 2005. Structural effects of hypermodified nucleosides in the *Escherichia coli* and human tRNA^{Lys} anticodon loop: the effect of nucleosides s2U, mcm5U, mcm5s2U, mnm5s2U, t6A, and ms2t6A. *Biochemistry*. 44(22):8078–8089.
- Dyubankova N, Sochacka E, Kraszewska K, Nawrot B, Herdewijn P, Lescrinier E. 2015. Contribution of dihydrouridine in folding of the D-arm in tRNA. *Org Biomol Chem*. 13(17):4960–4966.
- Emmerchts G, Herdewijn P, Rozenski J. 2005. Pseudouridine detection improvement by derivatization with methyl vinyl sulfone and capillary HPLC-mass spectrometry. *J Chromatogr B Analyt Technol Biomed Life Sci*. 825(2):233–238.
- Enroth C, Poulsen LD, Iversen S, Kirpekar F, Albrechtsen A, Vinther J. 2019. Detection of internal N7-methylguanosine (m7G) RNA modifications by mutational profiling sequencing. *Nucleic Acids Res*. 47(20):e126.
- Feederle R, Schepers A. 2017. Antibodies specific for nucleic acid modifications. *RNA Biol*. 14(9):1089–1098.
- Feng Y, Ma C-J, Ding J-H, Qi C-B, Xu X-J, Yuan B-F, Feng Y-Q. 2020. Chemical labeling – assisted mass spectrometry analysis for sensitive detection of cytidine dual modifications in RNA of mammals. *Anal Chim Acta*. 1098:56–65.
- Fernández-Costa C, Martínez-Bartolomé S, McClatchy DB, Saviola AJ, Yu N-K, Yates JR. 2020. Impact of the identification strategy on the reproducibility of the DDA and DIA results. *J Proteome Res*. 19(8):3153–3161.
- Fradin A, Gruhl H, Feldmann H. 1975. Mapping of yeast tRNAs by two-dimensional electrophoresis on polyacrylamide gels. *FEBS Lett*. 50(2):185–189.
- Gao Y, McLuckey SA. 2013. Electron transfer followed by collision-induced dissociation (NET-CID) for generating sequence information from backbone-modified oligonucleotide anions. *Rapid Commun Mass Spectrom*. 27(1):249–257.
- García-Campos MA, Edelheit S, Toth U, Safra M, Shachar R, Viukov S, Winkler R, Nir R, Lasman L, Brandis A, et al. 2019. Deciphering the “m6A Code” via antibody-independent quantitative profiling. *Cell*. 178(3):731–747.
- Gaston KW, Limbach PA. 2014. The identification and characterization of non-coding and coding RNAs and their modified nucleosides by mass spectrometry. *RNA Biol*. 11(12):1568–1585.
- Gato A, Catala M, Tisné C, Barraud P. 2021. A method to monitor the introduction of post-transcriptional modifications in tRNAs with NMR spectroscopy. *Methods Mol Biol*.
- Gaudin C, Nonin-Lecomte S, Tisné C, Corvaisier S, Bordeau V, Dardel F, Felden B. 2003. The tRNA-like domains of *E coli* and *A.aeolicus* transfer-messenger RNA: structural and functional studies. *J Mol Biol*. 331(2):457–471.
- Gehrke CW, Kuo KC, Zumwalt RW. 1980. Chromatography of nucleosides. *J Chromatogr*. 188(1):129–147.
- Gehrke CW, Patel AB. 1976. Gas-liquid chromatography of nucleosides. Derivatization and chromatography. *J Chromatogr*. 123(2):335–345.
- Gehrke CW, Ruyle CD. 1968. Gas-liquid chromatographic analysis of nucleic acid components. *J Chromatogr*. 38(4):473–491.
- Golubev A, Fatkhullin B, Khusainov I, Jenner L, Gabdulkhakov A, Validov S, Yusupova G, Yusupov M, Usachev K. 2020. Cryo-EM structure of the ribosome functional complex of the human pathogen *Staphylococcus aureus* at 3.2 Å resolution. *FEBS Lett*. 594(21):3551–3567.
- Goyon A, Zhang K. 2020. Characterization of antisense oligonucleotide impurities by ion-pairing reversed-phase and anion exchange chromatography coupled to hydrophilic interaction liquid chromatography/mass spectrometry using a versatile two-dimensional liquid chromatography setup. *Anal Chem*. 92(8):5944–5951.
- Grosjean H, Droogmans L, Roovers M, Keith G. 2007. Detection of enzymatic activity of transfer RNA modification enzymes using radiolabeled tRNA substrates. *Methods Enzymol*. 425:55–101.
- Grozhi AV, Olarerin-George AO, Sindelar M, Li X, Gross SS, Jaffrey SR. 2019. Antibody cross-reactivity accounts for widespread appearance of m1A in 5'UTRs. *Nat Commun*. 10(1):5126.
- Guo M, Li X, Zhang L, Liu D, Du W, Yin D, Lyu N, Zhao G, Guo C, Tang D. 2017. Accurate quantification of 5-Methylcytosine, 5-Hydroxymethylcytosine, 5-Formylcytosine, and 5-Carboxylcytosine in genomic DNA from breast cancer by chemical derivatization coupled with ultra performance liquid chromatography- electrospray quadrupole time of flight mass spectrometry analysis. *Oncotarget*. 8(53):91248–91257.
- Guo C, Xie C, Chen Q, Cao X, Guo M, Zheng S, Wang Y. 2018. A novel malic acid-enhanced method for the analysis of 5-methyl-2'-deoxycytidine, 5-hydroxymethyl-2'-deoxycytidine, 5-methylcytidine and 5-

- hydroxymethylcytidine in human urine using hydrophilic interaction liquid chromatography-tandem mass spectrometry. *Anal Chim Acta*. 1034:110–118.
- Gupta RC, Randerath K. 1979. Rapid print-readout technique for sequencing of RNA's containing modified nucleotides. *Nucleic Acids Res*. 6(11):3443–3458.
- Gupta RC, Randerath E, Randerath K. 1976. A double-labeling procedure for sequence analysis of picomole amounts of nonradioactive RNA fragments. *Nucleic Acids Res*. 3(11):2895–2914.
- Hagelskamp F, Borland K, Ramos J, Hendrick AG, Fu D, Kellner S. 2020. Broadly applicable oligonucleotide mass spectrometry for the analysis of RNA writers and erasers in vitro. *Nucleic Acids Res*. 48(7):e41.
- Han L, Phizicky EM. 2018. A rationale for tRNA modification circuits in the anticodon loop. *RNA*. 24(10):1277–1284.
- Hare DR, Ribeiro NS, Wemmer DE, Reid BR. 1985. Complete assignment of the imino protons of *Escherichia coli* valine transfer RNA: two-dimensional NMR studies in water. *Biochemistry*. 24(16):4300–4306.
- Hartstock K, Nilges BS, Ovcharenko A, Cornelissen NV, Püllen N, Lawrence-Dörner AM, Leidel SA, Rentmeister A. 2018. Enzymatic or in vivo installation of propargyl groups in combination with click chemistry for the enrichment and detection of methyltransferase target sites in RNA. *Angew Chem Int Ed Engl*. 57(21):6342–6346.
- Hartstock K, Rentmeister A. 2019. Mapping N6-methyladenosine (m6A) in RNA: established methods, remaining challenges, and emerging approaches. *Chemistry*. 25(14):3455–3464.
- Hauenschild R, Tserovski L, Schmid K, Thüring K, Winz ML, Sharma S, Entian KD, Wacheul L, Lafontaine DL, Anderson J, et al. 2015. The reverse transcription signature of N1-methyladenosine in RNA-Seq is sequence dependent. *Nucleic Acids Res*. 43(20):9950–9964.
- Heerschap A, Mellema JR, Janssen HG, Walters JA, Haasnoot CA, Hilbers CW. 1985. Imino-proton resonances of yeast tRNAPhe studied by two-dimensional nuclear Overhauser enhancement spectroscopy. *Eur J Biochem*. 149(3):649–655.
- Heiss M, Hagelskamp F, Marchand V, Motorin Y, Kellner S. 2021. Cell culture NAIL-MS allows insight into human tRNA and rRNA modification dynamics in vivo. *Nat Commun*. 12(1):389.
- Heiss M, Kellner S. 2017. Detection of nucleic acid modifications by chemical reagents. *RNA Biol*. 14(9):1166–1174.
- Heiss M, Reichle VF, Kellner S. 2017. Observing the fate of tRNA and its modifications by nucleic acid isotope labeling mass spectrometry: NAIL-MS. *RNA Biol*. 14(9):1260–1268.
- Helm M, Motorin Y. 2017. Detecting RNA modifications in the epitranscriptome: predict and validate. *Nat Rev Genet*. 18(5):275–291.
- Hilbers CW, Heerschap A, Haasnoot CA, Walters JA. 1983. The solution structure of yeast tRNAPhe as studied by nuclear Overhauser effects in NMR. *J Biomol Struct Dyn*. 1(1):183–207.
- Hoernes TP, Clementi N, Faserl K, Glasner H, Breuker K, Lindner H, Hüttenhofer A, Erlacher MD. 2016. Nucleotide modifications within bacterial messenger RNAs regulate their translation and are able to rewire the genetic code. *Nucleic Acids Res*. 44(2):852–862.
- Hong T, Yuan Y, Chen Z, Xi K, Wang T, Xie Y, He Z, Su H, Zhou Y, Tan ZJ, et al. 2018. Precise antibody-independent m6A identification via 4SedTTP-involved and FTO-assisted strategy at single-nucleotide resolution. *J Am Chem Soc*. 140(18):5886–5889.
- Hossain M, Limbach PA. 2007. Mass spectrometry-based detection of transfer RNAs by their signature endonuclease digestion products. *RNA*. 13(2):295–303.
- Huang W, Lan MD, Qi CB, Zheng SJ, Wei SZ, Yuan BF, Feng YQ. 2016. Formation and determination of the oxidation products of 5-methylcytosine in RNA. *Chem Sci*. 7(8):5495–5502.
- Huang TY, Liu J, Liang X, Hodges BD, McLuckey SA. 2008. Collision-induced dissociation of intact duplex and single-stranded siRNA anions. *Anal Chem*. 80(22):8501–8508.
- Huang TY, Liu J, McLuckey SA. 2010. Top-down tandem mass spectrometry of tRNA via ion trap collision-induced dissociation. *J Am Soc Mass Spectrom*. 21(6):890–898.
- Huang T-y, McLuckey SA. 2011. Gas-phase ion/ion reactions of rubrene cations and multiply charged DNA and RNA anions. *Int J Mass Spectrom*. 304(2–3):140–147.
- Incarnato D, Anselmi F, Morandi E, Neri F, Maldotti M, Rapelli S, Parlato C, Basile G, Oliviero S. 2017. High-throughput single-base resolution mapping of RNA 2'-O-methylated residues. *Nucleic Acids Res*. 45(3):1433–1441.
- Jackson LA, Anderson EJ, Roupheal NG, Roberts PC, Makhene M, Coler RN, McCullough MP, Chappell JD, Denison MR, Stevens LJ, et al. 2020. An mRNA vaccine against SARS-CoV-2 - preliminary report. *N Engl J Med*. 383(20):1920–1931.
- Jenjaroenpun P, Wongsurawat T, Wadley TD, Wassenaar TM, Liu J, Dai Q, Wanchai V, Akel NS, Jamshidi-Parsian A, Franco AT, et al. 2021. Decoding the epitranscriptional landscape from native RNA sequences. *Nucleic Acids Res*. 49(2):e7.
- Jia G, Fu Y, Zhao X, Dai Q, Zheng G, Yang Y, Yi C, Lindahl T, Pan T, Yang Y-G, et al. 2011. N6-Methyladenosine in nuclear RNA is a major substrate of the obesity-associated FTO. *Nat Chem Biol*. 7(12):885–887.
- Jiang T, Yu N, Kim J, Murgu JR, Kissai M, Ravichandran K, Miracco EJ, Presnyak V, Hua S. 2019. Oligonucleotide sequence mapping of large therapeutic mRNAs via parallel ribonuclease digestions and LC-MS/MS. *Anal Chem*. 91(13):8500–8506.
- Jora M, Borland K, Abernathy S, Zhao R, Kelley M, Kellner S, Addepalli B, Limbach PA. 2020. Chemical amination/iminination of carbonothiolated nucleosides during RNA hydrolysis. *Angew Chem Int Ed*. 60(8):3961–3966.
- Jora M, Lobue PA, Ross RL, Williams B, Addepalli B. 2019. Detection of ribonucleoside modifications by liquid chromatography coupled with mass spectrometry. *Biochim Biophys Acta Gene Regul Mech*. 1862(3):280–290.
- Kan LS, Ts'o POP, Haar Fvd, Sprinzl M, Cramer F. 1974. NMR study on the methyl and methylene proton resonances of tRNA Phe yeast. *Biochem Biophys Res Commun*. 59(1):22–29.
- Karikó K, Muramatsu H, Welsh FA, Ludwig J, Kato H, Akira S, Weissman D. 2008. Incorporation of pseudouridine into mRNA yields superior nonimmunogenic vector with increased translational capacity and biological stability. *Mol Ther*. 16(11):1833–1840.

- Kastrup RV, Schmidt PG. 1978. ¹H NMR of valine tRNA modified bases. Evidence for multiple conformations. *Nucleic Acids Res.* 5(1):257–269.
- Keith G. 1995. Mobilities of modified ribonucleotides on two-dimensional cellulose thin-layer chromatography. *Biochimie.* 77(1-2):142–144.
- Kellersberger KA, Yu E, Kruppa GH, Young MM, Fabris D. 2004. Top-down characterization of nucleic acids modified by structural probes using high-resolution tandem mass spectrometry and automated data interpretation. *Anal Chem.* 76(9):2438–2445.
- Kellner S, Burhenne J, Helm M. 2010. Detection of RNA modifications. *RNA Biol.* 7(2):237–247.
- Kellner S, Neumann J, Rosenkranz D, Lebedeva S, Ketting RF, Zischler H, Schneider D, Helm M. 2014. Profiling of RNA modifications by multiplexed stable isotope labelling. *Chem Commun.* 50(26):3516–3518.
- Kellner S, Ochel A, Thüning K, Spenkuch F, Neumann J, Sharma S, Entian K-D, Schneider D, Helm M. 2014. Absolute and relative quantification of RNA modifications via biosynthetic isotopomers. *Nucleic Acids Research.* 42(18):e142.
- Khoddami V, Yerra A, Mosbrugger TL, Fleming AM, Burrows CJ, Cairns BR. 2019. Transcriptome-wide profiling of multiple RNA modifications simultaneously at single-base resolution. *Proc Natl Acad Sci USA.* 116(14):6784–6789.
- Kim D, Lee JY, Yang JS, Kim JW, Kim VN, Chang H. 2020. The architecture of SARS-CoV-2 transcriptome. *Cell.* 181(4):914–921.
- Kimura S, Dedon PC, Waldor MK. 2020. Comparative tRNA sequencing and RNA mass spectrometry for surveying tRNA modifications. *Nat Chem Biol.* 16(9):964–972.
- Knutson SD, Ayele TM, Heemstra JM. 2018. Chemical labeling and affinity capture of inosine-containing RNAs using acrylamidofluorescein. *Bioconjug Chem.* 29(9):2899–2903.
- Koehler KM, Schmidt PG. 1973. NMR study of the modified base resonances of tRNA tyr-coli. *Biochem Biophys Res Commun.* 50(2):370–376.
- Kowalak JA, Pomerantz SC, Crain PF, McCloskey JA. 1993. A novel method for the determination of post-transcriptional modification in RNA by mass spectrometry. *Nucleic Acids Res.* 21(19):4577–4585.
- Kristen M, Plehn J, Marchand V, Friedland K, Motorin Y, Helm M, Werner S. 2020. Manganese ions individually alter the reverse transcription signature of modified ribonucleosides. *Genes.* 11(8):950.
- Krogh N, Nielsen H. 2019. Sequencing-based methods for detection and quantitation of ribose methylations in RNA. *Methods.* 156:5–15.
- Kumar RK, Davis DR. 1997. Synthesis and studies on the effect of 2-thiouridine and 4-thiouridine on sugar conformation and RNA duplex stability. *Nucleic Acids Res.* 25(6):1272–1280.
- Kumazawa Y, Yokogawa T, Tsurui H, Miura K, Watanabe K. 1992. Effect of the higher-order structure of tRNAs on the stability of hybrids with oligodeoxyribonucleotides: separation of tRNA by an efficient solution hybridization. *Nucleic Acids Res.* 20(9):2223–2232.
- Lakings DB, Gehrke CW. 1971. Analysis of base composition of RNA and DNA hydrolysates by gas-liquid chromatography. *J Chromatogr.* 62(3):347–367.
- Li L, Dai H, Nguyen AP, Gu W. 2020. A convenient strategy to clone modified/unmodified small RNA and mRNA for high throughput sequencing. *RNA Eng.* 26(2):218–227.
- Li N, El Zahar NM, Saad JG, van der Hage ERE, Bartlett MG. 2018. Alkylamine ion-pairing reagents and the chromatographic separation of oligonucleotides. *J Chromatogr A.* 1580:110–119.
- Li F, Su X, Bäurer S, Lämmerhofer M. 2020. Multiple heart-cutting mixed-mode chromatography-reversed-phase 2D-liquid chromatography method for separation and mass spectrometric characterization of synthetic oligonucleotides. *J Chromatogr A.* 1625:461338.
- Li X, Zhu P, Ma S, Song J, Bai J, Sun F, Yi C. 2015. Chemical pulldown reveals dynamic pseudouridylation of the mammalian transcriptome. *Nat Chem Biol.* 11(8):592–597. eng.
- Limbach PA, Crain PF, McCloskey JA. 1995. Characterization of oligonucleotides and nucleic acids by mass spectrometry. *Curr Opin Biotechnol.* 6(1):96–102.
- Lin S, Liu Q, Jiang YZ, Gregory RI. 2019. Nucleotide resolution profiling of m(7)G tRNA modification by TRAC-Seq. *Nat Protoc.* 14(11):3220–3242.
- Lin S, Liu Q, Lelyveld VS, Choe J, Szostak JW, Gregory RI. 2018. Mettl1/Wdr4-mediated m7G tRNA methylation is required for normal mRNA translation and embryonic stem cell self-renewal and differentiation. *Mol Cell.* 71(2):244–255.
- Lin H, Miyauchi K, Harada T, Okita R, Takeshita E, Komaki H, Fujioka K, Yagasaki H, Goto Y-I, Yanaka K, et al. 2018. CO₂-sensitive tRNA modification associated with human mitochondrial disease. *Nat Commun.* 9(1):1875.
- Linder B, Jaffrey SR. 2019. Discovering and mapping the modified nucleotides that comprise the epitranscriptome of mRNA. *Cold Spring Harb Perspect Biol.* 11(6):a032201.
- Liu H, Begik O, Lucas MC, Ramirez JM, Mason CE, Wiener D, Schwartz S, Mattick JS, Smith MA, Novoa EM. 2019. Accurate detection of m6A RNA modifications in native RNA sequences. *Nat Commun.* 10(1):4079.
- Liu J, Yue Y, Han D, Wang X, Fu Y, Zhang L, Jia G, Yu M, Lu Z, Deng X, et al. 2014. A METTL3-METTL14 complex mediates mammalian nuclear RNA N6-adenosine methylation. *Nat Chem Biol.* 10(2):93–95.
- Lobue PA, Jora M, Addepalli B, Limbach PA. 2019. Oligonucleotide analysis by hydrophilic interaction liquid chromatography-mass spectrometry in the absence of ion-pair reagents. *J Chromatogr A.* 1595:39–48.
- Lobue PA, Yu N, Jora M, Abernathy S, Limbach PA. 2019. Improved application of RNAModMapper - An RNA modification mapping software tool - For analysis of liquid chromatography tandem mass spectrometry (LC-MS/MS) data. *Methods.* 156:128–138.
- Lorenz DA, Sathe S, Einstein JM, Yeo GW. 2020. Direct RNA sequencing enables m6A detection in endogenous transcript isoforms at base-specific resolution. *RNA.* 26(1):19–28.
- Lovejoy AF, Riordan DP, Brown PO. 2014. Transcriptome-wide mapping of pseudouridines: pseudouridine synthases modify specific mRNAs in *S. cerevisiae*. *PLoS One.* 9(10):e110799.
- Maden BE. 2001. Mapping 2'-O-methyl groups in ribosomal RNA. *Methods.* 25(3):374–382.
- Marchand V, Ayadi L, Ernst FGM, Hertler J, Bourguignon-Igel V, Galvanin A, Kotter A, Helm M, Lafontaine DLJ, Motorin

- Y. 2018. AlkAniline-Seq: profiling of m7G and m3 C RNA modifications at single nucleotide resolution. *Angew Chem Int Ed Engl.* 57(51):16785–16790.
- Marchand V, Blanloeil-Oillo F, Helm M, Motorin Y. 2016. Illumina-based RiboMethSeq approach for mapping of 2'-O-Me residues in RNA. *Nucleic Acids Res.* 44(16):e135.
- Marchand V, Pichot F, Neybecker P, Ayadi L, Bourguignon-Igel V, Wacheul L, Lafontaine DLJ, Pinzano A, Helm M, Motorin Y. 2020. HydraPsiSeq: a method for systematic and quantitative mapping of pseudouridines in RNA. *Nucleic Acids Res.* 48(19):e110.
- Matsuzawa S, Wakata Y, Ebi F, Isobe M, Kurosawa N. 2019. Development and validation of monoclonal antibodies against N6-methyladenosine for the detection of RNA modifications. *PLoS One.* 14(10):e0223197.
- Matthiesen R, Kirpekar F. 2009. Identification of RNA molecules by specific enzyme digestion and mass spectrometry: software for and implementation of RNA mass mapping. *Nucleic Acids Res.* 37(6):e48.
- Matuszewski M, Wojciechowski J, Miyauchi K, Gdaniec Z, Wolf WM, Suzuki T, Sochacka E. 2017. A hydantoin isoform of cyclic N6-threonylcarbamoyladenine (ct6A) is present in tRNAs. *Nucleic Acids Res.* 45(4):2137–2149.
- McGinnis AC, Grubb EC, Bartlett MG. 2013. Systematic optimization of ion-pairing agents and hexafluoroisopropanol for enhanced electrospray ionization mass spectrometry of oligonucleotides. *Rapid Commun Mass Spectrom.* 27(23):2655–2664.
- McIntyre ABR, Gokhale NS, Cerchiatti L, Jaffrey SR, Horner SM, Mason CE. 2020. Limits in the detection of m6A changes using MeRIP/m6A-seq. *Sci Rep.* 10(1):6590.
- McLuckey SA, Van Berkel GJ, Glish GL. 1992. Tandem mass spectrometry of small, multiply charged oligonucleotides. *J Am Soc Mass Spectrom.* 3(1):60–70.
- Mengel-Jørgensen J, Kirpekar F. 2002. Detection of pseudouridine and other modifications in tRNA by cyanoethylation and MALDI mass spectrometry. *Nucleic Acids Res.* 30(23):e135.
- Mishima E, Jinno D, Akiyama Y, Itoh K, Nankumo S, Shima H, Kikuchi K, Takeuchi Y, Elkordy A, Suzuki T, et al. 2015. Immuno-northern blotting: detection of RNA modifications by using antibodies against modified nucleosides. *PLoS One.* 10(11):e0143756.
- Mo J, Håkansson K. 2006. Characterization of nucleic acid higher order structure by high-resolution tandem mass spectrometry. *Anal Bioanal Chem.* 386(3):675–681.
- Motorin Y, Helm M. 2019. Methods for RNA modification mapping using deep sequencing: established and new emerging technologies. *Genes.* 10(1):35.
- Motorin Y, Muller S, Behm-Ansmant I, Branlant C. 2007. Identification of modified residues in RNAs by reverse transcription-based methods. *Methods Enzymol.* 425:21–53.
- Mulligan MJ, Lyke KE, Kitchin N, Absalon J, Gurtman A, Lockhart S, Neuzil K, Raabe V, Bailey R, Swanson KA, et al. 2020. Phase I/II study of COVID-19 RNA vaccine BNT162b1 in adults. *Nature.* 586(7830):589–593.
- Nakayama H, Akiyama M, Taoka M, Yamauchi Y, Nobe Y, Ishikawa H, Takahashi N, Isobe T. 2009. Ariadne: a database search engine for identification and chemical analysis of RNA using tandem mass spectrometry data. *Nucleic Acids Res.* 37(6):e47.
- Nakayama H, Yamauchi Y, Nobe Y, Sato K, Takahashi N, Shalev-Benami M, Isobe T, Taoka M. 2019. Method for direct mass-spectrometry-based identification of monomethylated RNA nucleoside positional isomers and its application to the analysis of Leishmania rRNA. *Anal Chem.* 91(24):15634–15643.
- Natchiar SK, Myasnikov AG, Kratzat H, Hazemann I, Klaholz BP. 2017. Visualization of chemical modifications in the human 80S ribosome structure. *Nature.* 551(7681):472–477.
- Nyakas A, Blum LC, Stucki SR, Reymond J-L, Schürch S. 2013. OMA and OPA-software-supported mass spectra analysis of native and modified nucleic acids. *J Am Soc Mass Spectrom.* 24(2):249–256.
- Okada S, Ueda H, Noda Y, Suzuki T. 2019. Transcriptome-wide identification of A-to-I RNA editing sites using ICE-seq. *Methods.* 156:66–78.
- Ougland R, Zhang CM, Liiv A, Johansen RF, Seeberg E, Hou YM, Remme J, Falnes P. 2004. AlkB restores the biological function of mRNA and tRNA inactivated by chemical methylation. *Mol Cell.* 16(1):107–116.
- Pandolfini L, Barbieri I, Bannister AJ, Hendrick A, Andrews B, Webster N, Murat P, Mach P, Brandi R, Robson SC, et al. 2019. METTL1 Promotes let-7 MicroRNA Processing via m7G Methylation. *Mol Cell.* 74(6):1278–1290.
- Patterson KG, Rodicio LP, Limbach PA. 2001. Identification of the mass-silent post-transcriptionally modified nucleoside pseudouridine in RNA by matrix-assisted laser desorption/ionization mass spectrometry. *Nucleic Acids Res.* 29(10):E49.
- Paulines MJ, Wetzel C, Limbach PA. 2019. Using spectral matching to interpret LC-MS/MS data during RNA modification mapping. *J Mass Spectrom.* 54(11):906–914.
- Peters-Clarke TM, Quan Q, Brademan DR, Hebert AS, Westphall MS, Coon JJ. 2020. Ribonucleic acid sequence characterization by negative electron transfer dissociation mass spectrometry. *Anal Chem.* 92(6):4436–4444.
- Pomerantz SC, McCloskey JA. 1990. [44] Analysis of RNA hydrolyzates by liquid chromatography-mass spectrometry. *Methods Enzymol.* 193:796–824.
- Pomerantz SC, McCloskey JA. 2005. Detection of the common RNA nucleoside pseudouridine in mixtures of oligonucleotides by mass spectrometry. *Anal Chem.* 77(15):4687–4697.
- Ranaei-Siadat E, Fabret C, Seijo B, Dardel F, Grosjean H, Nonin-Lecomte S. 2013. RNA-methyltransferase TrmA is a dual-specific enzyme responsible for C5-methylation of uridine in both tmRNA and tRNA. *RNA Biol.* 10(4):572–578.
- Reichle VF, Kaiser S, Heiss M, Hagelskamp F, Borland K, Kellner S. 2018. Surpassing limits of static RNA modification analysis with dynamic NAIL-MS. *Methods.* 156:91–101.
- Reichle VF, Petrov DP, Weber V, Jung K, Kellner S. 2019. NAIL-MS reveals the repair of 2-methylthiocytidine by AlkB in *E. coli*. *Nat Commun.* 10(1):5600.
- Reichle VF, Weber V, Kellner S. 2018. NAIL-MS in *E. coli* determines the source and fate of methylation in tRNA. *ChemBioChem.* 19(24):2575–2583.
- Ringear M, Marchand V, Decroly E, Motorin Y, Bennasser Y. 2019. FTSJ3 is an RNA 2'-O-methyltransferase recruited by HIV to avoid innate immune sensing. *Nature.* 565(7740):500–504.

- Rost HL, Sachsenberg T, Aiche S, Bielow C, Weisser H, Aicheler F, Andreotti S, Ehrlich HC, Gutenbrunner P, Kenar E, et al. 2016. OpenMS: a flexible open-source software platform for mass spectrometry data analysis. *Nat Methods*. 13(9):741–748.
- Roy S, Redfield AG. 1983. Assignment of imino proton spectra of yeast phenylalanine transfer ribonucleic acid. *Biochemistry*. 22(6):1386–1390.
- Rozenski J, McCloskey JA. 2002. SOS: a simple interactive program for ab initio oligonucleotide sequencing by mass spectrometry. *J Am Soc Mass Spectrom*. 13(3):200–203.
- Ryvkin P, Leung YY, Silverman IM, Childress M, Valladares O, Dragomir I, Gregory BD, Wang LS. 2013. HAMR: high-throughput annotation of modified ribonucleotides. *RNA*. 19(12):1684–1692.
- Safra M, Sas-Chen A, Nir R, Winkler R, Nachshon A, Bar-Yaacov D, Erlacher M, Rossmanith W, Stern-Ginossar N, Schwartz S. 2017. The m1A landscape on cytosolic and mitochondrial mRNA at single-base resolution. *Nature*. 551(7679):251–255.
- Sakamoto K, Kawai G, Niimi T, Satoh T, Sekine M, Yamaizumi Z, Nishimura S, Miyazawa T, Yokoyama S. 1993. A modified uridine in the first position of the anticodon of a minor species of arginine tRNA, the argU gene product, from *Escherichia coli*. *Eur J Biochem*. 216(2):369–375.
- Sakurai M, Yano T, Kawabata H, Ueda H, Suzuki T. 2010. Inosine cyanoethylation identifies A-to-I RNA editing sites in the human transcriptome. *Nat Chem Biol*. 6(10):733–740.
- Sample PJ, Gaston KW, Alfonzo JD, Limbach PA. 2015. RoboOligo: software for mass spectrometry data to support manual and de novo sequencing of post-transcriptionally modified ribonucleic acids. *Nucleic Acids Res*. 43(10):e64.
- Sarin LP, Kienast SD, Leufken J, Ross RL, Dziergowska A, Debiec K, Sochacka E, Limbach PA, Fufezan C, Drexler HCA, et al. 2018. Nano LC-MS using capillary columns enables accurate quantification of modified ribonucleosides at low femtomol levels. *RNA*. 24(10):1403–1417.
- Sas-Chen A, Thomas JM, Matzov D, Taoka M, Nance KD, Nir R, Bryson KM, Shachar R, Liman GLS, Burkhart BW, et al. 2020. Dynamic RNA acetylation revealed by quantitative cross-evolutionary mapping. *Nature*. 583(7817):638–643.
- Schaefer M, Kapoor U, Jantsch MF. 2017. Understanding RNA modifications: the promises and technological bottlenecks of the ‘epitranscriptome’. *Open Biol*. 7(5):170077.
- Schneeberger EM, Breuker K. 2017. Native top-down mass spectrometry of TAR RNA in complexes with a wild-type tat peptide for binding site mapping. *Angew Chem Int Ed Engl*. 56(5):1254–1258.
- Schurch S. 2016. Characterization of nucleic acids by tandem mass spectrometry - The second decade (2004–2013): from DNA to RNA and modified sequences. *Mass Spectrom Rev*. 35(4):483–523.
- Schwartz S, Bernstein DA, Mumbach MR, Jovanovic M, Herbst RH, León-Ricardo BX, Engreitz JM, Guttman M, Satija R, Lander ES, et al. 2014. Transcriptome-wide mapping reveals widespread dynamic-regulated pseudouridylation of ncRNA and mRNA. *Cell*. 159(1):148–162.
- Shi H, Liu B, Nussbaumer F, Rangadurai A, Kreutz C, Al-Hashimi HM. 2019. NMR chemical exchange measurements reveal that N6-methyladenosine slows RNA annealing. *J Am Chem Soc*. 141(51):19988–19993.
- Shu X, Cao J, Cheng M, Xiang S, Gao M, Li T, Ying X, Wang F, Yue Y, Lu Z, et al. 2020. A metabolic labeling method detects m6A transcriptome-wide at single base resolution. *Nat Chem Biol*. 16(8):887–895.
- Slama K, Galliot A, Weichmann F, Hertler J, Feederle R, Meister G, Helm M. 2019. Determination of enrichment factors for modified RNA in MeRIP experiments. *Methods*. 156:102–109.
- Smith SI, Brodbelt JS. 2010. Characterization of oligodeoxynucleotides and modifications by 193 nm photodissociation and electron photodetachment dissociation. *Anal Chem*. 82(17):7218–7226.
- Smith SI, Brodbelt JS. 2011. Hybrid activation methods for elucidating nucleic acid modifications. *Anal Chem*. 83(1):303–310.
- Smith AM, Jain M, Mulroney L, Galalde DR, Akeson M. 2019. Reading canonical and modified nucleobases in 16S ribosomal RNA using nanopore native RNA sequencing. *PLoS One*. 14(5):e0216709.
- Solivio B, Yu N, Addepalli B, Limbach PA. 2018. Improving RNA modification mapping sequence coverage by LC-MS through a nonspecific RNase U2-E49A mutant. *Anal Chim Acta*. 1036:73–79.
- Stanley J, Vassilenko S. 1978. A different approach to RNA sequencing. *Nature*. 274(5666):87–89.
- Stojković V, Myasnikov AG, Young ID, Frost A, Fraser JS, Fujimori DG. 2020. Assessment of the nucleotide modifications in the high-resolution cryo-electron microscopy structure of the *Escherichia coli* 50S subunit. *Nucleic Acids Res*. 48(5):2723–2732.
- Streititz E, Rangadurai A, Plangger R, Kremser J, Juen MA, Tollinger M, Al-Hashimi HM, Kreutz C. 2018. 5-oxyacetic acid modification destabilizes double helical stem structures and favors anionic watson-crick like cmo5 U-G base pairs. *Chemistry*. 24(71):18903–18906.
- Stuart JW, Koshlap KM, Guenther R, Agris PF. 2003. Naturally-occurring modification restricts the anticodon domain conformational space of tRNA(Phe). *J Mol Biol*. 334(5):901–918.
- Sutton JM, Bartlett MG. 2020. Modeling cationic adduction of oligonucleotides using electrospray desorption ionization. *Rapid Commun Mass Spectrom*. 34(8):e8696.
- Suzuki T, Suzuki T. 2007. Chaplet column chromatography: isolation of a large set of individual RNAs in a single step. *Methods Enzymol*. 425:231–239.
- Suzuki T, Ueda H, Okada S, Sakurai M. 2015. Transcriptome-wide identification of adenosine-to-inosine editing using the ICE-seq method. *Nat Protoc*. 10(5):715–732.
- Tang Y, Zheng SJ, Qi CB, Feng YQ, Yuan BF. 2015. Sensitive and simultaneous determination of 5-methylcytosine and its oxidation products in genomic DNA by chemical derivatization coupled with liquid chromatography-tandem mass spectrometry analysis. *Anal Chem*. 87(6):3445–3452.
- Taoka M, Ikumi M, Nakayama H, Masaki S, Matsuda R, Nobe Y, Yamauchi Y, Takeda J, Takahashi N, Isobe T. 2010. In-gel digestion for mass spectrometric characterization of RNA from fluorescently stained polyacrylamide gels. *Anal Chem*. 82(18):7795–7803.
- Taoka M, Nobe Y, Yamaki Y, Sato K, Ishikawa H, Izumikawa K, Yamauchi Y, Hirota K, Nakayama H, Takahashi N, et al.

2018. Landscape of the complete RNA chemical modifications in the human 80S ribosome. *Nucleic Acids Res.* 46(18):9289–9298.
- Taoka M, Nobe Y, Yamaki Y, Yamauchi Y, Ishikawa H, Takahashi N, Nakayama H, Isobe T. 2016. The complete chemical structure of *Saccharomyces cerevisiae* rRNA: partial pseudouridylation of U2345 in 25S rRNA by snoRNA snR9. *Nucleic Acids Res.* 44(18):8951–8961.
- Taucher M, Breuker K. 2010. Top-down mass spectrometry for sequencing of larger (up to 61 nt) RNA by CAD and EDD. *J Am Soc Mass Spectrom.* 21(6):918–929.
- Taucher M, Breuker K. 2012. Characterization of modified RNA by top-down mass spectrometry. *Angew Chem Int Ed Engl.* 51(45):11289–11292.
- Thakur P, Estevez M, Lobue PA, Limbach PA, Addepalli B. 2020. Improved RNA modification mapping of cellular non-coding RNAs using C- and U-specific RNases. *Analyst.* 145(3):816–827.
- Thiaville JJ, Kellner SM, Yuan Y, Hutinet G, Thiaville PC, Jumpathong W, Mohapatra S, Brochier-Armanet C, Letarov AV, Hillebrand R, et al. 2016. Novel genomic island modifies DNA with 7-deazaguanine derivatives. *Proc Natl Acad Sci USA.* 113(11):E1452–E1459.
- Thomas JM, Briney CA, Nance KD, Lopez JE, Thorpe AL, Fox SD, Bortolin-Cavaille ML, Sas-Chen A, Arango D, Oberdoerffer S, et al. 2018. A chemical signature for cytidine acetylation in RNA. *J Am Chem Soc.* 140(40):12667–12670.
- Tisné C, Rigourd M, Marquet R, Ehresmann C, Dardel F. 2000. NMR and biochemical characterization of recombinant human tRNA(Lys)₃ expressed in *Escherichia coli*: identification of posttranscriptional nucleotide modifications required for efficient initiation of HIV-1 reverse transcription. *RNA.* 6(10):1403–1412.
- Tserovski L, Marchand V, Hauenschild R, Blanloeil-Oillo F, Helm M, Motorin Y. 2016. High-throughput sequencing for 1-methyladenosine (m(1)A) mapping in RNA. *Methods.* 107:110–121.
- Tuorto F, Liebers R, Musch T, Schaefer M, Hofmann S, Kellner S, Frye M, Helm M, Stoecklin G, Lyko F. 2012. RNA cytosine methylation by Dnmt2 and NSun2 promotes tRNA stability and protein synthesis. *Nat Struct Mol Biol.* 19(9):900–905.
- Vendeix FAP, Murphy t, Frank V, Cantara WA, Leszczyńska G, Gustilo EM, Sproat B, Malkiewicz A, Agris PF. 2012. Human tRNA(Lys₃)(UUU) is pre-structured by natural modifications for cognate and wobble codon binding through keto-enol tautomerism. *J Mol Biol.* 416(4):467–485.
- Vermeulen A, McCallum SA, Pardi A. 2005. Comparison of the global structure and dynamics of native and unmodified tRNAval. *Biochemistry.* 44(16):6024–6033.
- Vilfan ID, Tsai YC, Clark TA, Wegener J, Dai Q, Yi C, Pan T, Turner SW, Korlach J. 2013. Analysis of RNA base modification and structural rearrangement by single-molecule real-time detection of reverse transcription. *J Nanobiotechnology.* 11:8.
- Wein S, Andrews B, Sachsenberg T, Santos-Rosa H, Kohlbacher O, Kouzarides T, Garcia BA, Weissner H. 2020. A computational platform for high-throughput analysis of RNA sequences and modifications by mass spectrometry. *Nat Commun.* 11(1):926.
- Wurm JP, Meyer B, Bahr U, Held M, Frolow O, Kötter P, Engels JW, Heckel A, Karas M, Entian K-D, et al. 2010. The ribosome assembly factor Nep1 responsible for Bowen-Conradi syndrome is a pseudouridine-N1-specific methyltransferase. *Nucleic Acids Res.* 38(7):2387–2398.
- Xu L, Seki M. 2020. Recent advances in the detection of base modifications using the Nanopore sequencer. *J Hum Genet.* 65(1):25–33.
- Yamauchi Y, Nobe Y, Izumikawa K, Higo D, Yamagishi Y, Takahashi N, Nakayama H, Isobe T, Taoka M. 2016. A mass spectrometry-based method for direct determination of pseudouridine in RNA. *Nucleic Acids Res.* 44(6):e59.
- Yang J, Håkansson K. 2009. Characterization of oligodeoxynucleotide fragmentation pathways in infrared multiphoton dissociation and electron detachment dissociation by Fourier transform ion cyclotron double resonance. *Eur J Mass Spectrom.* 15(2):293–304.
- Yu N, Jora M, Solivio B, Thakur P, Acevedo-Rocha CG, Randau L, de Crecy-Lagard V, Addepalli B, Limbach PA. 2019. tRNA modification profiles and codon-decoding strategies in *Methanocaldococcus jannaschii*. *J Bacteriol.* 201(9):e00690.
- Yu N, Lobue PA, Cao X, Limbach PA. 2017. RNAModMapper: RNA modification mapping software for analysis of liquid chromatography tandem mass spectrometry data. *Anal Chem.* 89(20):10744–10752.
- Zhang F, Ge W, Ruan G, Cai X, Guo T. 2020. Data-independent acquisition mass spectrometry-based proteomics and software tools: a glimpse in 2020. *Proteomics.* 20(17–18):1900276.
- Zhang LS, Liu C, Ma H, Dai Q, Sun HL, Luo G, Zhang Z, Zhang L, Hu L, Dong X, et al. 2019. Transcriptome-wide mapping of internal N7-methylguanosine methylome in mammalian mRNA. *Mol Cell.* 74(6):1304–1316.
- Zhang N, Shi S, Jia TZ, Ziegler A, Yoo B, Yuan X, Li W, Zhang S. 2019. A general LC-MS-based RNA sequencing method for direct analysis of multiple-base modifications in RNA mixtures. *Nucleic Acids Res.* 47(20):e125.
- Zhao LY, Song J, Liu Y, Song CX, Yi C. 2020. Mapping the epigenetic modifications of DNA and RNA. *Protein Cell.* 11(11):792–808.
- Zhao X, Yu YT. 2004. Detection and quantitation of RNA base modifications. *RNA.* 10(6):996–1002.
- Zheng G, Dahl JA, Niu Y, Fedorcsak P, Huang CM, Li CJ, Vågbo CB, Shi Y, Wang WL, Song SH, et al. 2013. ALKBH5 is a mammalian RNA demethylase that impacts RNA metabolism and mouse fertility. *Mol Cell.* 49(1):18–29.
- Zheng G, Qin Y, Clark WC, Dai Q, Yi C, He C, Lambowitz AM, Pan T. 2015. Efficient and quantitative high-throughput tRNA sequencing. *Nat Methods.* 12(9):835–837.
- Zhou H, Kimsey IJ, Nikolova EN, Sathyamoorthy B, Grazioli G, McSally J, Bai T, Wunderlich CH, Kreutz C, Andricioaei I, et al. 2016. m(1)A and m(1)G disrupt A-RNA structure through the intrinsic instability of Hoogsteen base pairs. *Nat Struct Mol Biol.* 23(9):803–810.
- Zhou KI, Parisien M, Dai Q, Liu N, Diatchenko L, Sachleben JR, Pan T. 2016. N(6)-methyladenosine modification in a long noncoding RNA hairpin predisposes its conformation to protein binding. *J Mol Biol.* 428(5):822–833.
- Zhu Y, Pirnie SP, Carmichael GG. 2017. High-throughput and site-specific identification of 2'-O-methylation sites using ribose oxidation sequencing (RibOxi-seq). *RNA.* 23(8):1303–1314.
- Zubarev RA. 2013. The challenge of the proteome dynamic range and its implications for in-depth proteomics. *Proteomics.* 13(5):723–726.