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Editorial: Technologies for RNA Detection

Anca F. Savulescu[1], Renate Weizbauer[2], Marion Hogg[3, *] and Kif Liakath-Ali[4, 5, *]

[1]Division of Chemical, Systems & Synthetic Biology, Faculty of Health Sciences, Institute of Infectious Disease & Molecular Medicine, University of Cape Town, Cape Town, South Africa
[2]Department of Plant Biology, Carnegie Institution of Washington, Stanford, CA, USA
[3]SDepartment of Biosciences, School of Science and Technology, Nottingham Trent University, Clifton campus, Nottingham, UK
[4]School of Biological Sciences, University of Southampton, Southampton, UK
[5]Institute for Life Sciences, University of Southampton, Southampton, UK
*For correspondence: marion.hogg@ntu.ac.uk; kif.liakath-ali@soton.ac.uk

Ribonucleic acid (RNA) molecules are essential for multiple cellular processes, including protein synthesis, regulation of gene expression, and maintenance of chromosomal structure. To fulfill these key roles, RNAs interact directly and/or indirectly with other cellular biomolecules, such as proteins and additional RNAs, often forming complex structures. Identifying and characterizing the roles and structures of RNAs within these subcellular complexes and processes requires the application of highly advanced spatial and temporal techniques. Indeed, recent years have seen a plethora of single-cell-based RNA sequencing (RNA-seq) approaches, single-molecule RNA visualization methodologies, and artificial intelligence–based prediction models for RNA localization and structure. This special issue highlights some of the latest technological advances in RNA detection, visualization, RNA-seq methodologies, structural elucidation, and characterization of RNA–protein binding.

Techniques for RNA visualization and detection in cells and tissues

Subcellular localization of RNA molecules has been widely accepted in recent years as one of the strategies by which gene expression is regulated [1]. By localizing specific RNA transcripts in defined subcellular spaces, specialized cells such as neurons, epithelial cells, and fibroblasts ensure timely translation based on the cell's needs [1]. Furthermore, RNA expression and subcellular distribution may differ between cells of the same type in different cell states and between defined cell populations within a tissue [2–4]. Thus, technologies enabling visualization and quantification of RNA transcripts in subcellular domains, cells, and tissues are critical for elucidating complex subcellular processes, cell functions, and states. In this special issue, several methods aimed at the detection, visualization, and quantification of mRNAs in cells and tissues, as well as the colocalization of mRNAs and proteins, are presented. O'Hanlon and Wu developed a protocol to visualize RNA transcripts in live cells using aptamers, which are genetically encodable and fluorogenic [5]. The protocol is applied to several cell lines, showcasing its generalizability. The authors include an analytical framework aimed at distinguishing real signals, quantifying RNA foci, comparing healthy and pathogenic RNAs, quantifying subcellular localized RNA transcripts, and measuring RNA stability and half-life. Another aptamer-based protocol by Wierzba et al. utilizes novel RNA-targeting probes that incorporate a peptide nucleic acid (PNA)-based linker with Riboglow-based probes [6,7]. These novel probes are shown to enhance probe affinity and specificity to RNA and successfully detect RNA subcellular localization and dynamics. This comprehensive protocol provides details on all stages of the generation and utilization of PNA-based probes. Additionally, the protocol offers an increased dynamic range for a stress granule assay used to assess the ability of a fluorescent tag to detect RNA localization.

The ability to colocalize biomolecules in a single sample aids the characterization of processes where biological molecules interact with other biomolecules or are localized to a subcellular anchor. For instance, visualizing colocalization of RNA transcripts with protein subcellular markers advances our understanding of RNA subcellular distribution and gene expression

patterns. In this special issue, several protocols describe methodologies to efficiently visualize and quantify colocalization of RNA transcripts with other biomolecules. Sierras et al. combined RNA-FISH and antibody staining to simultaneously detect RNAs and hematopoietic and stromal cell surface markers, respectively, in formalin-fixed paraffin-embedded (FFPE) bone marrow biopsy samples [8]. The use of this protocol has clarified gene expression patterns and heterogeneity in acute lymphoblastic leukemia. Similarly, a FISH-based protocol aimed at detecting mRNAs in neuronal tissue was developed by Becher et al. [9]. Here, too, the authors studied the subcellular localization of RNA transcripts of interest by combining FISH with the visualization of cellular markers using immunohistochemistry (IHC) in rodent neuronal tissue. The authors describe a detailed analytical pipeline developed in ImageJ to quantify mRNA puncta and the area covered by IHC in the same image [9].

Kankanamalage et al. approach RNA and protein co-detection in complex samples using a flow cytometry–based method [10]. They implement the Thermofisher's PrimeFlow™ assay, which combines flow cytometry with branched DNA (bDNA)-based in situ hybridization, on mouse spleen samples. Furthermore, by integrating PrimeFlow™ with cell immunophenotyping, luciferase transgene mRNA and protein expression are detected in spleen samples treated with luciferase mRNA-lipid nanoparticles (LNPs) in vivo [10]. This protocol has the potential to advance the detection of transgene mRNA and protein in pre-clinical samples, aiding in the development of RNA-based therapeutics.

Finally, a technique for detecting RNA molecules without requiring amplification is presented by Hu et al. Here, the authors developed an optimized and enhanced split-crRNA Cas12a assay, termed SCas12aV2 [11]. By combining Cas12a with a split crRNA, the detection of RNA molecules is achieved without amplification. This protocol describes the preparation of the SCas12a system and demonstrates its ability to detect long-chain RNA transcripts with complex secondary structures from clinical samples [12]. The method can successfully distinguish pre-miRNA from mature miRNA levels [13], positioning SCas12aV2 as a powerful addition to the growing toolkit for sensitive and amplification-free RNA detection.

Bioinformatics approaches for gene expression data analysis

There is an abundance of studies in the RNA field that integrate computational tools to study various aspects of RNA biology. In this special issue, several protocols using bioinformatics and computational biology tools for RNA gene expression analysis are presented. These address complementary dimensions of RNA biology, including functional enrichment, detection of discistronic transcripts, and alternative splicing analysis, across diverse biological systems, ranging from bacteria to plants and human gliomas. Eden and Vetrivel developed a protocol to construct custom R annotation packages using genomic data [14]. They showcase the utility of this workflow by annotating *Mycobacterium tuberculosis* (*Mtb*) H37Rv and characterizing the transcriptional response of *Mtb* to rifampicin treatment. This user-friendly computational protocol supports functional enrichment and biological interpretation of RNA-seq datasets, particularly for organisms that remain poorly annotated. In another protocol, Zheng et al. focus on the detection of dicistronic tRNA-mRNA transcripts from short-read RNA-seq datasets in plants [15]. Their model, named DiRT v2.0, is an improvement to DiRT v1.0, expanding the range of discistronic transcripts detected. The authors use this protocol to detect tRNA and tRNA-like structures of discistronic transcripts from two plant species. This assists in the elucidation of these transcripts' roles in RNA systemic mobility through the plant's vascular tissues. An additional computational protocol for analyzing RNA-seq data focuses on alternative splicing in pediatric high-grade gliomas with H3.3K27M mutation [16]. Here, the authors identify alternative splicing events, visualize splicing patterns, and conduct differential alternative splicing analysis in SMART-Seq2 data. Although shown in gliomas, this computational workflow can be utilized to analyze alternative splicing in other biological and disease contexts.

Techniques for advancing RNA sequencing

Since their development, next-generation sequencing (NGS) and RNA-seq have consistently been among the most popular techniques for detecting and quantifying RNA transcripts in biological samples. They aid in characterizing gene expression patterns, alternative splicing, and other RNA biology-related aspects, and they have been applied in multiple biological sample types and contexts. Technologies advancing different steps in sequencing approaches are highly useful, as they allow higher resolution and generalizability of these approaches. Lamont et al. developed Transcript-Capture, a novel protocol for extracting bacterial RNA from infected host samples [17]. Here, bacterial-specific RNA reads are enriched prior to sequencing via biotinylating base-pair DNA probes, capturing the entire bacterial genome. This is followed by hybridization of these probes to the cDNA of NGS sequencing libraries from host samples. Using this protocol, the authors were able to

capture *Mtb* genome activity in infected host samples. Transcript-Capture can be adapted to other bacterial species, as well as viruses and fungi, to characterize genomic activity of pathogens within their hosts.
Reverse transcriptases (RTs) convert RNA into cDNA, being essential in some RNA-seq approaches. For tRNA-focused sequencing pipelines, specific RTs that can deal with complex secondary structures and extensive post-translational modifications of these RNA molecules are required. MarathonRT is one such RT, offering the advantage of reading through RNA secondary structures and chemical modifications [18]. Pedor et al. developed a protocol to isolate MarathonRT for RNA-seq applications and validated their protocol in a mim-tRNAseq pipeline [19]. This protocol offers a few advantages compared to existing protocols, including producing a stable and highly active MarathonRT, reducing the purification time, and being cost-effective. Furthermore, the protocol also features a novel assay for measuring the enzymatic activity of the isolated MarathonRT [19].

Technologies for characterization of RNA–protein interactions

Identifying and characterizing RNA–protein interactions has been the subject of a large body of literature over the past two decades. Among other roles, these interactions function in the post-translational regulation of gene expression. Electrophoretic mobility shift assays (EMSAs) are a gold-standard approach to study binding affinities of RNA–protein interactions. McQuarrie and Soller present a novel protocol for assessing RNA–protein binding and complex formation using EMSA [20]. Here, they use ^{32}P-radiolabeled RNA and recombinant RNA-binding proteins in *E. coli*. This protocol should be of use to researchers looking to utilize the EMSA approach to characterize RNA–protein interactions in various biological contexts, in a reproducible and standardized manner.

Advances in methods for RNA structure determination

RNA molecules can fold into dynamic complex structures, which often contribute to their cellular functions. One of the standard approaches for elucidating RNA structures, especially those based on long-range RNA interactions, relies on proximity ligation. SPLASH is one such method, capturing RNA–RNA interactions via base-pairing [21]. Wang et al. developed a modification to the SPLASH method, termed HiCapR, by incorporating a targeted enrichment step post-cDNA library construction, enabling capture of low-abundance targets [22]. HiCapR was applied to study the structure and dynamics of the HIV RNA genome and seems to be well-suited for this purpose, given the low amounts of RNA in complex viral structures.

Summary

This special issue brings together a diverse collection of experimental and computational protocols that advance the study of RNA biology, from single-molecule visualization and amplification-free detection to improved RNA-seq workflows, bioinformatic pipelines, and structural mapping approaches. Collectively, these methodologies enable precise interrogation of RNA localization, dynamics, gene expression, RNA–protein interactions, and higher-order RNA structures in systems ranging from bacteria and plants to mammalian tissues and human disease models. By integrating innovations in imaging, sequencing, enzymology, and computational analysis, this issue highlights the rapidly expanding toolkit that is driving a deeper, more mechanistic understanding of RNA-mediated cellular processes.

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