



Nanopore Direct RNA Sequencing and the Epitranscriptome: Advances in Mapping Native RNA Landscapes

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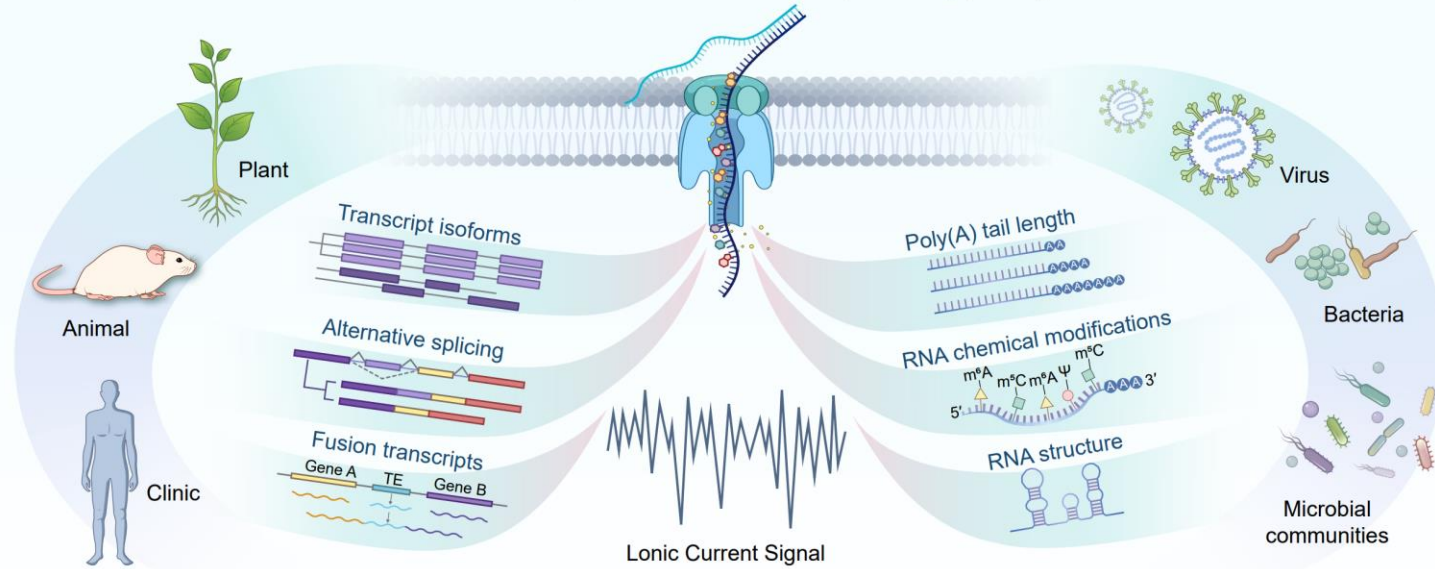
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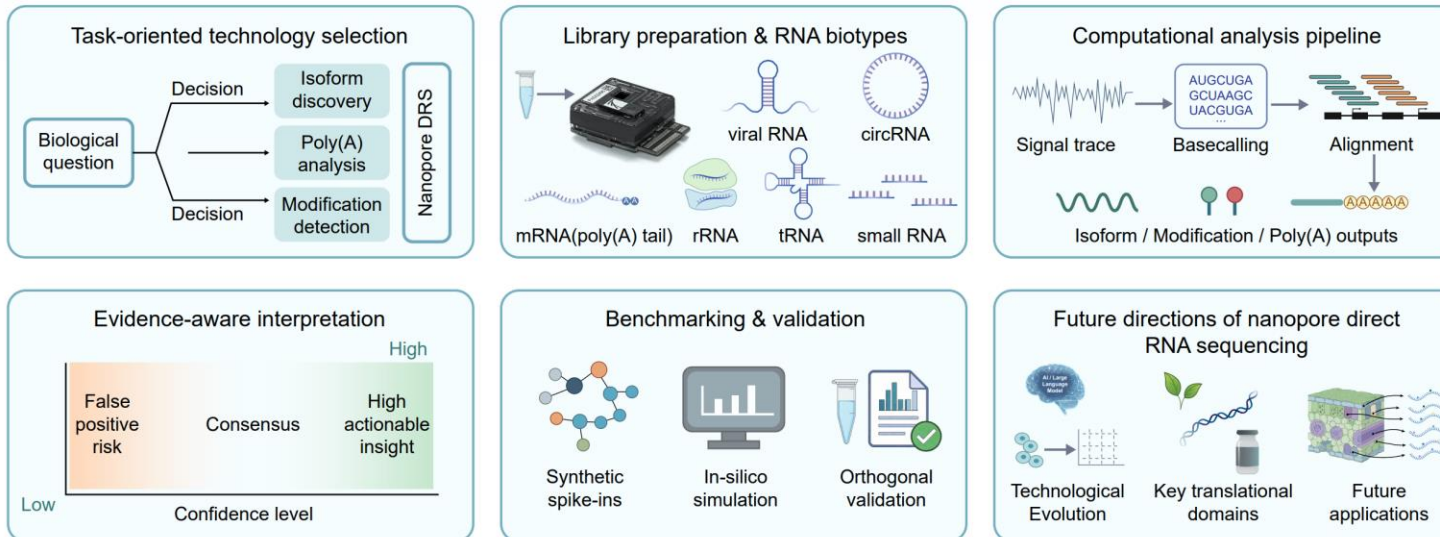


Overview

Nanopore direct RNA sequencing(DRS)



- Direct RNA sequencing (DRS) is a transformative RNA technology that has emerged over the past decade, leveraging long-read nanopore platforms to directly sequence native RNA molecules.
- This review targets global researchers in RNA biology and RNA modification. By summarizing over 700 DRS studies and methods papers, it provides a practical guide from experimental design and data analysis to multi-omics integration, and outlines key directions for the next 5–10 years.
- It highlights how DRS captures full-length transcripts, poly(A) tails, and RNA modifications at single-molecule resolution, offering a “one run, multilayer readout” framework for decoding RNA regulatory networks.



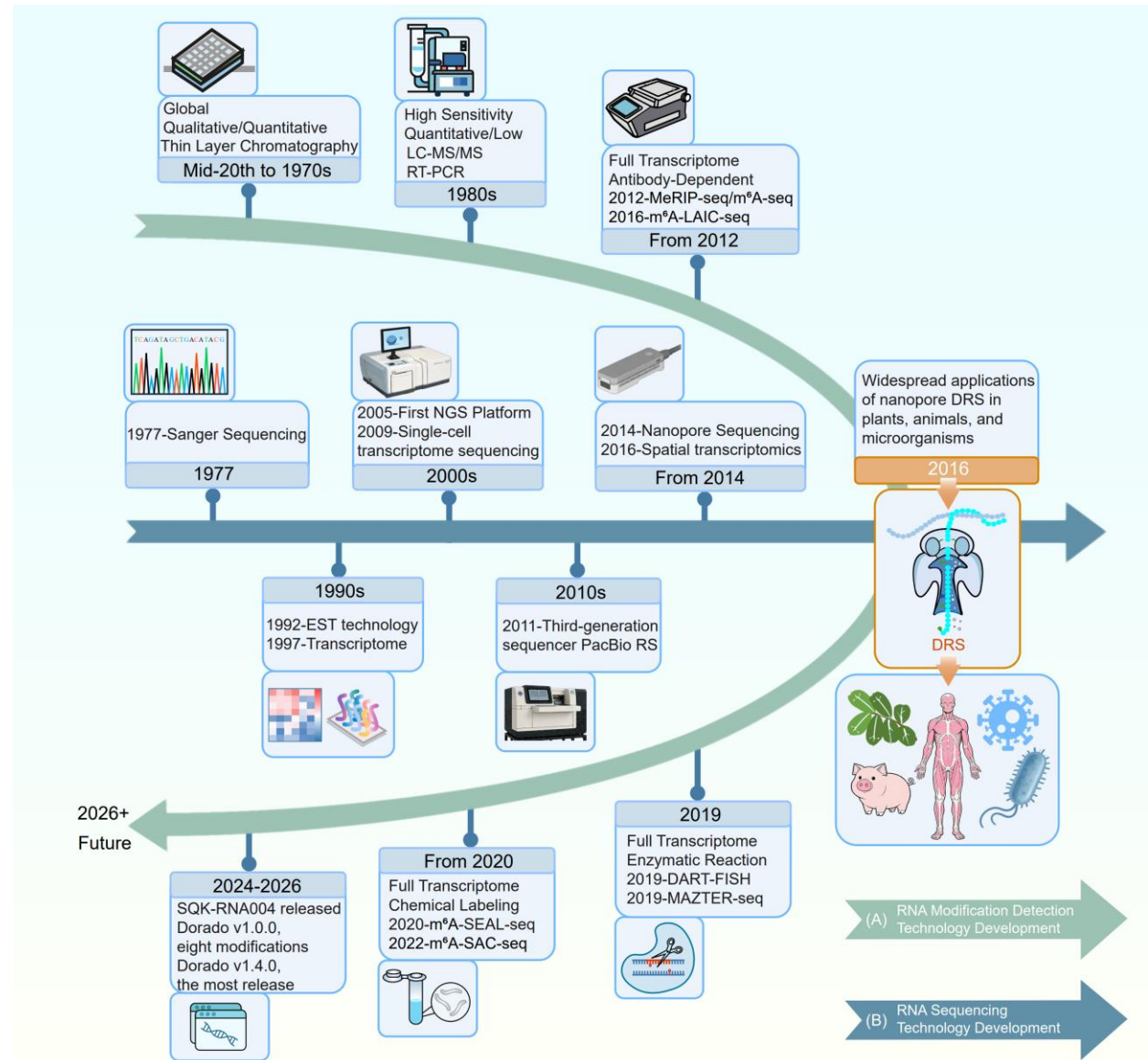


Highlight

- ❑ This article emphasizes that nanopore direct RNA sequencing can simultaneously resolve multiple layers of information from native RNA molecules at single-molecule resolution.
- ❑ This article systematically compares nanopore direct RNA sequencing with short-read, long-read, and modification-specific approaches.
- ❑ This article adopts a task-oriented perspective to clarify the advantages of nanopore direct RNA sequencing.
- ❑ This article emphasizes confidence grading in the interpretation of nanopore direct RNA sequencing results.
- ❑ This article proposes a layered benchmarking and validation framework.



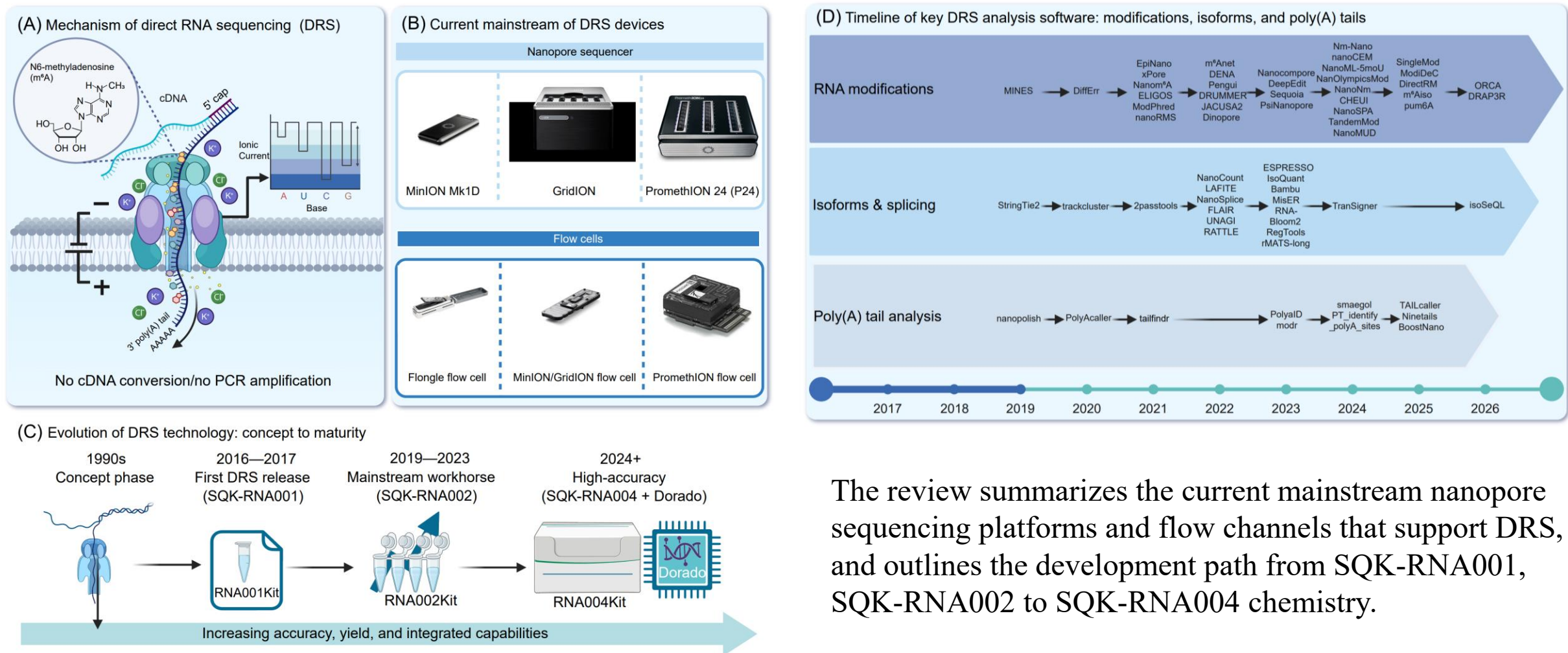
The Evolution of Nanopore Direct RNA Sequencing



The emergence of DRS enables researchers to directly link transcript structure, modification information, and poly(A) tail features on native RNA molecules.

Figure 1. Timeline of major innovations in RNA sequencing and RNA modification technologies

Overview of Oxford nanopore direct RNA sequencing (DRS) technology, platforms, and software



The review summarizes the current mainstream nanopore sequencing platforms and flow channels that support DRS, and outlines the development path from SQK-RNA001, SQK-RNA002 to SQK-RNA004 chemistry.

Figure 2. Overview of DRS technology, platforms, and software

Comparison of RNA sequencing strategies and the unique advantages of nanopore DRS

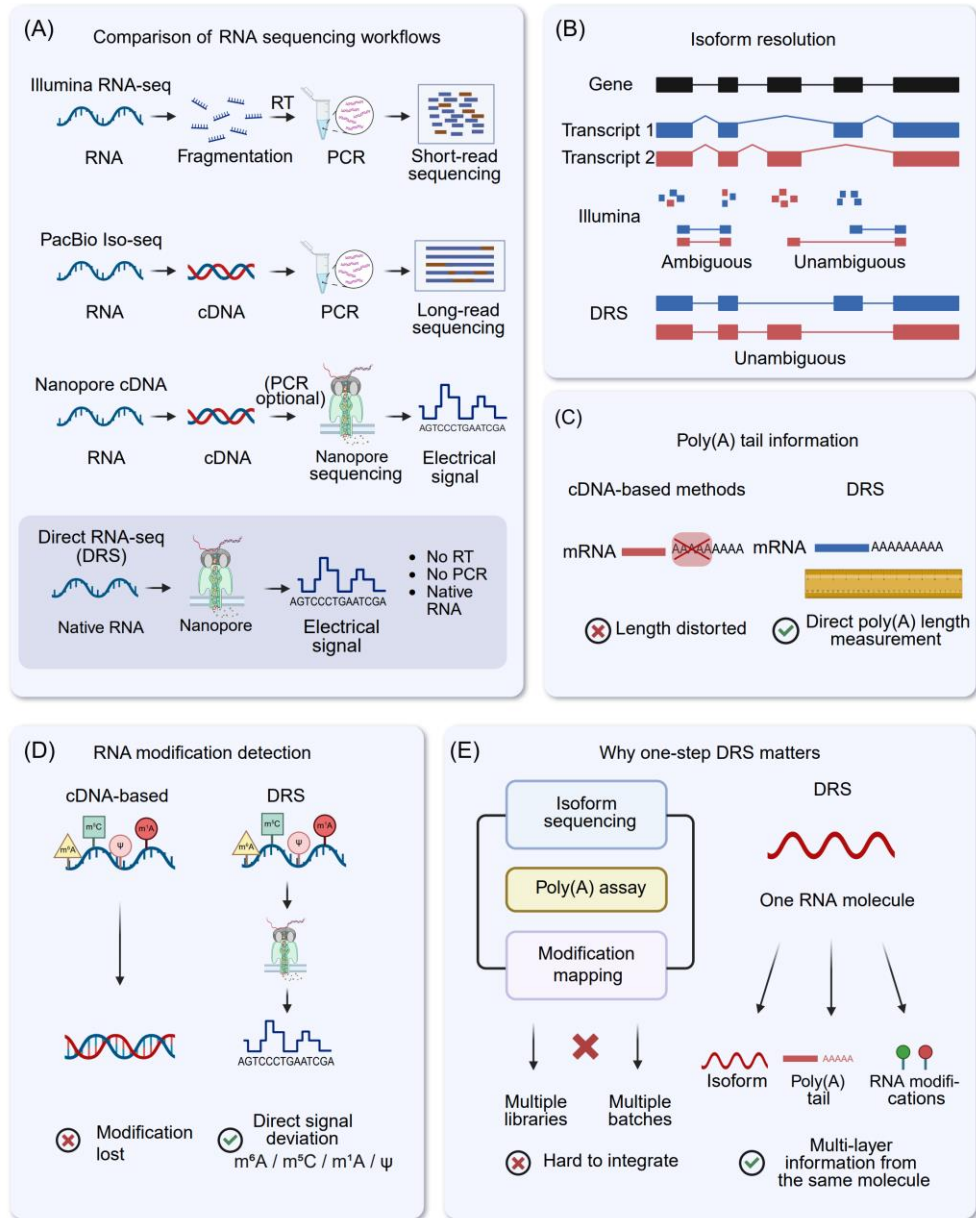


Table Scenario-oriented comparison of nanopore DRS and other RNA sequencing approaches.

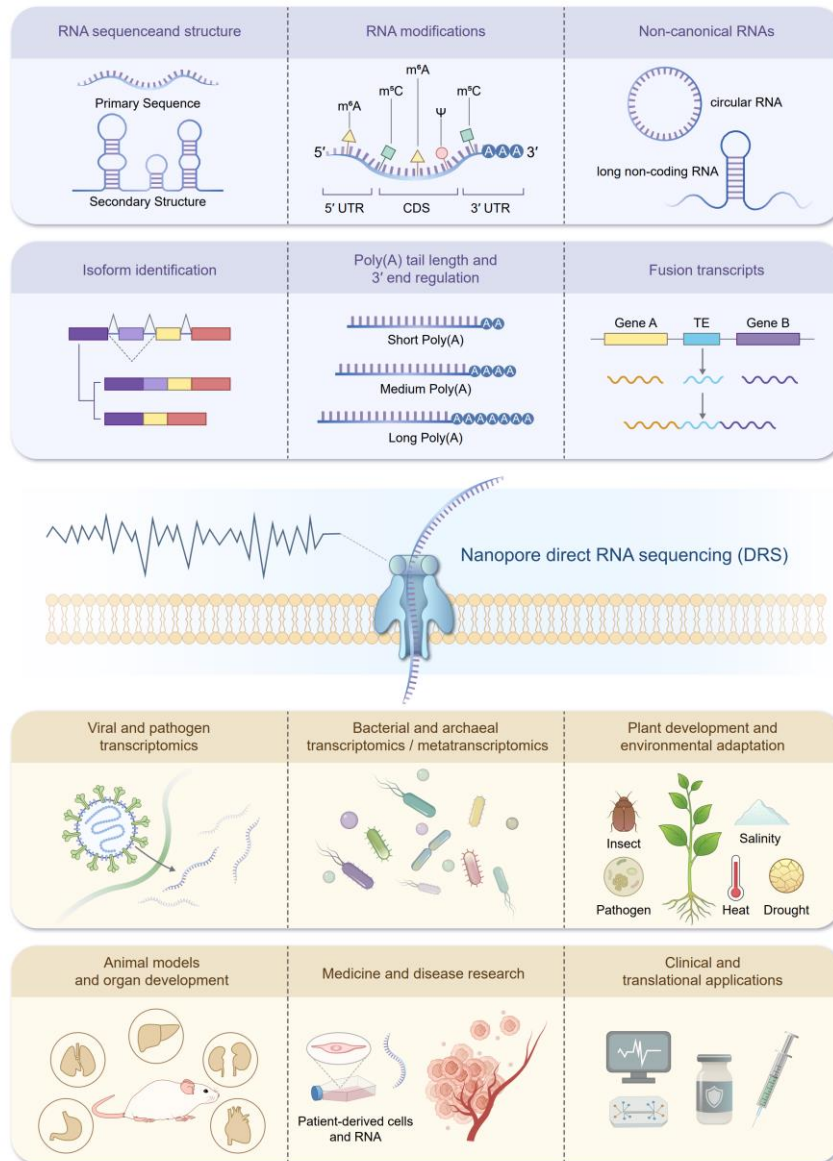
Application / research objective	NGS RNA-seq	ONT cDNA sequencing	PacBio Iso-Seq	Tail Iso-Seq	DRS
Large-scale gene expression quantification	+++	++	+	++	+
Differential gene expression analysis	+++	++	+	++	++
Isoform discovery / novel transcript annotation	+	+++	+++	+++	++
Isoform quantification	+++	++	++	++	+
Differential isoform usage / isoform switching	+	++	+++	++	++
Alternative splicing analysis	+	++	+++	++	+++
Fusion transcript detection	+	++	+++	++	+++
Allele-specific analysis	+	+++	+++	+++	+++
APA / 3' end usage analysis	+	++	++	+++	+++
Poly(A) tail length estimation	-	+	+	+++	+++
RNA modification profiling (m6A)	+	-	-	-	+++
RNA modification profiling (non-m6A)	+	-	-	-	++
Simultaneous analysis of isoform structure, poly(A), and RNA modifications on the same molecule	-	-	-	+	+++
Low-input or limited clinical samples	+++	++	++	++	+
Very short RNAs / small RNAs	+++	+	+	+	-
High-accuracy reference transcriptome construction	+	++	+++	++	++
Cost-sensitive population-scale studies	+++	++	+	++	+

Scoring system: +++, preferred; ++, suitable; +, possible but with notable limitations; -, generally not preferred.

Figure 3. Comparison of RNA sequencing strategies and features captured by DRS



Advantages and biological applications of DRS

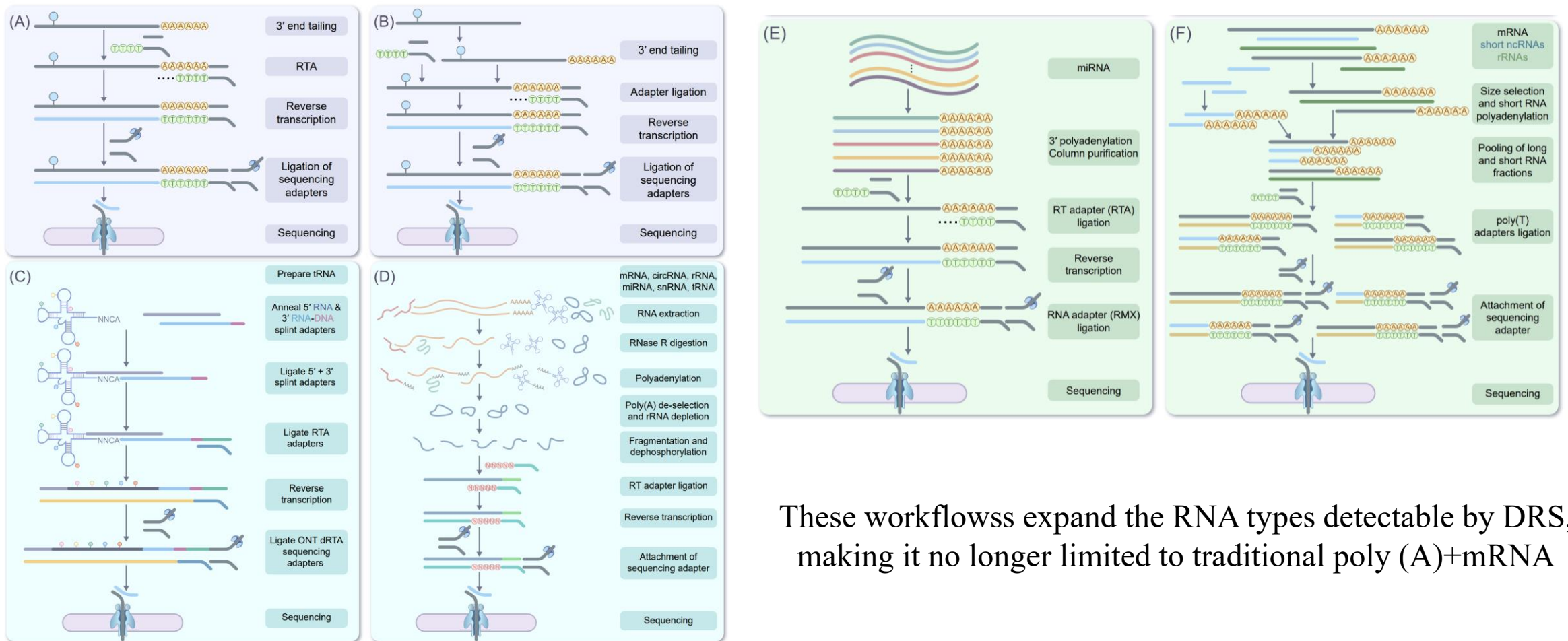


- Nanopore DRS can be used to analyze transcriptomes of viruses and pathogens, bacteria and archaea, plant development and environmental response, animal organ development, disease-related RNA regulation, and RNA drug quality control.
- Especially in the fields of viruses, cancer, plant stress response, and RNA therapy, DRS can provide natural RNA multi-layer information that is difficult to obtain with traditional methods.

Figure 4. Advantages and biological applications of DRS



Schematic workflows for DRS of diverse RNA classes



These workflows expand the RNA types detectable by DRS, making it no longer limited to traditional poly (A)+mRNA

Figure 5. Schematic workflows for DRS of diverse RNA classes

End-to-end computational workflow for Oxford Nanopore DRS data analysis and downstream applications

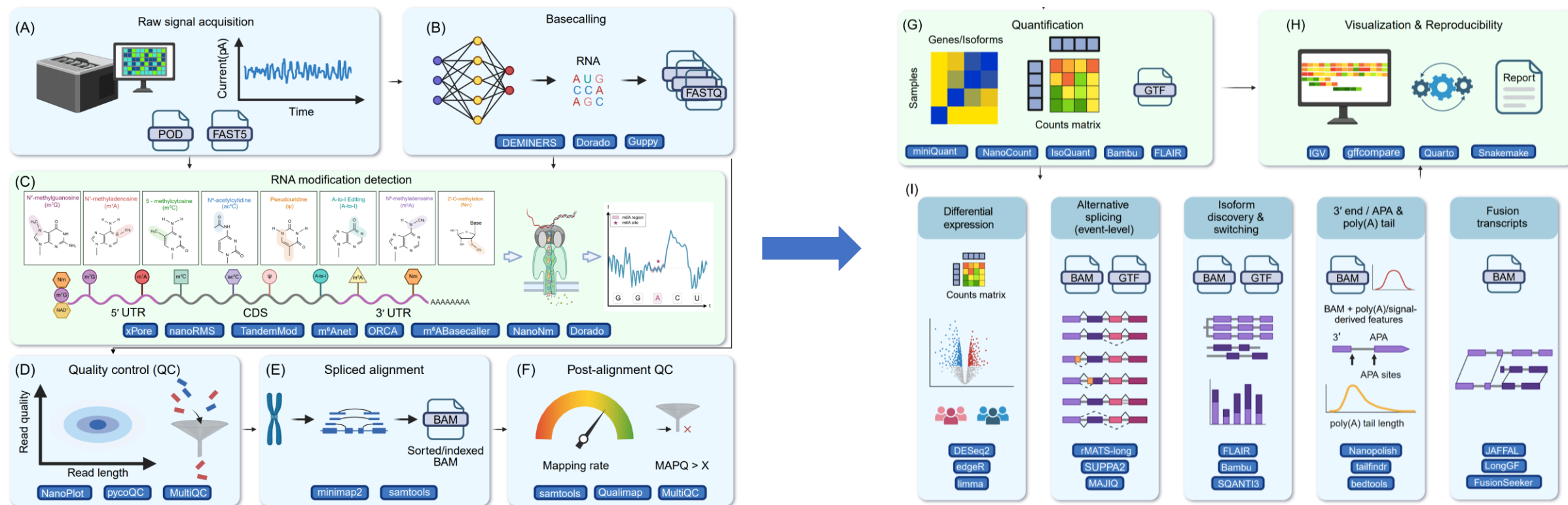
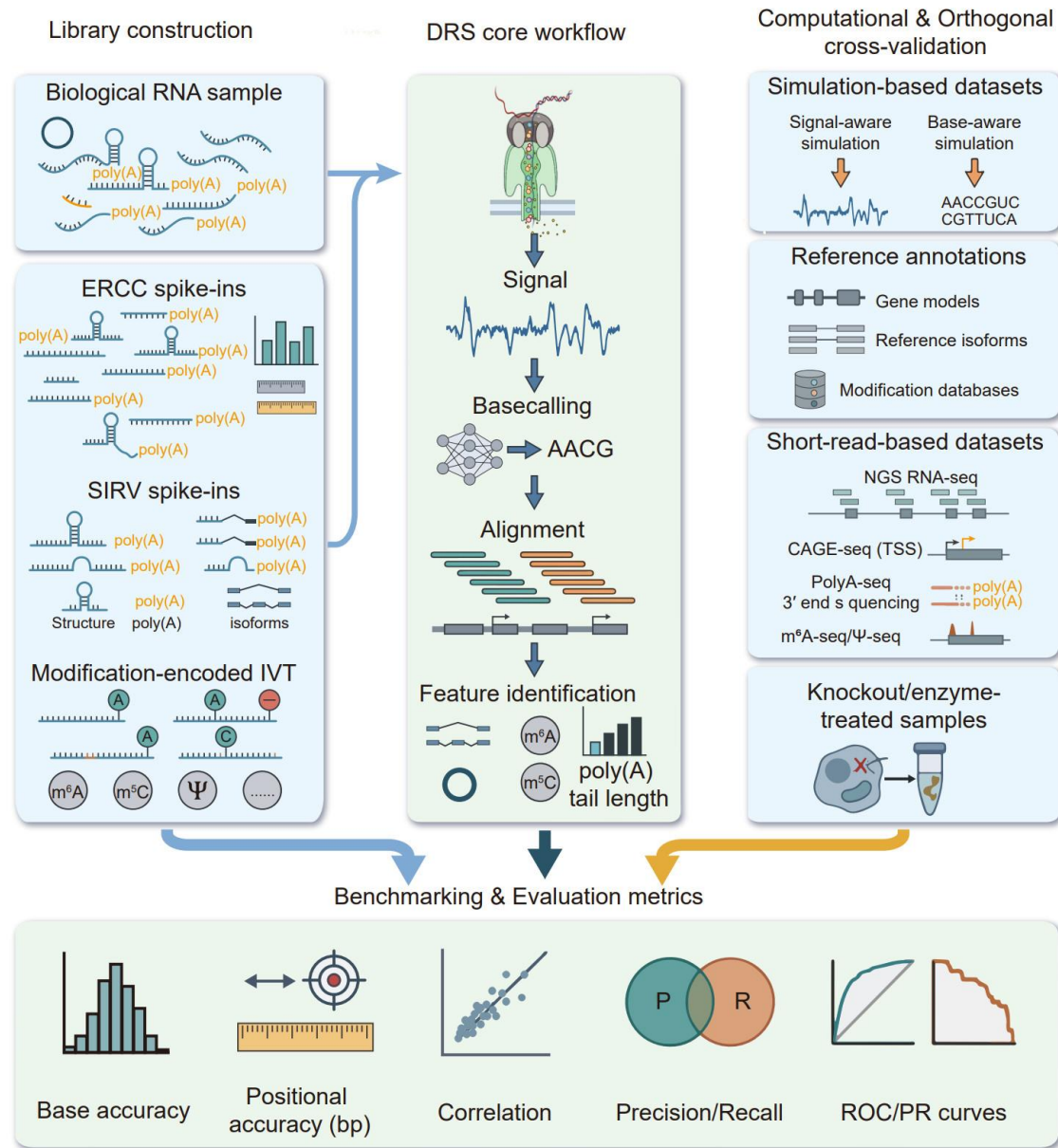


Figure 6. End-to-end computational workflow for DRS data analysis and downstream applications





Experimental design and multi-layer benchmarking framework for DRS

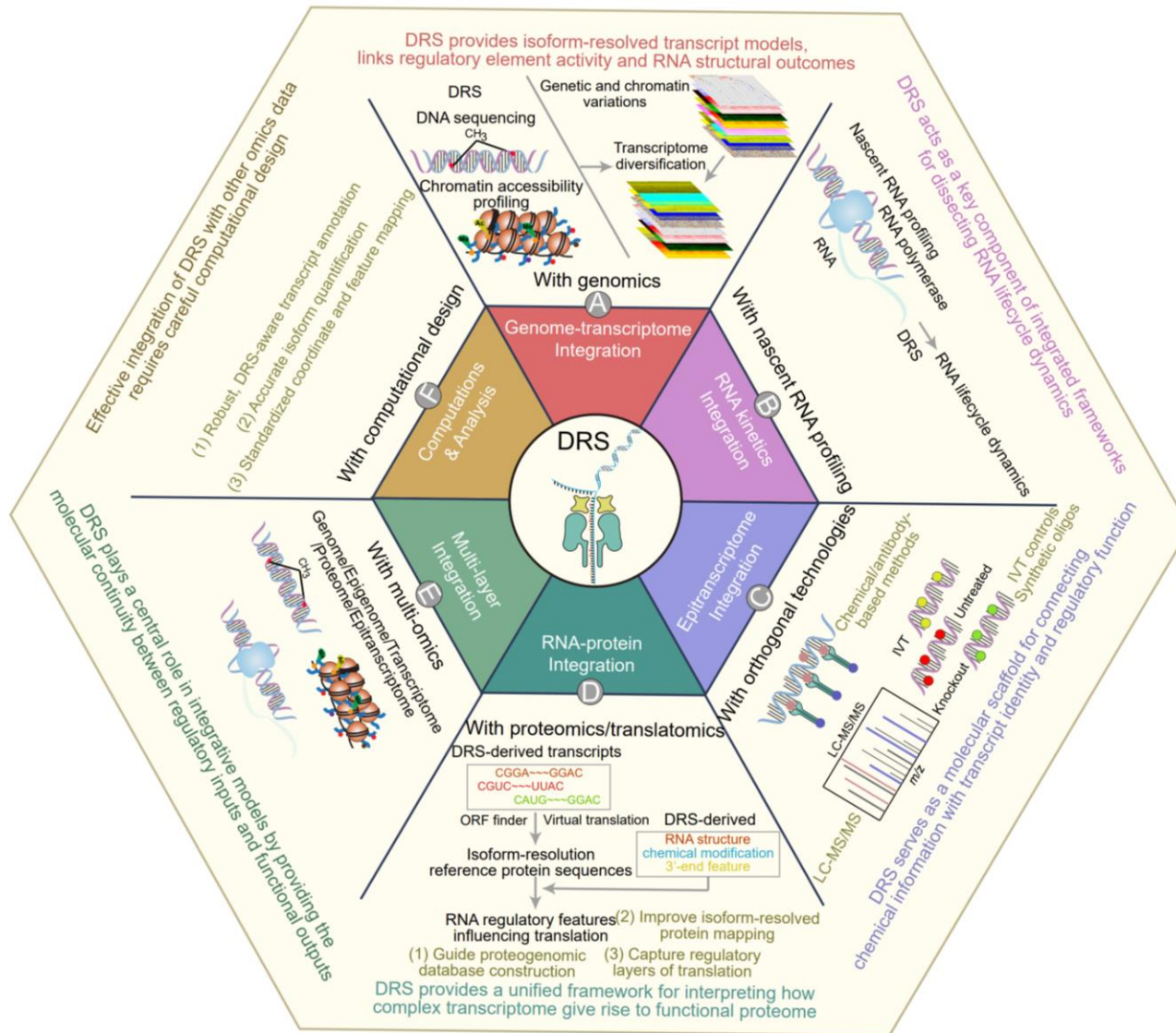


Standardization and validation are particularly crucial as DRS research spans multiple levels of analysis, including raw current signals, base recognition, alignment, transcript reconstruction, poly (A) tail estimation, and RNA modification detection. The article proposes that the DRS analysis results should be evaluated using multiple types of evidence, including ERCC, SIRV, modified coding IVT spike in, simulated data, reference annotations, short read length sequencing, 5' / 3' end sequencing, knockout or enzyme treated samples, etc. Different tasks should use different indicators, such as baseline accuracy, positional accuracy, correlation, precision/recall, ROC/PR curve, and consistency between replicates.

Figure 8. Experimental design and multi-layer benchmarking framework for DRS



Nanopore direct RNA sequencing as a central hub for multi-omics integration

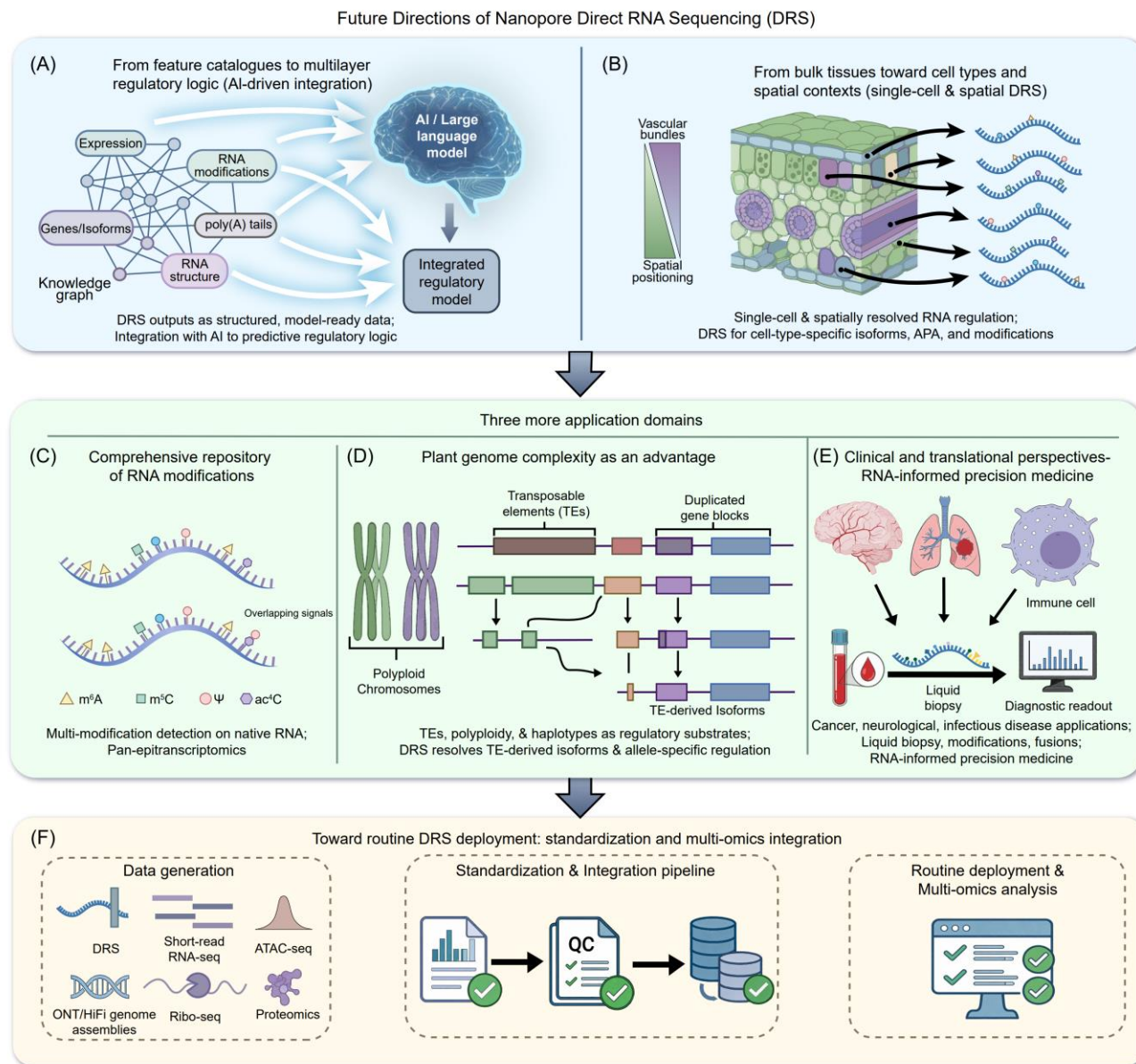


By integrating with DNA sequencing, ATAC-seq, short-read RNA-seq, metabolic labeling, LC-MS/MS, ribosome profiling, and proteomics, DRS is expected to help researchers trace regulatory information from genomic variation and chromatin states to RNA processing, RNA modifications, translational efficiency, and protein output, thereby enabling the construction of a more comprehensive RNA-centered regulatory network.

Figure 9. Nanopore direct RNA sequencing as a central hub for multi-omics integration



Outlook of nanopore DRS



DRS still faces several challenges, including high sample input requirements, insufficient detection of short RNAs, incomplete 5'-end coverage, relatively high sequencing error rates, model-dependent modification identification, and inadequate standardization. Its reliability therefore needs to be continuously improved through hardware optimization, algorithmic advances, the development of reference standards, and orthogonal validation.

Figure 10. Outlook of nanopore DRS applications



Online eBook and codes

Nanopore DRS guide

Search docs

Preface

Research Highlight

Introduction

Nanopore direct RNA sequencing: fundamental principles and developmental trajectory

Comparative analysis of DRS technology and traditional sequencing technology

The biological landscape from a DRS perspective

Typical application scenarios and breakthrough discoveries of DRS technology

Design, standardization and optimization of DRS workflows

DRS data analysis

Designing, validating, and benchmarking DRS: an integrated framework

Application-oriented integration of nanopore DRS with multi-omics

Challenges and technical improvements of DRS

Outlook: toward integrative, haplotype-

Home / Preface

Preface

RNA regulation is central to gene expression, encompassing RNA modifications, transcript architecture, alternative splicing, and poly(A) tail dynamics. Conventional sequencing depends on cDNA synthesis, which fragments RNA and uncouples these regulatory layers. In contrast, nanopore direct RNA sequencing (DRS) uniquely enables high throughput native RNA sequencing, preserving full length transcripts, RNA modifications, and poly(A) features simultaneously. Despite rapid advances, the field still lacks a comprehensive, unified review that connects technology, computation, and applications.

This supplement to the review "Nanopore Direct RNA Sequencing and the Epitranscriptome: Toward a Complete Map of RNA Regulation" is inspired by technical field guides that aim to make a rapidly evolving technology accessible and detail to further understanding of the exhibits.

Research Highlight

- Overview of nanopore direct RNA sequencing principles and evolution.

Published Examples of analytical visualizations commonly used in Nanopore Direct RNA sequencing (DRS) studies (Tianyuan Zhang)

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code 232.5 KB

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06.Sample-wise_Poly(A)_Le... 2.67 KB

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Bioinformatics Examples of analytical visualizations commonly used in Nanopore Direct RNA sequencing (DRS) studies

Tianyuan Zhang

Examples of analytical visualizations commonly used in Nanopore Direct RNA sequencing (DRS) studies. The code and data will continue to be updated in the future, which has helped researchers analyze DRS research data

nanopore direct RNA sequencing DRS RNA-seq epitranscriptome

Capsule

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Online e-book: https://zhangtianyuan666.github.io/DRS_doc

CodeOcean repository : <https://doi.org/10.24433/CO.3183037.v1>



Summary

- ❑ Nanopore direct RNA sequencing leverages the advantages of native RNA single-molecule long-read sequencing, driving transcriptomics from single-layer expression analysis toward multi-layer information integration.
- ❑ Direct RNA sequencing holds broad application potential in complex transcriptome analysis, disease feature discovery, and multi-omics integration.
- ❑ The reliable application of DRS still requires appropriate experimental design, well-established computational workflows, advanced analytical tools, evidence-aware interpretation, and multi-layer benchmarking and validation frameworks. In practical studies, DRS should be evaluated from three key perspectives: application context, confidence of results, and validation strategy.

Tianyuan Zhang, Jia Li, Chao Tang, You Wu, Hao wu, Xi-Tong Zhu, Ziyang Luo, et al. 2026. Nanopore direct RNA sequencing and the epitranscriptome: Advances in mapping native RNA landscapes. *iMeta* 5: e70136.

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