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Biphasic Liquid Microjunction Extraction for Profiling Neuronal RNA Modifications by Liquid Chromatography–Tandem Mass Spectrometry

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Abstract

RNA modifications are emerging as critical players in the spatiotemporal regulation of gene expression. Although liquid chromatography–tandem mass spectrometry (LC–MS/MS) enables the simultaneous quantification of numerous enzymatically modified RNAs in a biological sample, conventional RNA extraction and enzymatic digestion protocols that are employed prior to analysis have precluded the application of this technique for small-volume samples. In this study, a biphasic liquid microjunction (LMJ) extraction system using coaxial capillaries that direct and aspirate extraction solvents onto a ~350 μm -diameter sample spot was developed and applied for the extraction of RNA from individual cell clusters in the central nervous system of the marine mollusk *Aplysia californica*. To maximize RNA recoveries, optimized extraction solvents consisting of 10% methanol and chloroform were evaluated under dynamic and static extraction conditions. An MS-compatible RNA digestion buffer was developed to minimize the number of sample transfer steps and facilitate the direct enzymatic digestion of extracted RNA within the sample collection tube. Compared to RNA extraction using a conventional phenol-chloroform method, the LMJ-based method provided 3-fold greater coverage of the neuronal epitranscriptome for similar amounts of tissue and also produced mRNA of sufficient purity for reverse transcription polymerase chain reaction amplification. Using this approach, the expression of RNA-modifying enzymes in a given neuronal cell cluster can be characterized and simultaneously correlated with the LC–MS/MS analysis of RNA modifications within the same subset of neurons.

Graphical Abstract

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Supporting Information

Reagent details, Supporting Figures S1–S22, and Tables S1–S4, as noted in the text (PDF).

Introduction

The canonical nucleosides of RNA undergo myriad post-transcriptional modifications to form what is collectively known as the epitranscriptome. To date, over 30 classes of RNA modifications have been discovered, rendering a diverse library of more than 100 structurally distinct modified nucleosides that include methylation, acetylation, heavy atom addition, and even hypermodification of the substrate RNA with metabolites or complex heterocycles.^{1,2} Both coding and non-coding RNAs undergo enzymatic modifications that influence the physicochemical properties of the resulting biopolymer, such as higher order structure,^{3,4} resistance to degradation,⁵ and translation efficiency.^{6,7} Modifications are often deposited in sequence-specific contexts by enzymatic “writers” for subsequent interaction with cellular machinery. Select RNA modifications may also be targeted for enzymatic removal by modification “erasers,” resulting in a dynamic cellular epitranscriptome that serves as an additional layer of gene expression regulation.⁸

Recent advances in analytical methodologies that enable the detection of RNA modifications have greatly expanded our understanding of the roles that modified RNAs play in cell development,⁹ homeostasis,¹⁰ and disease.¹¹ However, most of these approaches involve antibodies or chemical derivatization steps that target specific modifications prior to downstream RNA sequencing and are thus limited to the analysis of a single modification per experiment. Moreover, the indirect detection of RNA modifications on the basis of RNA-seq enrichment profiles or reverse transcription signatures presents a challenge for discerning modification sites from noise or false-positive signals.^{12,13} In contrast, mass spectrometry (MS) permits the simultaneous and unambiguous direct detection of multiple RNA modifications. MS can be used to distinguish modified and unmodified RNAs on the basis of their molecular mass, enabling capture of the functional interplay within the suite of modified RNAs in a biological sample. For isobaric modifications (e.g., positional isomers of nucleoside methylations), tandem mass spectrometry (MS/MS) and/or separation techniques, such as liquid chromatography (LC) or ion mobility spectrometry, can be used to expand the coverage of the epitranscriptome within a single experiment.^{14–17} Provided an adequate amount of RNA material is available, modifications can be mapped with single nucleotide resolution to specific positions within a given sequence.^{18–20} However, obtaining

adequate quantities of sufficiently pure RNA for MS characterization presents a formidable challenge, particularly for rare and small-volume samples.

RNA extraction is an important step in the analytical workflow for studying RNA modifications and often dictates the quality of downstream analysis. Classical RNA isolation methods rely on liquid-liquid extraction (LLE) of biological samples with phenolic solutions and chloroform to induce differential partitioning of proteins, lipids, and nucleic acids between an aqueous layer and the immiscible organic phase.²¹ LLE approaches are compatible with standard laboratory equipment and readily scalable for large quantities of cells/tissue to furnish high yields of RNA. Despite the utility of LLE, this technique involves a number of tedious sample-handling and centrifugation steps that are difficult to automate. These drawbacks are especially problematic when working with extremely small samples such as individual cells or cell clusters that must be analyzed using numerous replicates to substantiate conclusions about their biology. Moreover, the multiple sample-transfer steps in LLE procedures invite sample loss with each successive manipulation, diminishing the amount of RNA recovered from volume-limited samples.²²

In order to address the demands of small-volume measurements, microextraction techniques have been developed for RNA isolation that minimize the number of sample-transfer steps, thereby improving analyte recoveries. For example, a comparison of solid phase microextraction (SPME) and conventional LLE for the isolation of yeast mRNA revealed superior recoveries for the SPME technique as determined by reverse transcription-quantitative polymerase chain reaction (RT-qPCR).²³ Similarly higher yields of RNA from bacterial cell lysate were reported when using a polydimethylsiloxane microchip-based liquid-liquid microextraction method compared to conventional off-chip purification.²⁴ In addition to providing improved RNA recoveries relative to traditional approaches, microfluidic probes have been designed and applied for the spatially resolved extraction of RNA from layers of adherent cells.^{25,26} However, the primary means of RNA detection following the aforementioned microextraction methods involves RT-PCR amplification, precluding detection of the vast majority of RNA modifications. Given the emerging roles of RNA modifications in cell-cell signaling,²⁷ cell differentiation,²⁸ and response to changes in chemical microenvironments,²⁹ a method that combines the advantages of spatially-resolved RNA microextraction with a detection platform that is capable of simultaneously profiling multiple RNA modifications is highly desirable. Such an advancement would be particularly powerful for studying central nervous system (CNS) tissue, where RNA modifications have been identified as putative regulators of myelination,³⁰ translation of learning-related mRNA,³¹ and neuronal excitability.³² Moreover, the investigation of a given CNS phenotype typically involves a specific network of neurons that may be spatially diffuse and challenging to examine without a careful manipulation of the targeted cells.

Liquid microjunction (LMJ) surface sampling has the potential to accomplish this task. LMJ extraction uses a microextraction probe to precisely apply and collect an extraction solution from small-volume samples and has previously been implemented for the analysis of proteins³³ and lipids³⁴ from tissue samples, and metabolites from individual cells.³⁵ Inspired by LMJ surface sampling, we created an RNA extraction system that incorporates two immiscible liquid phases that impinge on cell clusters deposited on a surface to extract

analyte RNA for subsequent digestion and detection of modified nucleosides by LC–MS/MS. The optimized method minimizes the number of sample-transfer steps by facilitating direct digestion of RNA within the sample collection tube using an MS-compatible buffer. The LMJ-based RNA extraction approach enabled the detection of 6 ± 3 RNA modifications in neuronal cell clusters from the animal model *Aplysia californica*, representing a 3-fold greater coverage of the neural epitranscriptome compared to conventional LLE. In addition, we show that LMJ-based RNA extraction renders mRNA of sufficient purity for RT-PCR. Using this approach, the expression of two RNA methyltransferases in neuronal cell clusters was determined and simultaneously correlated with LC–MS/MS analysis of RNA modifications from the same subset of neurons.

Experimental

Reagents.

Unless otherwise indicated, reagents (see the Supporting Information) were used without further purification.

Animals.

A. californica weighing 150–200 g were obtained from the National Resource for *Aplysia* (Miami, FL) and housed in an aquarium with circulating, aerated artificial seawater (Instant Ocean, Blacksburg, VA) maintained at 14 °C. Prior to dissection, animals were anesthetized by injection with 0.33 M MgCl₂ (~40% volume/body weight) directly into the body cavity.

In vitro Transcription.

Synthesis of a heavy isotope-labeled RNA was performed using T7 RNA polymerase and a 92-nucleotide DNA template purchased from IDT (Coralville, IA). The template sequence (Table S1) included a T7 promoter region with 2 subsequent guanine nucleotides to facilitate efficient transcription. In vitro-transcribed RNA was purified by polyacrylamide gel electrophoresis (PAGE) prior to use.

LMJ Extraction.

A modified LMJ extraction probe was operated as described previously.³⁵ Briefly, fluid was directed through two coaxial capillaries via an IDEX MicroTee Assembly with Mounting Hole (IDEX Health and Science, Rohnert Park, CA, Part #P-875). The outer capillary (250 µm/350 µm inner/outer diameter) length was 3.5 cm and the inner capillary (100 µm/170 µm inner/outer diameter) length was 30 cm (Polymicro Technologies, Phoenix, AZ). The inner diameter of the MicroTee is approximately 150 µm; to accommodate the inner extraction capillary, a round circuit board drill (bit size: 0.008 in) was used to expand the through-hole. To facilitate biphasic extraction, an additional IDEX MicroTee Assembly (Part #P-890) was used to simultaneously infuse two solvents into the LMJ probe via a single fluid line (Figure 1A). Solutions were delivered with a PHD 2000 syringe pump at 1.5 µL min⁻¹ (Harvard Apparatus, Holliston, MA) to the combinatorial MicroTee and the combined fluid was aspirated through the LMJ probe and into the sample chamber with –10 to –16 inHg from a diaphragm vacuum/pressure pump (Cole-Parmer, Vernon Hills, IL). Following extraction of RNA from the sample, extraction solvents were continuously flowed through the LMJ

system for 2 min to transfer any remaining sample from the inner capillary to the sample collection tube. The inner capillary was replaced after approximately 9 extractions. The probe was positioned with three linear stages (Zaber Technologies, Vancouver, BC, Canada) controlled with the in-house written software, microMS.³⁶

RNA Digestion.

For RNA digestion to generate nucleosides, 6 μL of 0.365 M ammonium acetate (pH 9.2) were added, followed by an enzyme mixture containing alkaline phosphatase (0.99 U), phosphodiesterase I (0.1 U), and benzonase (25 U). Next, 10 μg of bovine serum albumin (BSA) were added to each sample in order to prevent adsorption of nucleic acids onto the walls of the sample tube. Pentostatin was also added (0.495 μg) to inhibit the activity of trace amounts of deaminases. RNA extracts (typically between 10–15 μL) were then combined into the sample tube and the mixture diluted with nuclease-free water to a total volume of 30 μL . RNA was hydrolyzed at 37 $^{\circ}\text{C}$ for 3 h and analyzed either by PAGE or LC–MS/MS. For LC–MS/MS, the aqueous phase containing nucleosides was transferred to an autosampler vial, avoiding transfer of the chloroform layer.

LC–MS/MS.

Nucleoside separations were performed on a Dionex Ultimate 3000 nanoLC system (Thermo Scientific, Waltham, MA) equipped with an autosampler and thermostatted column compartment using an Acclaim RSLC 120 C18 150 $\times$ 2.1 mm, 2.2 μm column (Thermo Scientific). The flow rate was set to 170 $\mu\text{L min}^{-1}$ for yeast tRNA digests and 250 $\mu\text{L min}^{-1}$ for *Aplysia* RNA digests, unless otherwise noted. The column compartment was maintained at 36 ± 1 $^{\circ}\text{C}$. The compositions of mobile phases A and B were 5 mM ammonium acetate (pH 5.6) and 60/40 mobile phase A/acetonitrile, respectively. Gradient elution was performed under the following conditions: 0–5 min 1% B, 5% B at 9 min, 7% B at 11 min, 10% B at 13 min, 15% B at 32 min, 40% B at 38 min, 50% B at 43 min, 75% B at 50 min, 100% B at 56 min with a 7 min hold at 100% B, and a 12-min column re-equilibration step at 1% B. The LC system (excluding the column) was flushed with 10% o-phosphoric acid after every ~100 injections to remove contaminating alkali metal salts.

An Impact HD UHR QqTOF-MS mass spectrometer (Bruker Corp., Billerica, MA) with an electrospray ionization source was operated in positive mode with collision induced dissociation (CID) for the collection of MS² data. Capillary voltage was 4 kV with the N₂ drying gas flow rate and temperature set to 9 L min⁻¹ and 275 $^{\circ}\text{C}$, respectively. Data were acquired from m/z 110–600 with a cycle time of 3 s. A preferred mass list constructed from the exact masses of known modified nucleosides according to the MODOMICS database² was used for selection of precursor ions for CID fragmentation. The m/z values of the canonical nucleosides A, G, and C were excluded from MS² analysis.

Extracted ion chromatograms (EICs) were plotted in Compass DataAnalysis (Bruker) and peaks integrated manually. The identities of modified nucleosides were confirmed by comparison of a given compound's retention time to a standard, as well as matching diagnostic MS² ions to database values.² A two-tailed Student's t-test with Bonferroni correction for multiple hypotheses was used to determine whether significant differences

existed between the mean peak areas for nucleosides for different RNA digestion buffer conditions. P-values $<0.05/n$ were considered significant, where n is the number of comparisons.

RT-PCR.

An aliquot of RNA extract (typically 2–5 μL) was converted to cDNA using oligo dT primers (IDT, 0.1 $\mu\text{g } \mu\text{L}^{-1}$) according to the manufacturer's instructions. Next, 2 μL of cDNA were transferred to a clean PCR tube and mixed with 10 μL of 2 $\times$ Power SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA), 0.6 μL of forward and reverse primers (10 μM each), and nuclease-free water to a final volume of 20 μL . Primer sequences are shown in Table S1. Amplification was performed using a Techne Personal Thermal Cycler (Bio-Techne, Minneapolis, MN) with the following temperature program: initial denaturation at 95 $^{\circ}\text{C}$ for 3 min followed by 40 cycles of denaturation at 94 $^{\circ}\text{C}$ for 20 s, annealing at 53 $^{\circ}\text{C}$ for 30 s, and extension at 60 $^{\circ}\text{C}$ for 30 s. Amplicons were visualized on a 1% agarose gel using SYBR Safe DNA Gel Stain (Life Technologies, Carlsbad, CA) and a blueBox S Transilluminator (Amplify, Cambridge, MA).

Results and Discussion

Biphasic LMJ Extraction of RNA.

The extraction of RNA from small-volume, mass-limited samples requires special considerations to ensure that the maximum amount of analyte material is recovered. Ideally, analytical measurements are performed directly on the sample extract without precipitation/washing steps in order to minimize sample loss. LMJ extraction accomplishes this task while also maintaining the capacity for semi-automated and spatially resolved RNA isolations. We implemented a LMJ extraction system similar to previous designs^{35,37} consisting of two concentric capillaries: an outer capillary through which extraction solvent(s) are delivered using a syringe pump and an inner capillary that is connected to a vacuum chamber to facilitate aspiration of the sample extract. Since typical LLE solvent systems consist of an aqueous extraction reagent and chloroform to extract RNA from biological samples, we initially outfitted the LMJ system with two syringes, one containing an aqueous methanol solution and the other, chloroform. Figure 1A shows a schematic of the biphasic LMJ extraction system setup and workflow used for the analysis of RNA modifications.

To optimize the LMJ extraction procedure, we used a total tRNA sample from baker's yeast. Setting the syringe pump and vacuum apparatus to 1.5 $\mu\text{L min}^{-1}$, and between -10 and -16 inHg, respectively, ensured that continuous contact was made between the impinging extraction liquids and the sample (deposited on a microscope slide) during sample collection without leakage of the extraction liquid across the slide. Figure 1B shows a dried sample spot (1.5 μL deposited) of tRNA (9 $\mu\text{g } \mu\text{L}^{-1}$) after LMJ extraction for 2 min. An additional 2 min of extraction liquid flow through the capillary was included post-extraction to ensure analyte RNA was transferred to the sample tube (11 ± 1 μL collected). Upon removal of the sample tube from the vacuum chamber, two separate phases were observed corresponding to chloroform (bottom) and aqueous (top). The diameter of the resulting LMJ extraction footprint was ~ 350 μm .

We initially investigated the RNA extraction performance of a series of extraction solvents that are capable of denaturing cell components. Various concentrations of aqueous guanidinium chloride, aqueous methanol, and a commercially available RNA extraction reagent (TRIzol) were among the solvents tested with the biphasic LMJ method using chloroform as the immiscible organic solvent. Guanidinium chloride clogged the inner capillary of the LMJ system due to deposition of salt crystals (Figure S1). An extraction solvent based on methanol produced moderate extraction yields and minimized clogging of the LMJ apparatus. A 10% methanol extraction solvent exhibited comparable tRNA recoveries to deionized water while avoiding the analyte carryover observed at higher methanol concentrations (Table S2). The TRIzol reagent yielded high RNA concentrations (Table S3), but low signals for nucleosides following enzymatic digestion and LC–MS/MS analysis compared to the 10% methanol solution, likely due to contamination of the extract with TRIzol reagent, resulting in diminished activity of the digestive enzymes (Figure 1C). Based on these results, a 10% methanol extraction solvent was selected for subsequent experiments.

Investigating Dynamic and Static LMJ Extraction Modes.

We operated the LMJ apparatus in two modes: (1) the dynamic extraction mode described above (Figure 1A) that involves continuous flow and aspiration of extraction solvents contacting the sample spot; and (2) a static extraction mode that involves the application of extraction solvents onto a sample, a delay time with no flow of extraction liquid to facilitate mass transfer into the aqueous layer, and subsequent recovery of the extract via aspiration (Figure S2). We compared these extraction methods by varying the extraction times and determining the total RNA yield from a dried tRNA sample spot (13.5 μg total) using UV spectrophotometry. For the dynamic extraction mode, 5 locations across the dried tRNA spot were sampled for equal duration and the extracts deposited in a single PCR tube. Both extraction modes were immediately followed by an additional 2 min of extraction liquid flowing through the LMJ system. The static extraction mode recovered $10.9 \pm 0.9 \mu\text{g}$ of the yeast tRNA ($81 \pm 7\%$ extraction efficiency) in just 10 s, whereas the dynamic mode recovered less than 1 μg in the same timeframe (Figure S3A). After a 2 min extraction, the dynamic extraction mode yielded $3.9 \pm 0.4 \mu\text{g}$ of RNA. Little to no increase in RNA yield was observed for longer extractions. While this corresponds to an extraction efficiency of $29 \pm 3\%$, the 5 locations extracted during dynamic extraction only sampled approximately 25% of the surface area of the dried RNA spot compared to complete surface coverage by static extraction. When normalized for the amount of surface area sampled (RNA recovered per mm^2), Figure S3B shows that dynamic extraction surpasses static LMJ in extraction performance at longer extraction times. Additionally, the dynamic extraction afforded spatial resolution on the order of $\sim 350 \mu\text{m}$, whereas the static extraction resolution was limited to the diameter of the extraction solvent droplet ($\sim 3 \text{ mm}$).

MS-Compatible Buffer for Direct Digestion of RNA from LMJ Extracts.

Non-volatile buffer salts are a common component of RNA digestion buffers that facilitate enzymatic activity. However, successive injections of salt-containing buffers into LC–MS systems may diminish the performance or even damage instrumentation if not adequately removed from samples prior to analysis. Precipitation or buffer exchange approaches are

commonly used to resuspend analytes in MS-compatible buffers, but these procedures often lead to sample losses that are prohibitive for the analysis of small-volume samples. To minimize the number of sample handling steps, we sought to develop an RNA digestion buffer that could be readily interfaced to LMJ-based RNA extraction.

We first investigated the feasibility of using a volatile ammonium acetate-based buffer for the digestion of a tRNA standard using PAGE to monitor the enzymatic hydrolysis. Figure 2A shows a comparison of the gel results obtained following tRNA digestion in either non-volatile Tris buffer (pH 8) or an ammonium acetate buffer (pH 9.2), with digestion times varying from 1 h to 3 h. As shown in lanes 7 and 8, incomplete digestion of the tRNA is observed for 1 h or 2 h time points when using the ammonium acetate buffer. No detectable intact tRNA remained after 3 h of digestion, which was similar to the tRNA digestion time profile observed when using the Tris buffer. Complete digestion of tRNA was also observed when varying the pH of the Tris or ammonium acetate digestion mixtures from pH 8 to 9.2 (Figure S4, lanes 4–8). Ammonium bicarbonate was also tested as an additional MS-compatible buffer salt at pH 9.2, similarly resulting in complete digestion after 3 h (Figure S4, lane 9).

Samples were also analyzed by LC–MS/MS to compare the levels of modified nucleosides detected following tRNA digestion in the studied buffers. EICs were plotted with a 0.01 Da mass window for the 14 yeast tRNA modifications detected with a signal-to-noise ratio greater than 10. Initially, peak areas for modified nucleosides from tRNA digested with the ammonium acetate buffer exhibited poor reproducibility (Table S4), potentially due to analyte adsorption onto the polypropylene sample tubes. Relative standard deviation (RSD) values for inosine were particularly high (36%), suggesting in vitro deamination of adenosine.³⁸ Upon adding BSA and the deaminase inhibitor pentostatin to the reaction mixture, triplicate analysis of yeast tRNA digests resulted in RSD values below 10% (Table S4). Figure 2B, C shows a comparison of representative EICs obtained for several modified nucleosides following digestion using either the optimized ammonium acetate buffer or the non-volatile Tris buffer. Triplicate analysis of the tRNA digests revealed no statistical difference between the mean peak areas of the modified nucleosides (Figure 2C).

We then used an in vitro transcribed RNA standard to investigate whether the newly optimized buffer would introduce RNA modification artifacts when mixed with the aqueous methanol LMJ extraction solvent. The RNA standard was designed to mimic *A. californica* tRNA^{Gln}(UUG) and prepared using heavy isotope-labeled nucleoside triphosphates for the detection of exogenous RNA modifications during the LMJ-based workflow. The heavy RNA standard was purified by PAGE before use (Figure S5). Following LMJ extraction of yeast tRNA, the heavy RNA standard was spiked into the sample tube and subjected to downstream digestion and LC–MS/MS analysis. Heavy isotopes of the canonical nucleosides were readily detected, but no signals were observed for isotopologues of methylated or deaminated nucleosides (Figure S6A,B). These results demonstrate the compatibility of the optimized digestion buffer and LMJ extraction solvent, rendering the system suitable for characterization of endogenous RNA modifications.

LMJ Extraction of Neuronal RNAs and Comparison to Conventional LLE.

For practical application of the LMJ method, we extracted RNA from the buccal ganglion of the well-established neurobiological model *A. californica*. This CNS tissue is divided into two approximately symmetrical hemiganglia (ca. 2–3 mm diameter, ~500 neurons³⁹) joined by the buccal commissure (Figure 3A). After severing the commissure, the hemiganglia were subjected to 10, 15, 20, and 25 min extractions. Figure S7A–C shows photographs of a typical hemiganglion before and after LMJ extraction. The amount of nucleic acid recovered from the hemiganglia was quantified by UV detection and was consistent over the extraction times studied, indicating that a 10 min extraction per sample was suitable (Figure S8). The total volume recovered after the 10 min extraction was $10 \pm 1 \mu\text{L}$, which is approximately 3-fold lower than expected, perhaps due to partial evaporation of the extract *in vacuo*.

We then compared the RNA extraction performance of the optimized LMJ approach to conventional LLE using LC–MS/MS. Symmetrical buccal hemiganglia from a given animal were separated, manually desheathed, and subjected to either LMJ extraction or LLE. Conventional LLE was performed using TRIzol reagent according to the manufacturer's instructions, with the RNA sample resuspended in a final volume of 10 μL . For LMJ extraction, 5 sampling and extraction events were performed (2 min each) across each hemiganglion. The MS-compatible RNA digestion buffer was used for extracts derived from both LLE and the LMJ apparatus. Figure 3B shows examples of EICs for cytidine as well as modified nucleosides obtained following digestion and LC–MS/MS analysis of RNA extracted using both techniques. The signal intensities for nucleosides were greater for the LMJ extraction compared to LLE, likely due to superior RNA yields that also permitted collection of MS/MS spectra. For example, Figure 3C shows the MS/MS spectrum generated for 2'-O-methyladenosine (Am), which facilitated positive identification of the modified nucleoside by matching the fragment ion at m/z 136 to the expected fragment reported in RNA modification databases.^{1,2} After 6 replicates (hemiganglia from 6 animals), conventional LLE detected just 2 ± 0 modified nucleosides (Table 1). In contrast, the LMJ approach rendered sufficient RNA for detection of 6 ± 3 modified nucleosides. Pseudouridine (Ψ) and inosine (I) were detected in all samples by both extraction methods and each are thought to play roles in neuronal response to environmental stimuli.^{10,40} Positional isomers of methyladenosine, including $m^1\text{A}$, Am, and $m^6\text{A}$, were not detected when using conventional LLE for sample preparation, but these three modified nucleosides were observed simultaneously in 4 of 6 buccal hemiganglion extracts when using LMJ-based extraction. The modified nucleosides 5-methylcytidine ($m^5\text{C}$), 2'-O-methylcytidine (Cm), 2-methylguanosine ($m^2\text{G}$), and 2'-O-methylguanosine (Gm) were detected in hemiganglia from 2 animals using LMJ extraction, while 7-methylguanosine ($m^7\text{G}$) was observed in 3 of 6 hemiganglia. EICs and mass spectra for the modified nucleosides detected in the hemiganglia from 6 animals are shown in Figures S9–S22. While it is difficult to ascertain the reason for the variability in the number of RNA modifications detected from the 6 hemiganglion extracts, it is conceivable that this phenomenon results from either sample loss while processing the pairs of hemiganglia or biological differences between animals.

The approximately 3-fold improvement that LMJ-based extraction afforded in detection coverage of RNA modifications over conventional LLE may facilitate better understanding of the unique RNA modification profiles of cell subpopulations. However, it is important to note that a typical eukaryotic cell contains approximately 20–30 unique RNA modifications,⁴¹ indicating that apart from the 6 ± 3 modifications we identified, a substantial group of modified nucleosides may have been undetected. Since our experiments involved the extraction of RNA from a small number of cells (~500 neurons for each buccal hemiganglion), it is not surprising that characterization of the epitranscriptome was incomplete. Similar coverage of RNA modifications was reported recently where 8 modified ribonucleosides were detected from 100 HeLa cells using conventional RNA extraction and a capillary electrophoresis–MS method.⁴² Given that modified nucleosides span a wide range of cellular concentrations, they are difficult to detect by LC–MS unless millions of cells are pooled together for analysis,^{43,44} emphasizing the importance of developing new approaches for the analysis of RNA modifications.

Simultaneous Characterization of RNA Methyltransferase Expression and Modified Nucleosides.

One core challenge in the analysis of minute sample quantities is the pressing need to collect the maximum amount of chemical information from a limited sample volume. MS methods are capable of simultaneously characterizing multiple RNA modifications, which may be complementary to additional analytical techniques to address complex biological questions. For example, it is unclear whether the expression of a given RNA-modifying enzyme is correlated with the quantity of modification that is deposited on a given RNA subtype. To begin addressing this question, we performed LMJ extraction on buccal hemiganglia from the *Aplysia* CNS and subjected aliquots of the extract to RT-PCR, as well as RNA digestion and LC–MS/MS. A schematic of the workflow is shown in Figure 4A. RT-PCR primers were designed to target two RNA-modifying enzymes: 7-methylguanosine tRNA methyltransferase (m^7G TMT), which catalyzes methylation of guanosine in tRNA, and DNMT2, which methylates cytidine. RNA extracts from LLE and static and dynamic LMJ extraction were analyzed by RT-PCR and the amplification products detected by agarose gel electrophoresis. Conventional LLE produced amplicons of moderate intensity for DNMT2 and a more intense band for m^7G TMT, whereas dynamic LMJ extraction did not render sufficient mRNA for detection of either methyltransferase (Figure 4B). However, the static LMJ extraction mode resulted in faint bands for both RNA-modifying enzymes. Guanidinium thiocyanate is a component of the TRIzol reagent used in LLE and is a stronger denaturing agent than the 10% methanol solution used for LMJ. This difference in enzyme denaturation may have contributed to the more intense bands observed for LLE due to inactivation of cellular RNases that would otherwise degrade the target mRNAs. Such RNA degradation would have less impact on nucleoside analysis by LC–MS/MS, where RNA hydrolysis is desirable. Figure 4C shows examples of EICs for 3 modified nucleosides detected, m^1A , I, and m^7G . These results demonstrate the feasibility of correlating the expression of RNA-modifying enzymes in a given neuronal cell cluster with the MS quantification of RNA modifications within the same subset of neurons.

Conclusions

We have developed a biphasic LMJ-based method for the extraction of RNA and subsequent LC–MS/MS profiling of RNA modifications from individual neuronal cell clusters in the CNS of *A. californica*. We optimized three key parameters—the extraction solvent, vacuum pressure, and extraction time—to facilitate the MS-compatible extraction and digestion of RNA from small-volume samples. The semi-automated LMJ approach ultimately provided 3-fold greater coverage of the epitranscriptome compared to conventional phenol-chloroform LLE for the same amount of CNS tissue. Moreover, RT-PCR analysis of RNA methyltransferase expression could be performed on the same LMJ extract, dramatically increasing the amount of chemical information acquired from the neuronal sample. In the brain, even adjacent cells have distinct functional roles, and so the ultimate analytical goal is to characterize the epitranscriptome cell-by-cell, requiring advances in analytical performance before such measurements become feasible. The LMJ RNA extraction platform we describe here enables us to simultaneously analyze an increasing number of RNA modifications and link them to the expression of their respective RNA-modifying enzymes. In future work, quantitative measurements of RNA modifications and RNA-modifying enzymes will be conducted to evaluate the epitranscriptomic heterogeneity of CNS tissue.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1.

(A) Schematic of the LMJ RNA extraction setup and analytical workflow. (B) Dried tRNA sample spot and the extraction footprint left by the LMJ probe following extraction. (C) Representative EICs for several modified nucleosides following digestion of tRNA samples spiked with 10% methanol (black trace) or TRIZOL (red trace) aqueous extraction solvents. EICs for the positional isomers of methyladenosine and methylcytidine were at m/z 282.12 $\pm$ 0.01 and m/z 258.11 $\pm$ 0.01, respectively.

Figure 2.

Comparison of yeast total tRNA digestion using a Tris buffer or MS-compatible ammonium acetate buffer. (A) PAGE of intact yeast total tRNA standard (lane 1), nucleosides standard (lane 2), digestion in Tris buffer for 3 h (lanes 3 and 4), and digestion in ammonium acetate buffer for 3 h (lanes 5 and 6), 2 h (lane 7), and 1 h (lane 8). (B) Overlays of representative EICs for several modified nucleosides obtained for the studied digestion conditions. EICs for positional isomers of methyladenosine (m/z 282.12), methylcytidine (m/z 258.11), and methylguanosine (m/z 298.12), as well as 5-aminomethyl-2-thiouridine (nm⁵s²U, m/z 290.08) and N2,N2-dimethylguanosine (m^{2,2}G, m/z 312.13), are plotted with a ± 0.01 m/z window. Mean peak areas ($n = 3$) are shown in panel (C) where error bars represent standard deviation. No significant differences ($p > 0.05/n$) were observed for modified nucleoside peak areas under the two conditions studied.

Figure 3.

Analysis of neuronal RNA modifications from CNS tissue of *Aplysia californica*. (A) Schematic of the animal's CNS, along with a photograph of the buccal ganglion. (B) EICs corresponding to the canonical nucleoside cytidine ($m/z\ 244.09 \pm 0.01$), the modified nucleoside inosine ($m/z\ 269.09 \pm 0.01$), and positional isomers of methyladenosine ($m/z\ 282.12 \pm 0.01$) detected using either LMJ RNA extraction or conventional LLE prior to RNA digestion and LC-MS/MS analysis. (C) Representative MS/MS spectrum for 2'-O-methyladenosine (Am) obtained following LMJ-based extraction.

Figure 4.

(A) Workflow for simultaneous LC-MS detection of modified nucleosides and RT-PCR analysis of mRNA for two RNA methyltransferases from *A. californica*. Identified B cells from the buccal ganglion are labeled B3–B5. (B) Agarose gel electrophoresis following RT-PCR of two RNA methyltransferases, namely, DNMT2 (lanes denoted with 1) and m^7G TMT (lanes denoted with 2). Lanes were further grouped and marked with a letter (a–c) indicating the extraction method used: (a) dynamic LMJ extraction, (b) conventional LLE, and (c) static LMJ extraction. H4 ctrl represents a positive control. (C) EICs for 1-methyladenosine (m^1A), inosine (I), and 7-methylguanosine (m^7G) were generated from the same static LMJ extraction aliquot used for the results shown in lanes c1 and c2.

Table 1.

Modified ribonucleosides detected in the buccal hemiganglia from 6 *Aplysia* using LMJ or conventional LLE.

Animal	Ψ	I	m ¹ A	Am	m ⁶ A	m ⁵ C	Cm	m ⁷ G	Gm	m ² G
1	+/+ ^a	+/+	+/-	+/-	+/-	+/-	+/-	+/-	-/-	+/-
2	+/+	+/+	+/-	+/-	+/-	-/-	+/-	+/-	+/-	-/-
3	+/+	+/+	+/-	+/-	+/-	+/-	-/-	-/-	-/-	-/-
4	+/+	+/+	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-
5	+/+	+/+	+/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-
6	+/+	+/+	+/-	+/-	+/-	-/-	-/-	+/-	+/-	+/-

^aExtraction by LMJ/LLE; modification detected (+) or not detected (-) on the basis of retention time matching to a standard and/or MS/MS spectrum.