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

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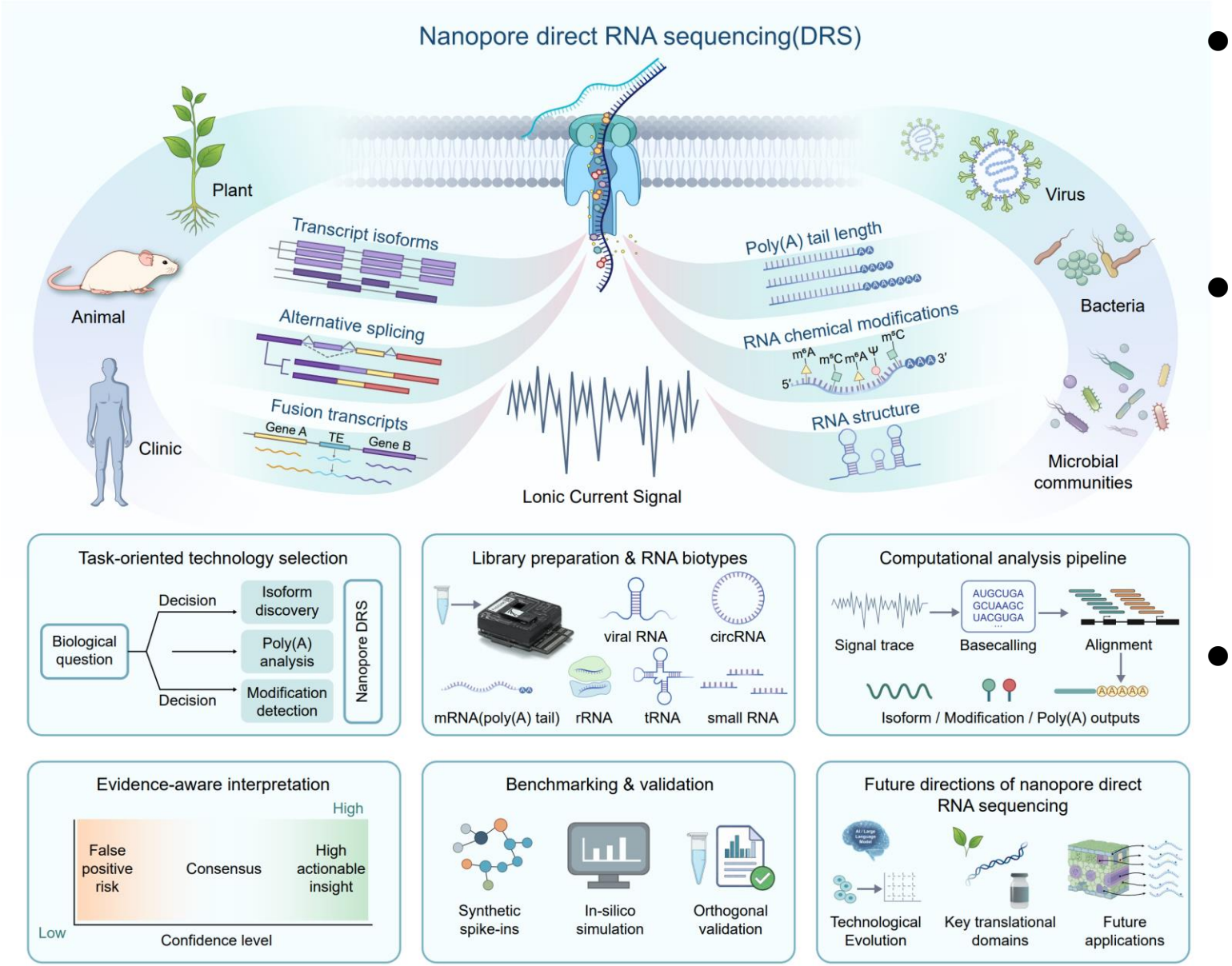
# Overview

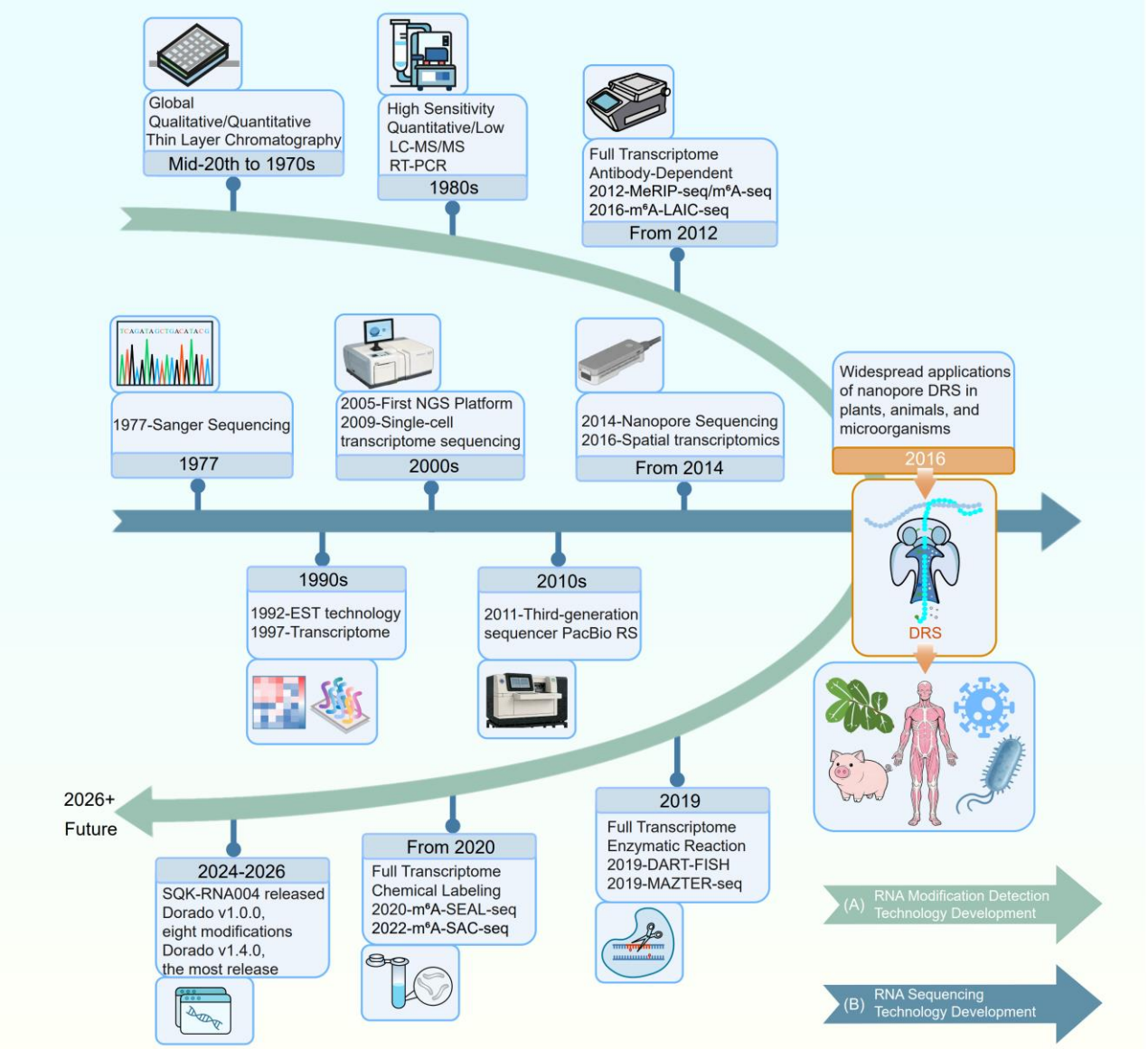



- Direct RNA sequencing (DRS) is a transformative RNA technology that has emerged over the past decade, leveraging long-read nanopore platforms to directly sequence native RNA molecules.
- This review targets global researchers in RNA biology and RNA modification. By summarizing over 700 DRS studies 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.

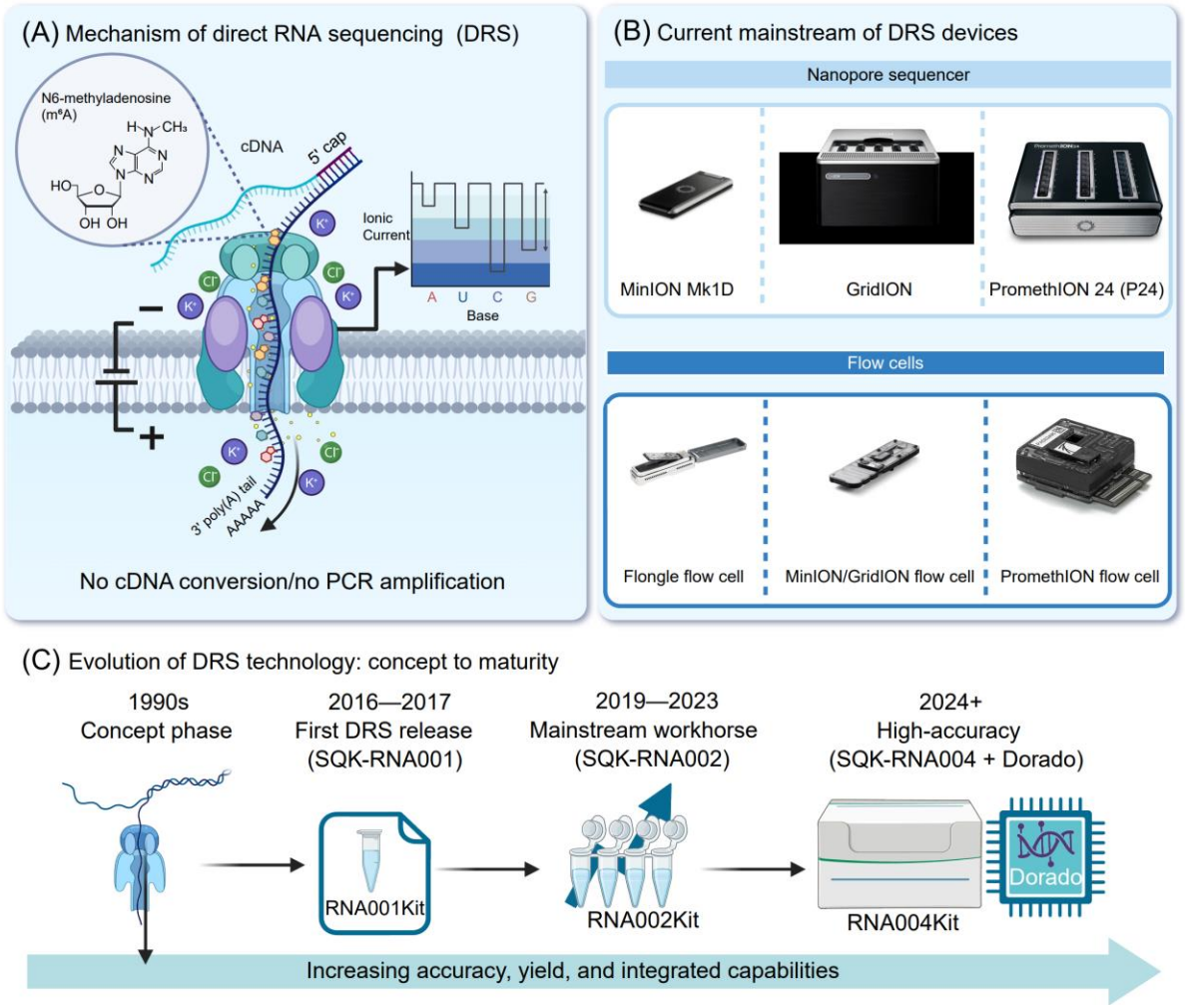
# The Evolution of Nanopore Direct RNA Sequencing




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

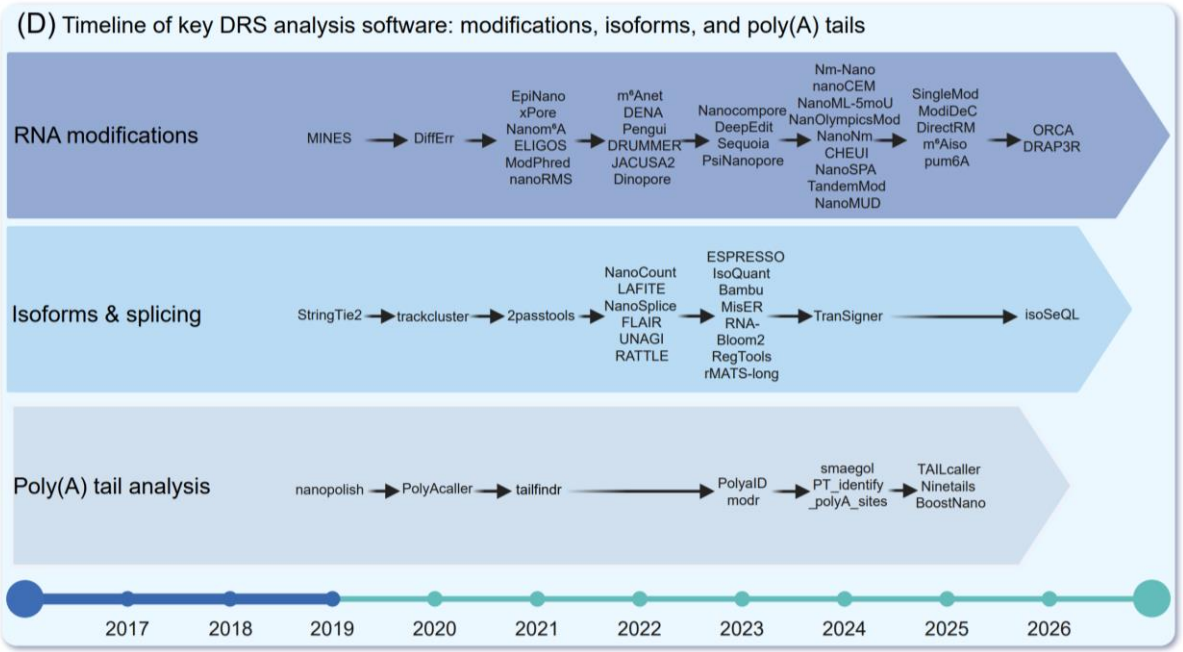
Figure 1. Timeline of major innovations in RNA sequencing and RNA modification technologies

# 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

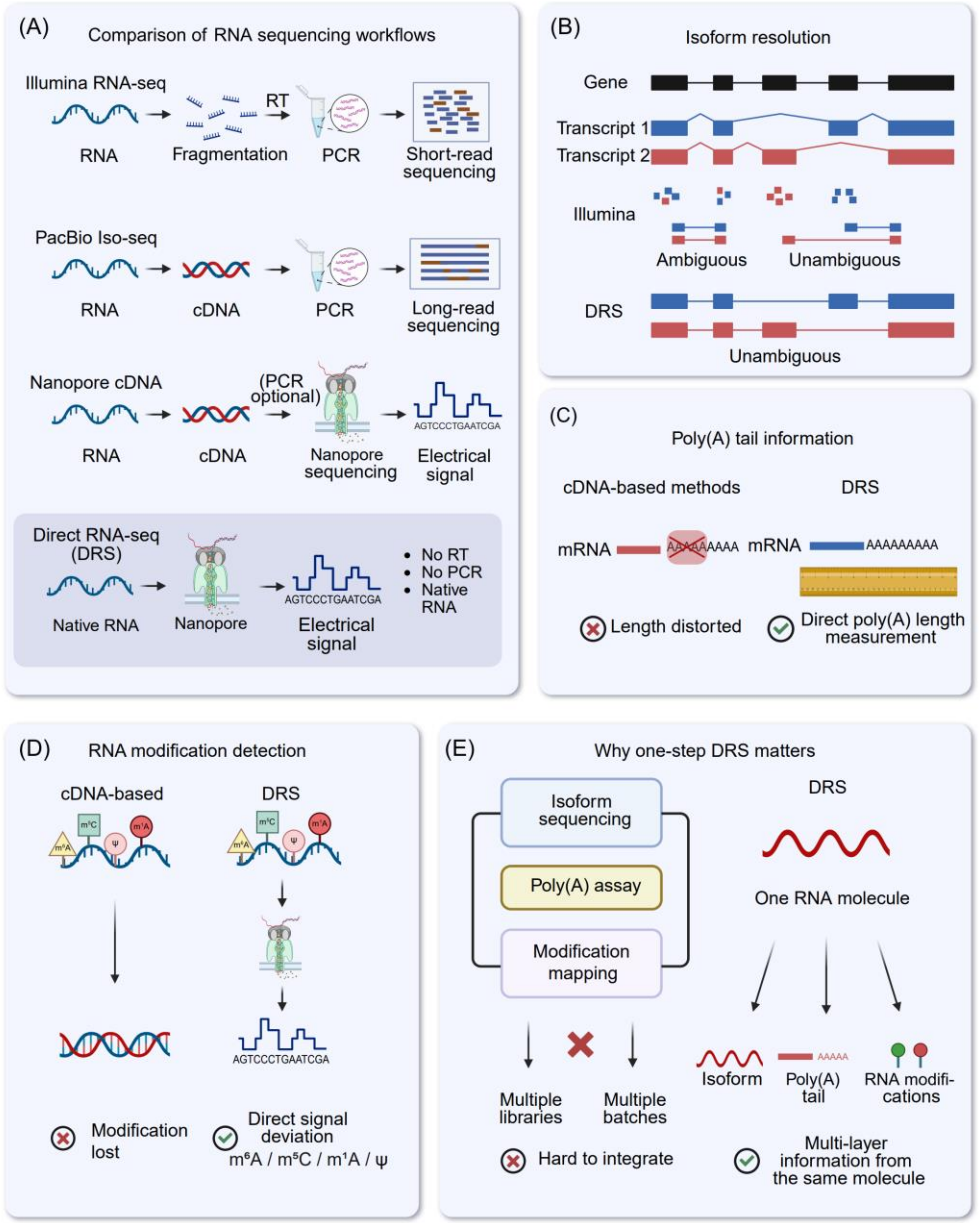


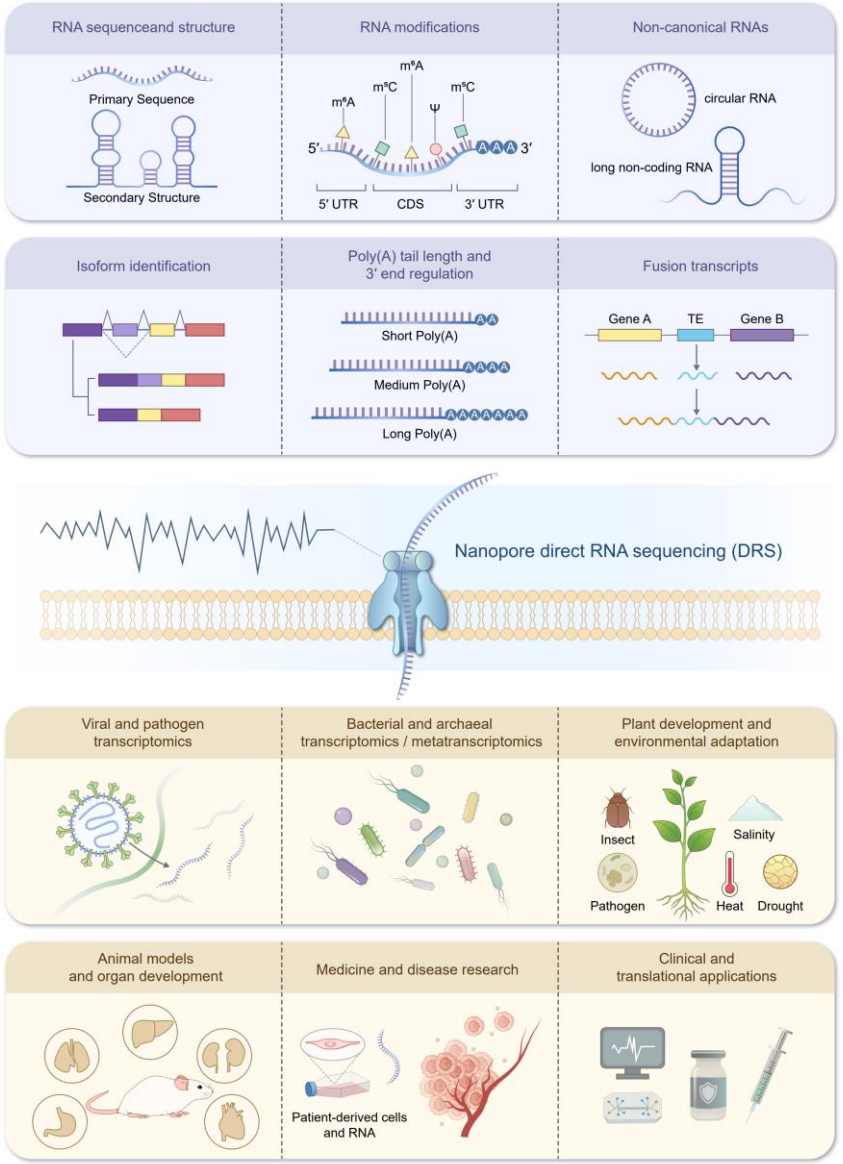

Table Scenario-oriented comparison of nanopore DRS and other RNA sequencing approaches.

| 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

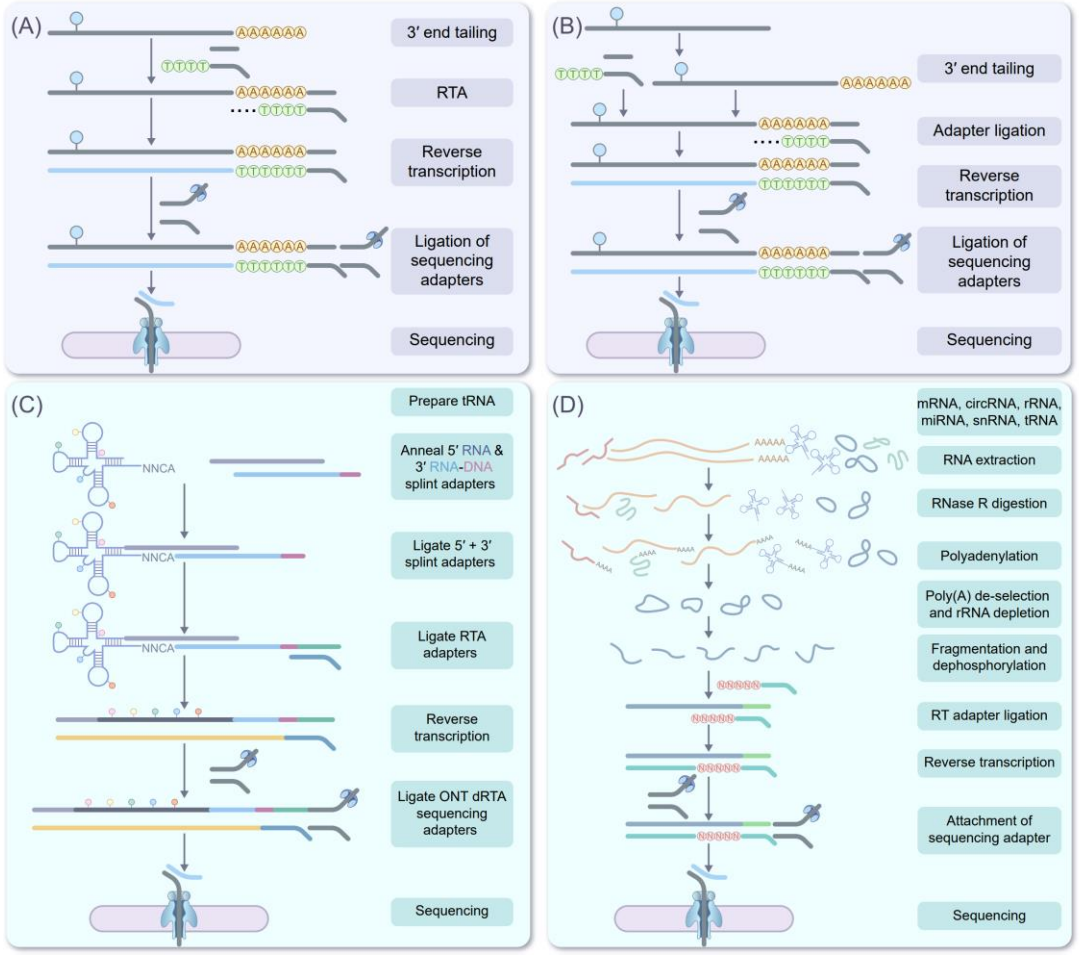
# Advantages and biological applications of DRS

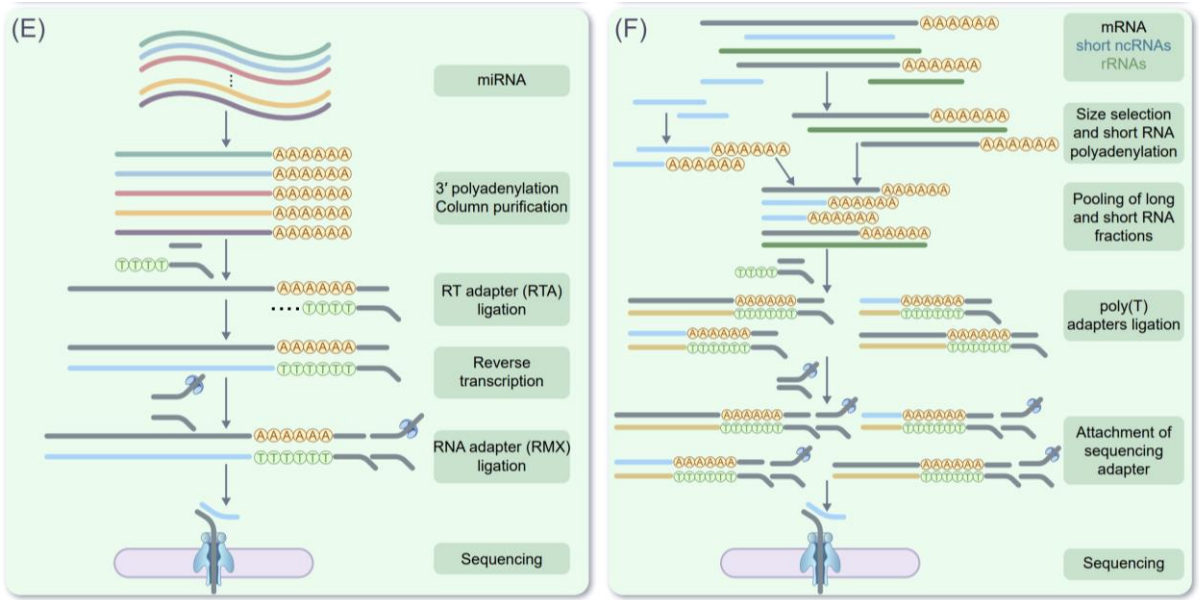



- Nanopore DRS can be used to analyze transcriptomes of viruses and pathogens, bacteria and archaea, plant development and environmental response, animal organ development, disease-related RNA regulation, and RNA drug quality control.
- Especially in the fields of viruses, cancer, plant stress response, 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 workflowss 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

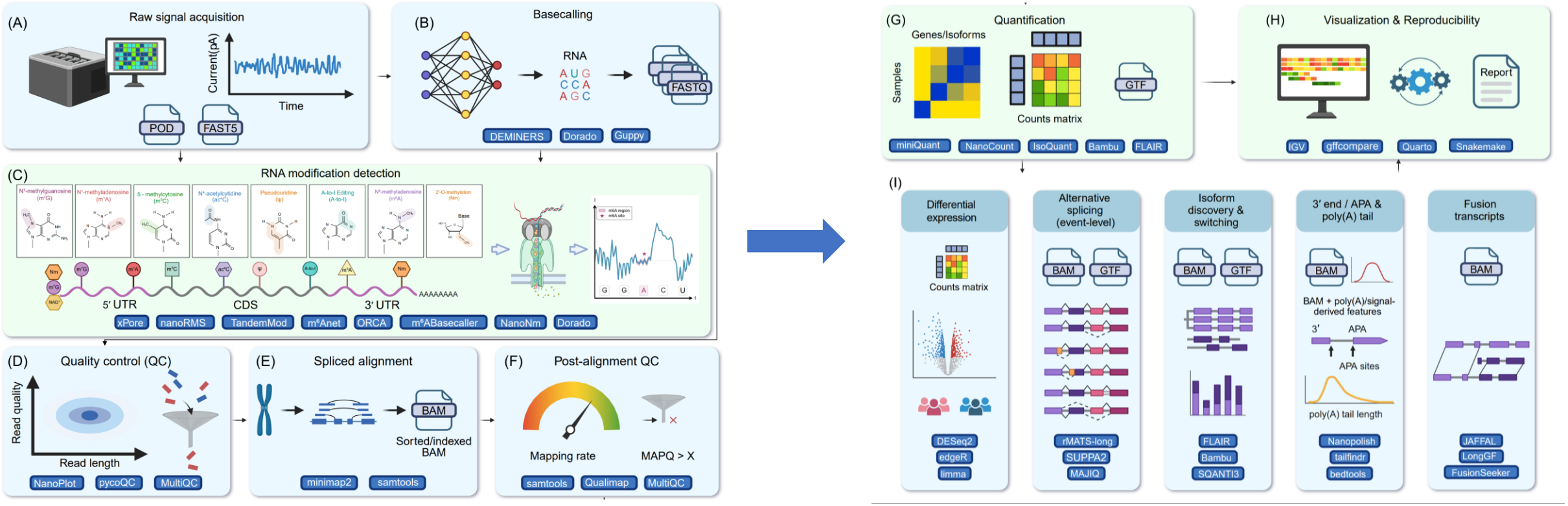



Figure 6. End-to-end computational workflow for DRS data analysis and downstream applications

# Representative high-level analytical visualizations commonly used in DRS studies

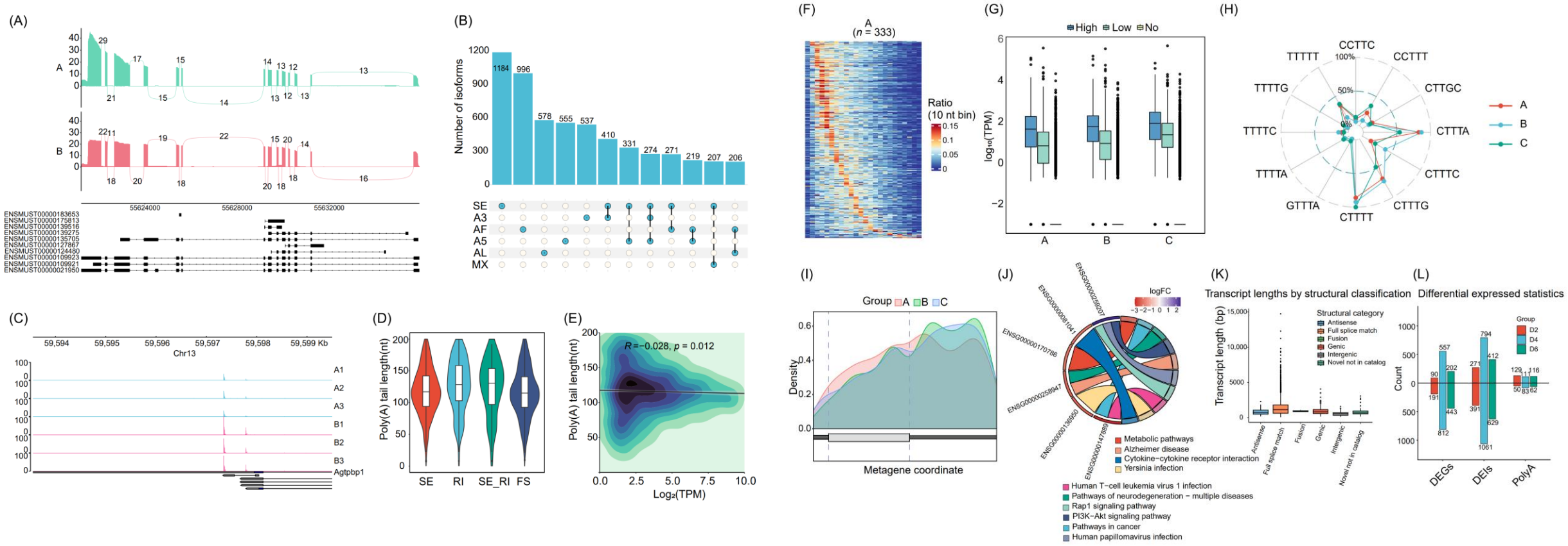
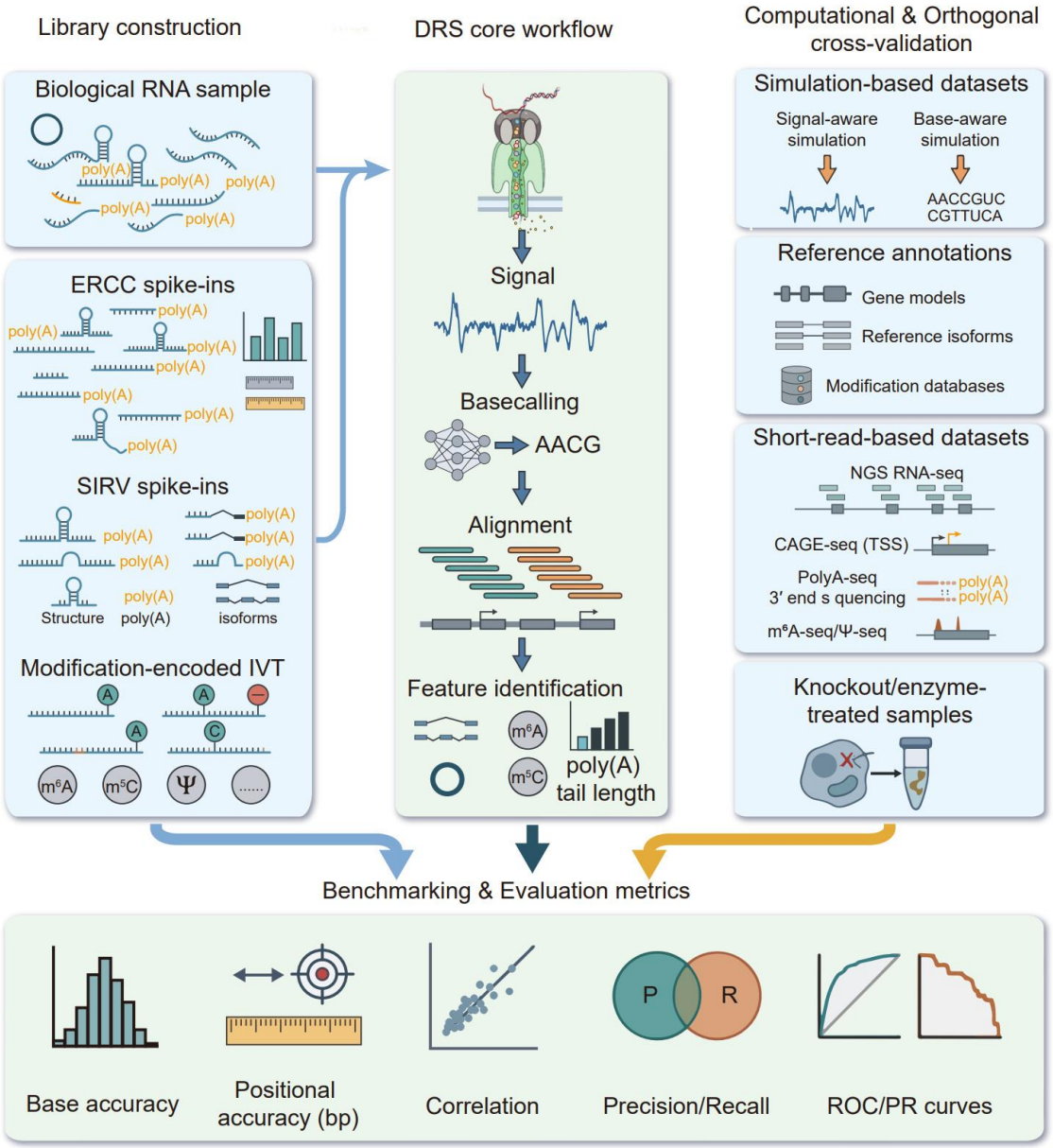



Figure 7. Examples of analytical visualizations commonly used in DRS studies

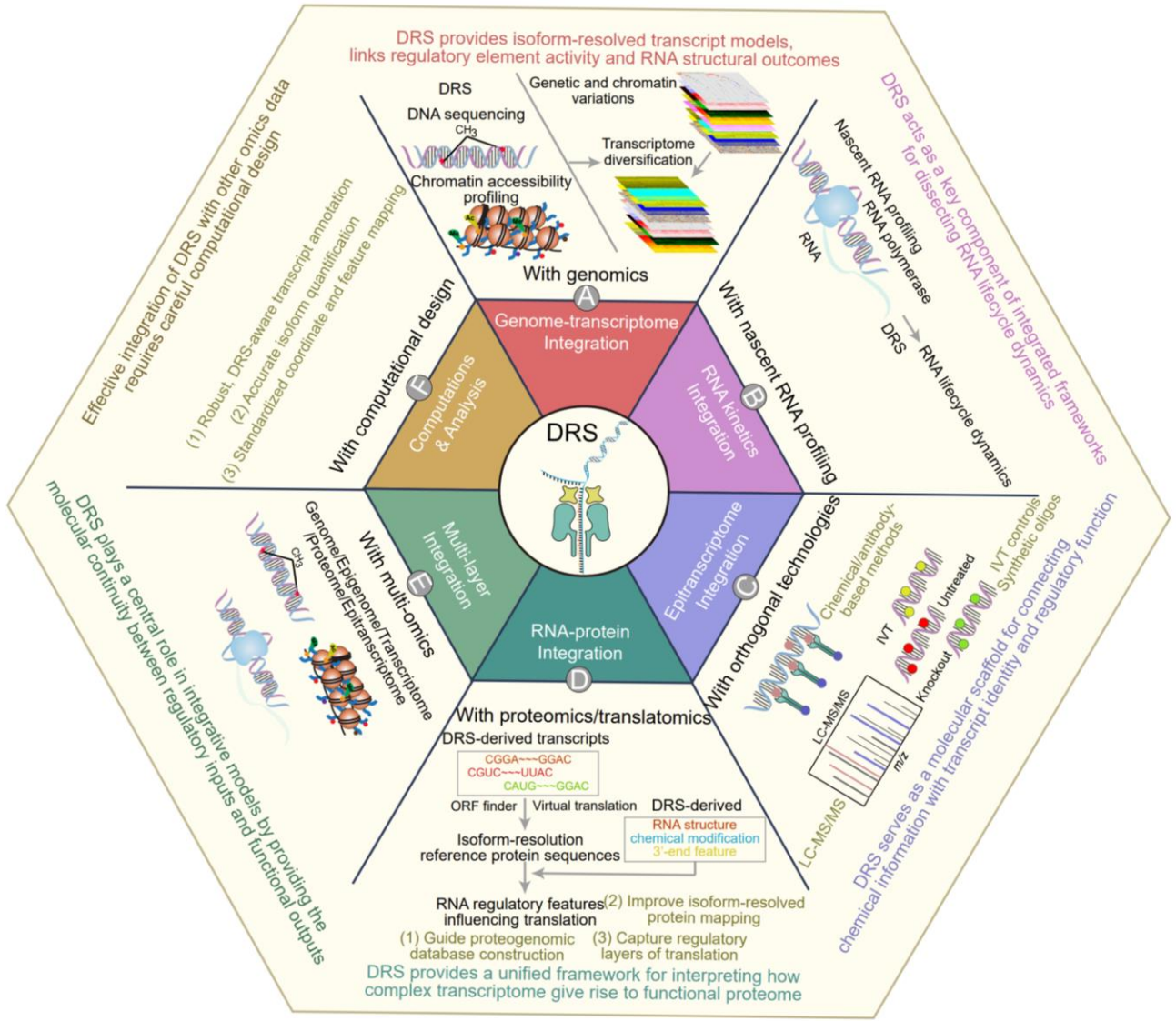
# Experimental design and multi-layer benchmarking framework for DRS




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

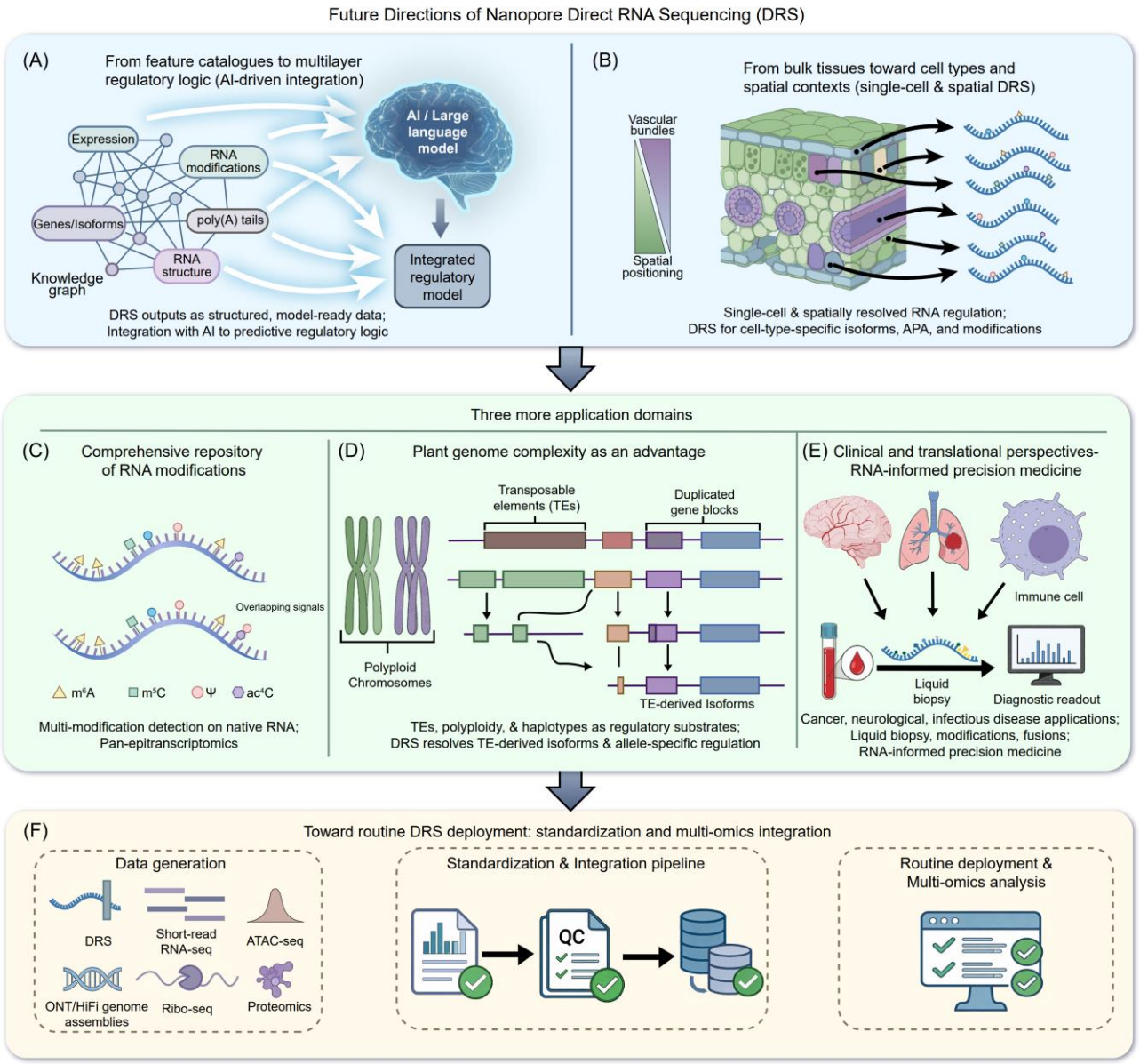
# Nanopore direct RNA sequencing as a central hub for multi-omics integration




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

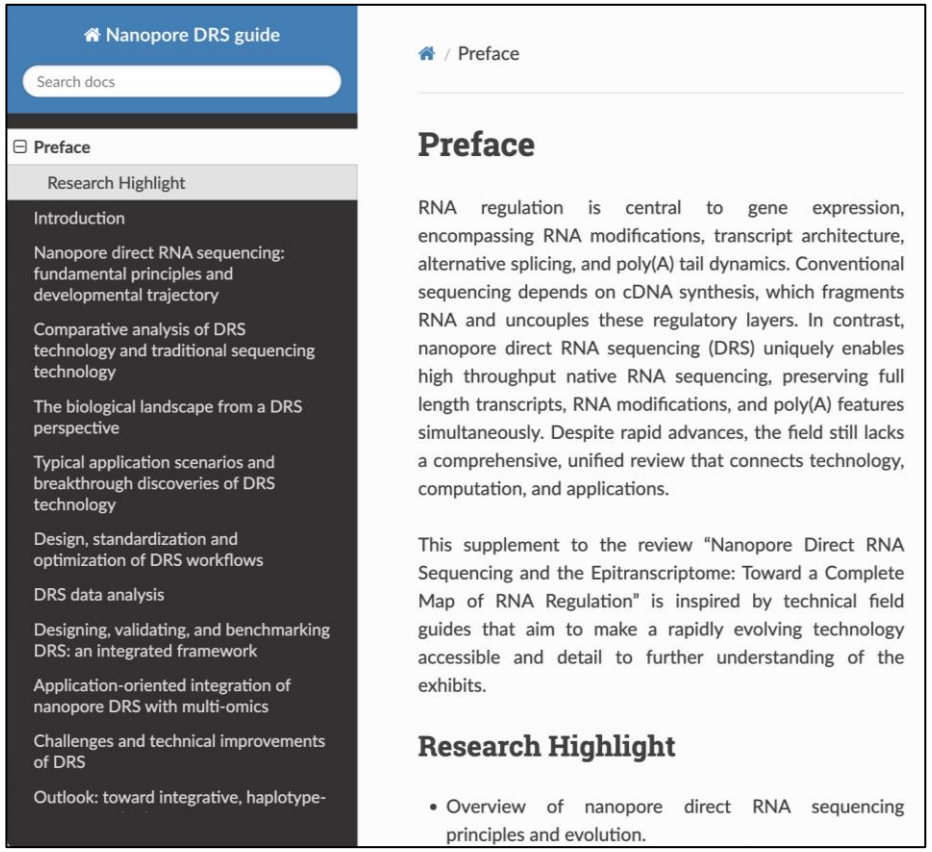
# Outlook of nanopore DRS

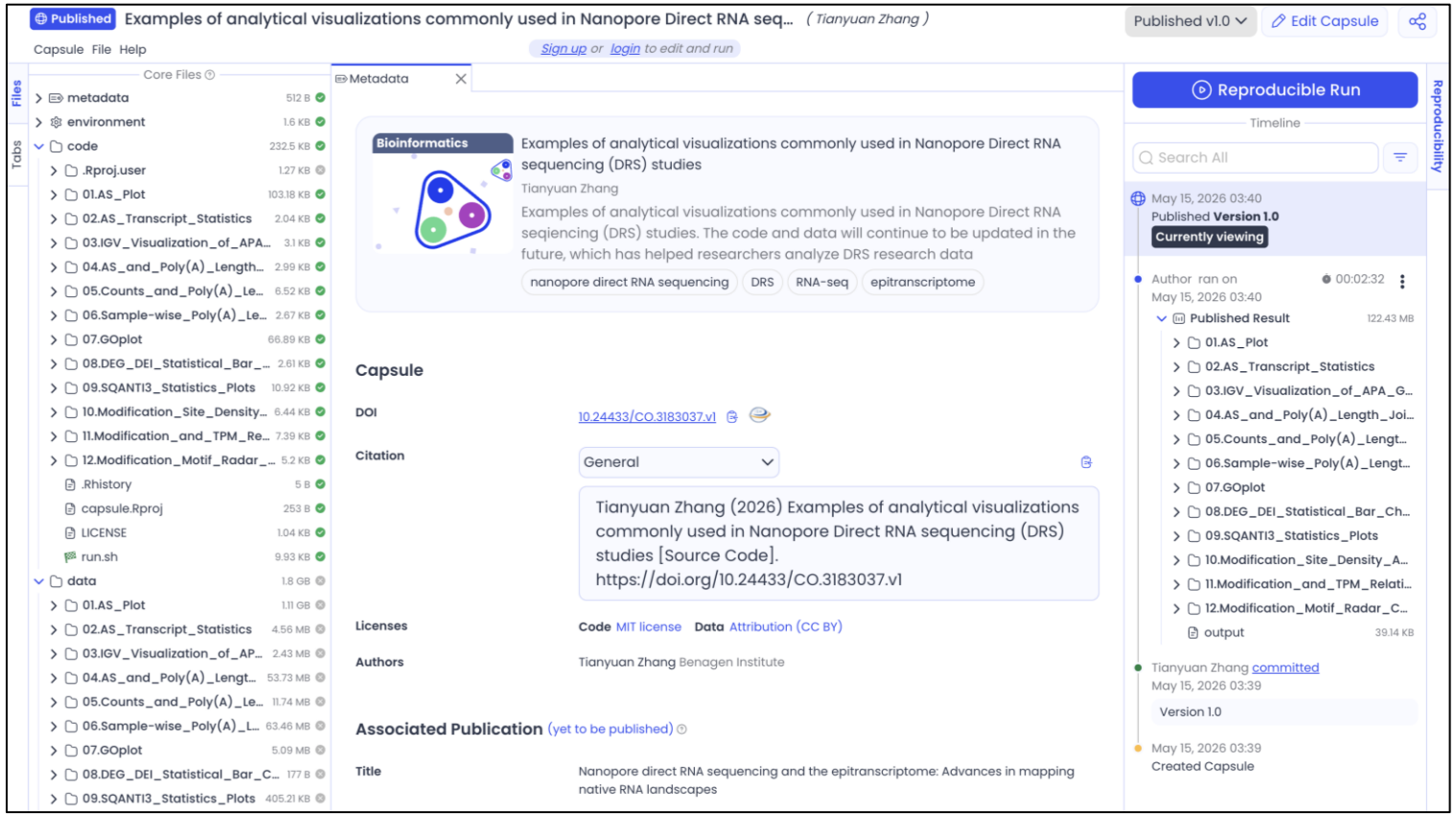



DRS still faces several challenges, including high sample input requirements, insufficient detection of short RNAs, incomplete 5′-end coverage, relatively high sequencing error rates, model-dependent modification identification, and inadequate standardization. Its reliability therefore needs to be continuously 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




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.

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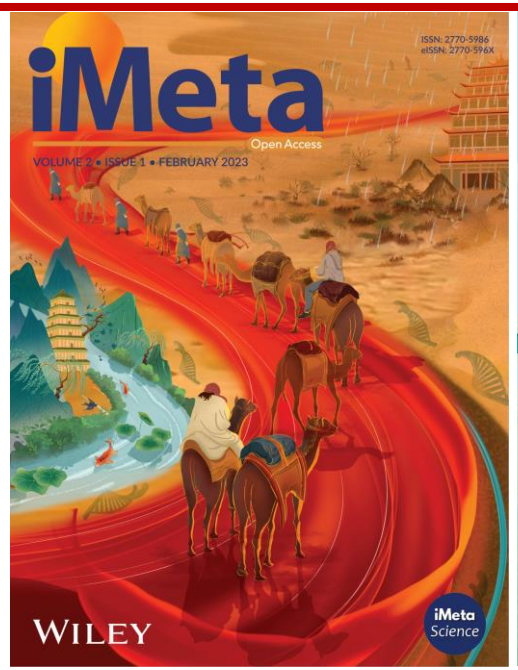


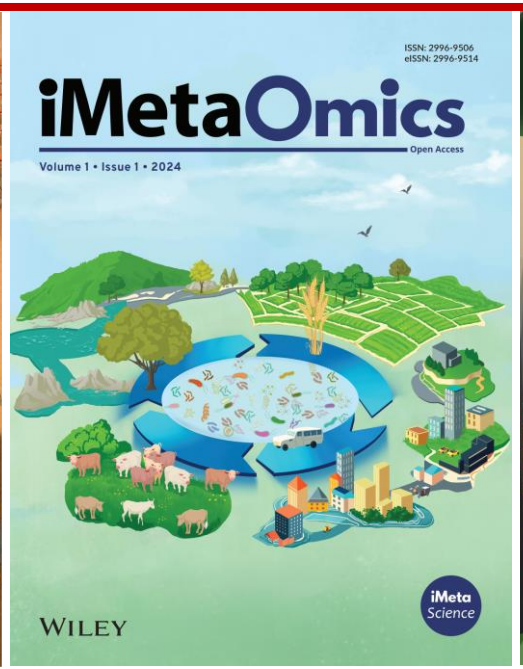





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