TRAINING COURSE IN

Computational Methods for Epitranscriptomics

Bari, 26th-28th April 2023

Overview of transcriptomics and epitranscriptomics

General overview on state-of-the-art approaches in transcriptome research

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Credits: Tommaso Leonardi & Luca Pandolfini

TRANSCRIPTOMICS IN A NUTSHELL

Transcriptomics is the study of the 'transcriptome,' a term now widely understood to mean the complete set of all the ribonucleic acid (RNA) molecules (transcripts) expressed in some given entity, such as a cell, tissue, or organism.

Applications for Transcriptomics:

Study gene expression and regulation (DEGs)	**Identify novel transcripts (de novo)**
Investigate RNA modifications	**Uncover alternative splicing events**

MULTIMODAL approach

In recent years, advances in sequencing technology, bioinformatics, and computational tools have revolutionized the field of transcriptomics.

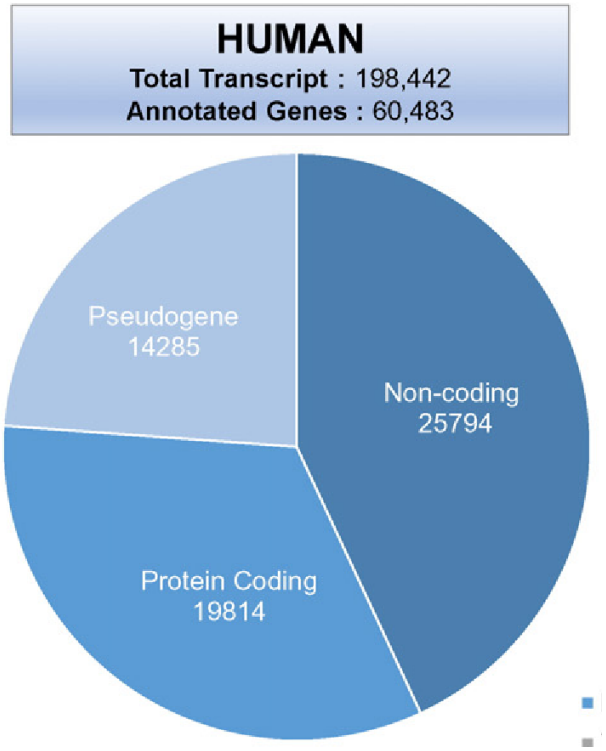
David Skerrett-Byrne Anthony, et al. Transcriptomics.
In: Encyclopedia of Cell Biology (Second Edition), 2023:363-371.

DECIPHERING TRANSCRIPTOME COMPLEXITY

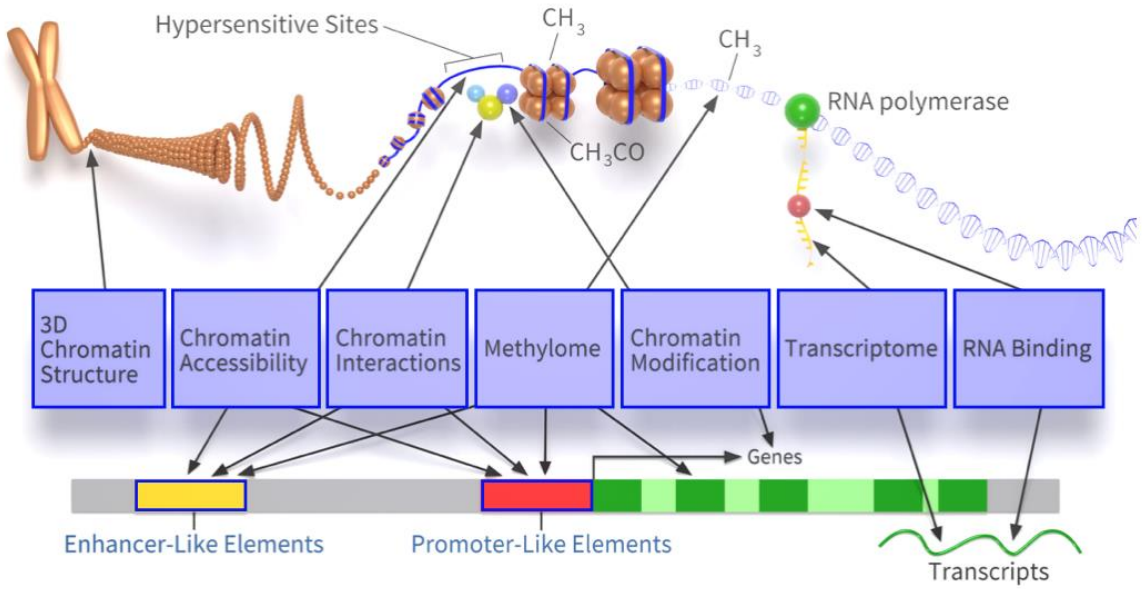
Large Scale **Consortia** and tailored **NGS approaches** helped highlight functions and regulation of the human genome

>> ***ENCODE*** - *Encyclopedia of DNA Elements*
https://www.encodeproject.org

>> ***FANTOM*** - *Functional Annotation of Mammalian Genome*
https://fantom.gsc.riken.jp



DATABASE: GENCODE

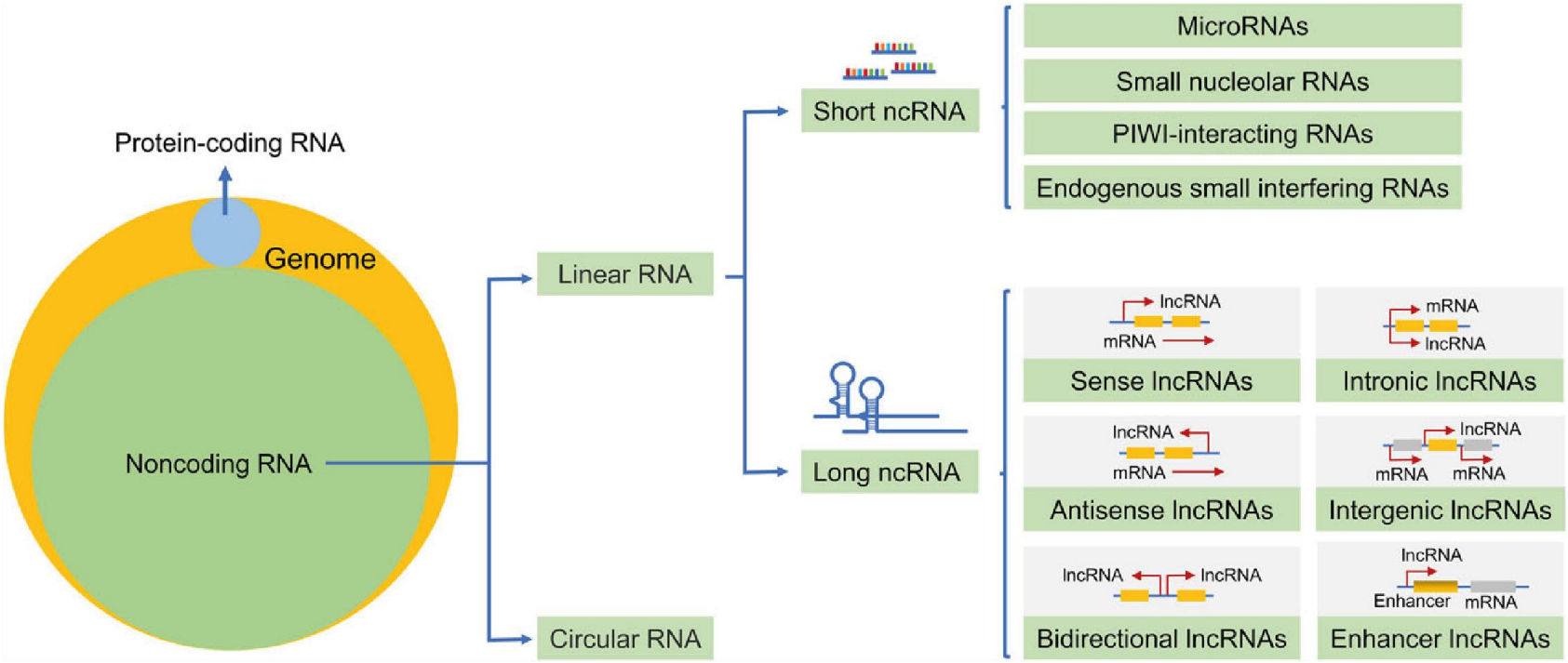


Non-coding DNA elements
promoters, enhancers, insulators

Coding and Non-coding transcripts
small or long by size

DON'T JUST CALL IT «RNA»: *MIND THE BIOTYPES!*

Technological advances have unravelled the remarkable complexity of RNA species



Many biotypes, many functions

Sophisticated regulatory system for gene expression

- Coordination of expression
- Spatio-temporal regulation
- Precision of gene dosage

Zhang et al., Journal of Experimental & Clinical Cancer Research, 2020

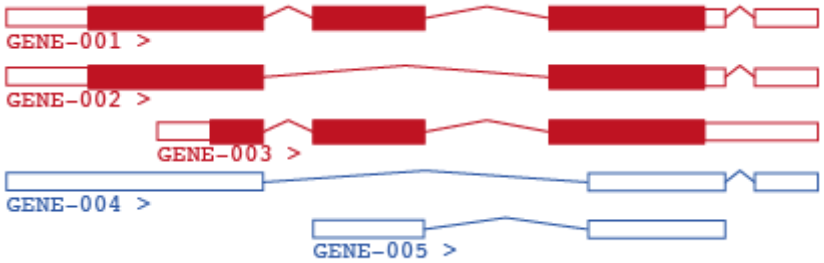
Li J and Liu C (2019). *Front. Genet.* 10:496

TRANSCRIPTOME REPOSITORIES

GENCODE

- **ENCODE** project
- **>> highly curated**
- 58721 human genes (206,694 transcripts
  - Protein coding 19,940 >> 83,124 RNA
  - Long ncRNAs 16,066 >> 29,566 RNA
  - Small ncRNAs 7,577
- RNA biotype >> transcript origin, processing type, localization or or coding potential

Gene and transcript variants



** five transcripts, some **coding (red)** and **non-coding (blue)***

ENSEMBL

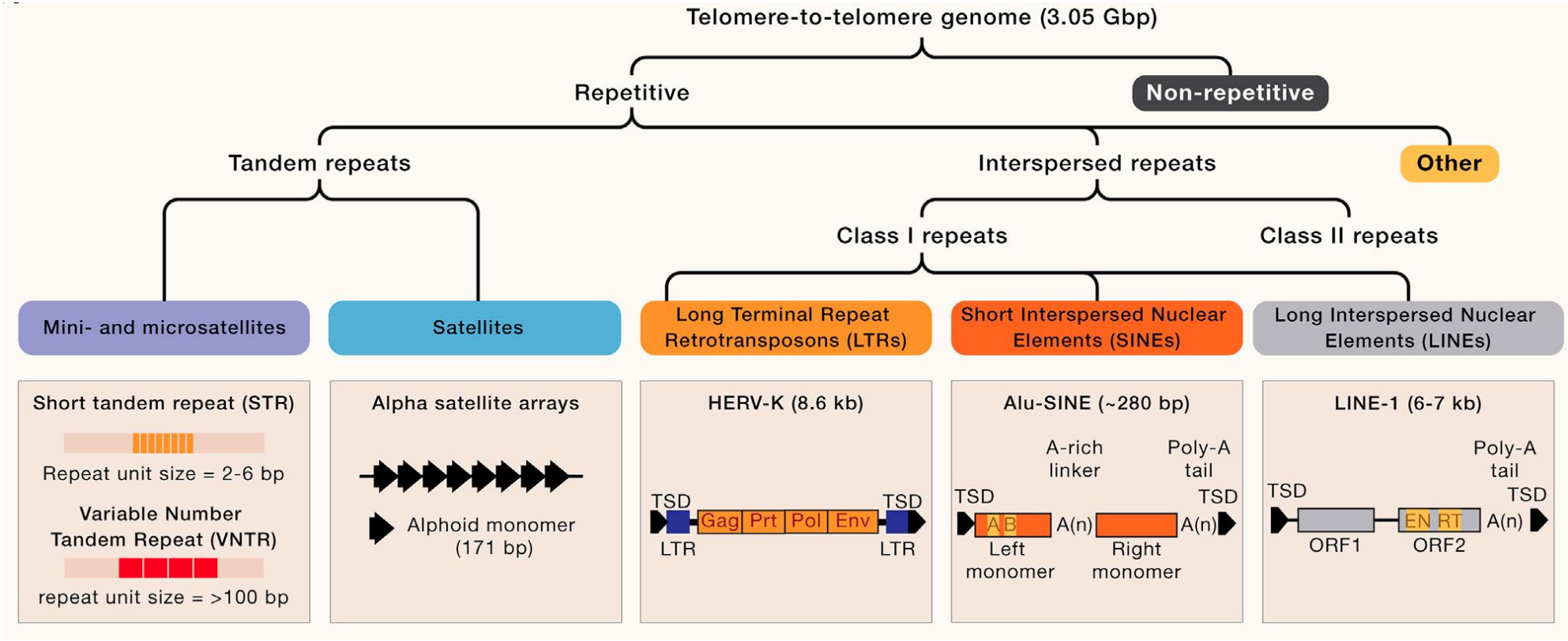
- part of the **ENCODE** project
- **HAVANA**= manually curated annotation (high trust)
- **Transcript Support Level** (TSL) is a method to highlight the well-supported vs. poorly-supported transcript
- **Gene** classification:
  - Coding (with >1 ORF)
  - Processed transcript (lncRNA, ncRNA, pseudogenes)
  - IG and TR Genes (variants)
  - TEC (to be experimentally confirmed)
- **Transcript classification**

Types of ncRNA

Abbreviation	Definition
tRNA	transfer RNA
Mt-tRNA	transfer RNA located in the mitochondrial g
rRNA	ribosomal RNA
scRNA	small cytoplasmic RNA
snRNA	small nuclear RNA
snoRNA	small nucleolar RNA
miRNA	microRNA precursors
misc_RNA	miscellaneous other RNA
lincRNA	Long intergenic non-coding RNAs

COMPLEXITY OF TRANSCRIPTOME: REPETITIVE ELEMENTS

The non-coding landscape is further expanded by multiple DNA repetitive elements that are transcribed into non-coding RNAs.



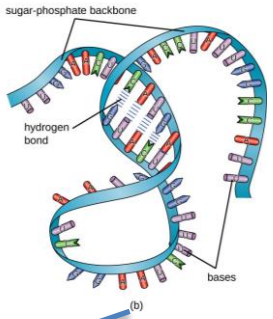
Haws SA., Cell. 2022

From a computational perspective, repeats create ambiguities in alignment and assembly, which, in turn, can produce biases and errors when interpreting results.

Treangen, T., Salzberg, S. *Nat Rev Genet* **13**, 36-46 (2012)

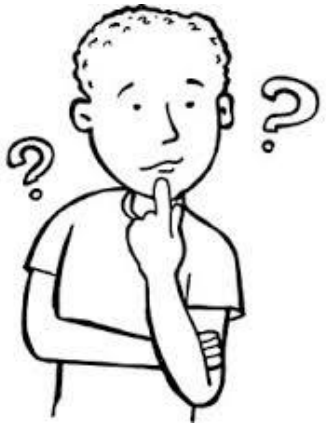
TO CODE *OR* NOT TO CODE?

BEFORE

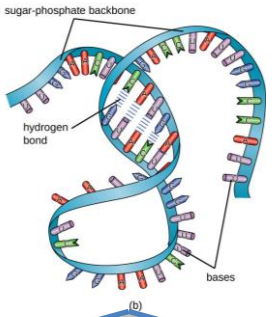


coding

Functional



NOW



coding

Functional

non-coding

Really functional?

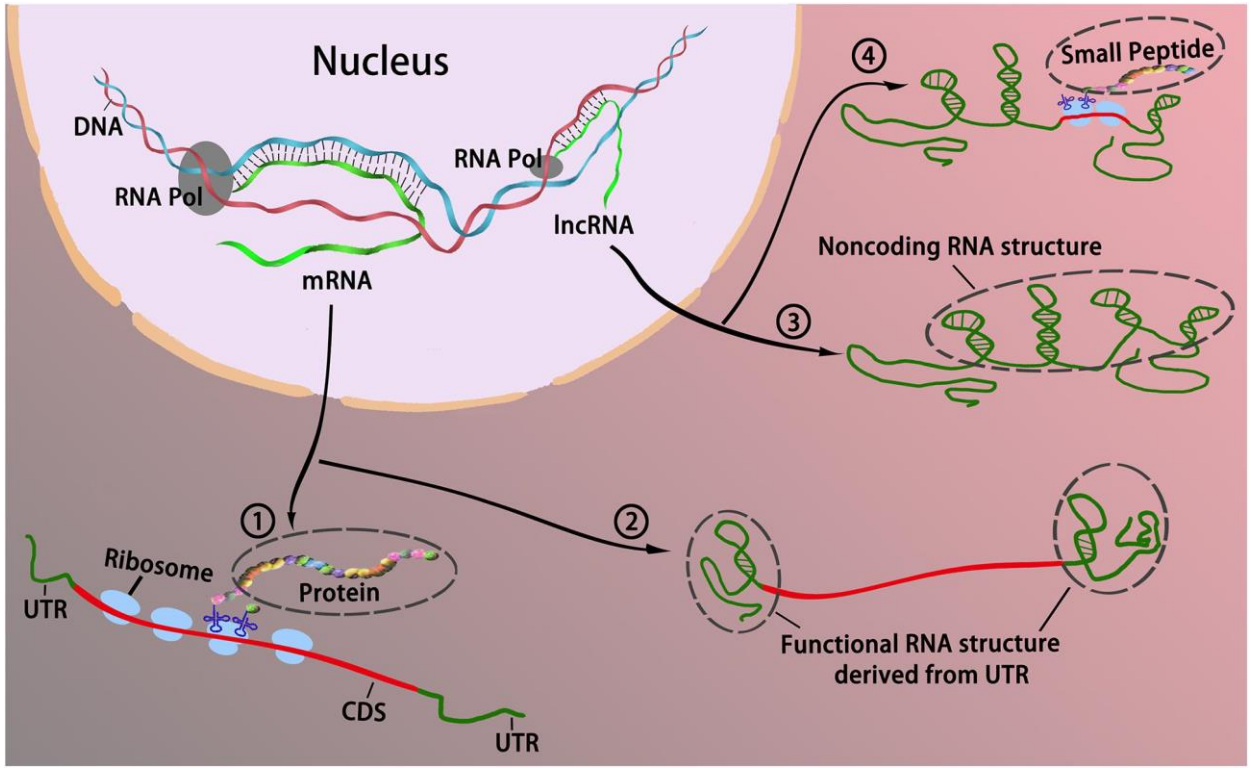
Coding vs Non-coding: biology doesn't care !!

DON'T RELY TOO MUCH ON DEFINITONS!

We should care on what is functional and on what has a role in a phenotype/process control (it depends on the context, too)

TO CODE *OR* NOT TO CODE? BIFUNCTIONAL RNAs

RNA molecules may possess both coding and non-coding functions, evolved to diversify phenotypes or produce convergent effects.



Li et al. Frontiers in Genetics 2019; doi.org/10.3389/fgene.2019.00496

Small ORFs into ncRNAs

Functional peptides

ARTICLE

https://doi.org/10.1038/s41467-019-09754-1 OPEN

LncRNA EPR controls epithelial proliferation by coordinating *Cdkn1a* transcription and mRNA decay response to TGF-β

Martina Rossi[1,2], Gabriele Bucci[3], Dario Rizzotto[4], Domenico Bordo[1], Matteo J. Marzi[5], Margherita Puppo[1,2], Arielle Flinois[6], Domenica Spadaro[6], Sandra Citi[6], Laura Emionite[7], Michele Cilli[7], Francesco Nicassio[5], Alberto Inga[4], Paola Briata[1] & Roberto Gherzi[1]

Non-coding functions into mRNAs

5' UTRs or 3'UTRs

circRNAs

ARTICLE

DOI: 10.1038/s41467-018-05182-9 OPEN

Endogenous transcripts control miRNA levels and activity in mammalian cells by target-directed miRNA degradation

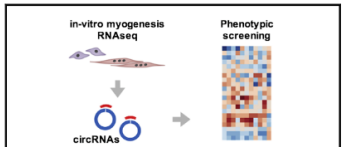
Francesco Ghini[1], Carmela Rubolino[1], Montserrat Climent[1], Ines Simeone[1], Matteo J. Marzi[1] & Francesco Nicassio[1]

Article

Molecular Cell

Circ-ZNF609 Is a Circular RNA that Can Be Translated and Functions in Myogenesis

Graphical Abstract



Authors

Ivano Legnini, Gaia Di Timoteo, Francesca Rossi, ..., Pietro Laneve, Nikolaus Rajewsky, Irene Bozzoni

Correspondence

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In Brief

Legnini et al. identified circ-ZNF609, a

THE TECHNOLOGY – THE STORY SO FAR

In the past decades, several sequencing technologies have entered the research practice, generating new opportunities and challenges

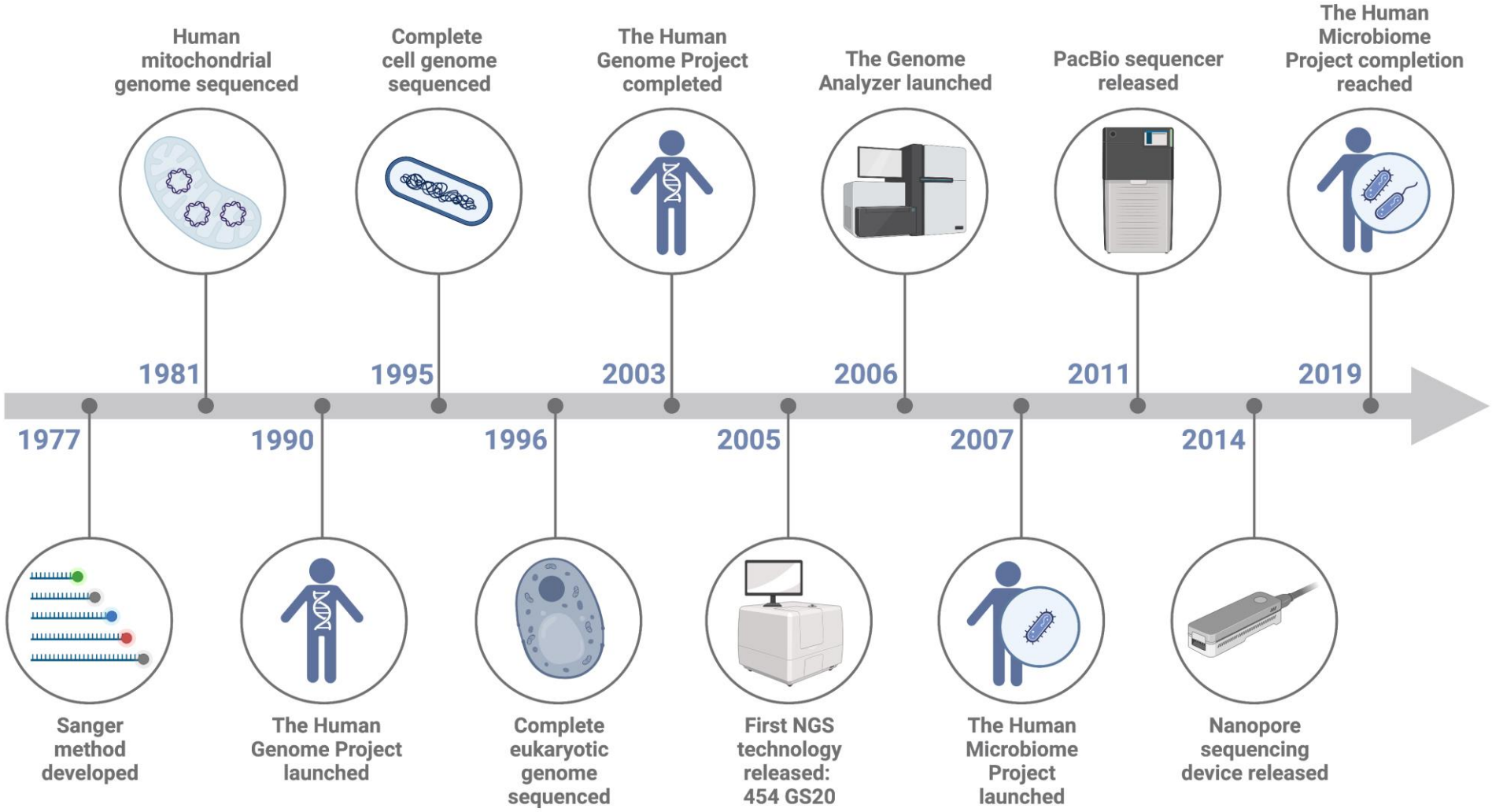
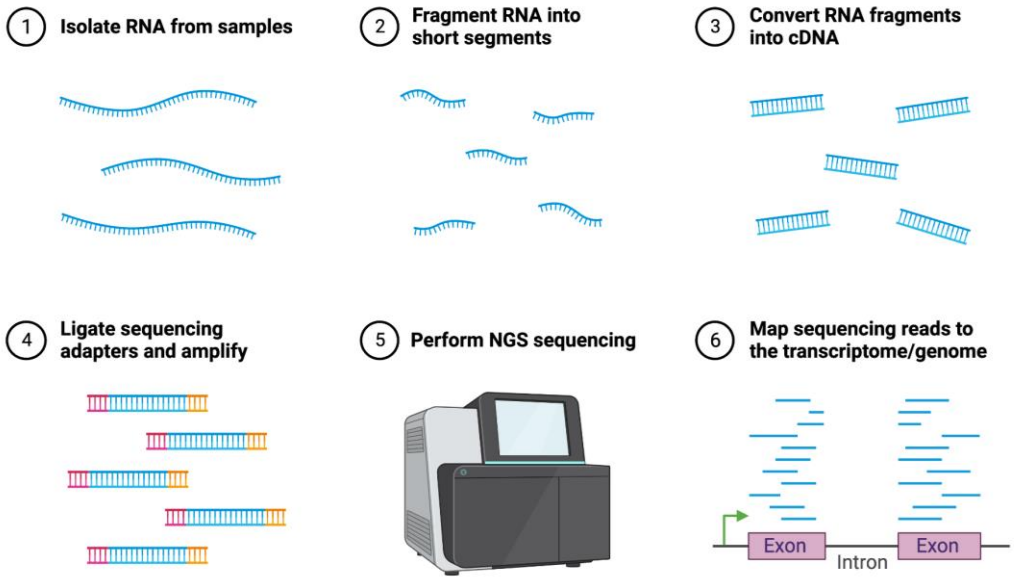


Image from Biorender

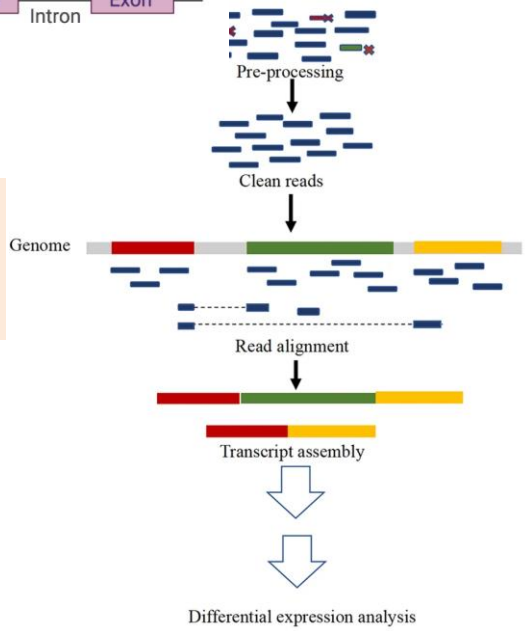
ILLUMINA *SHORT-READ* SEQUENCING

RNA Sequencing



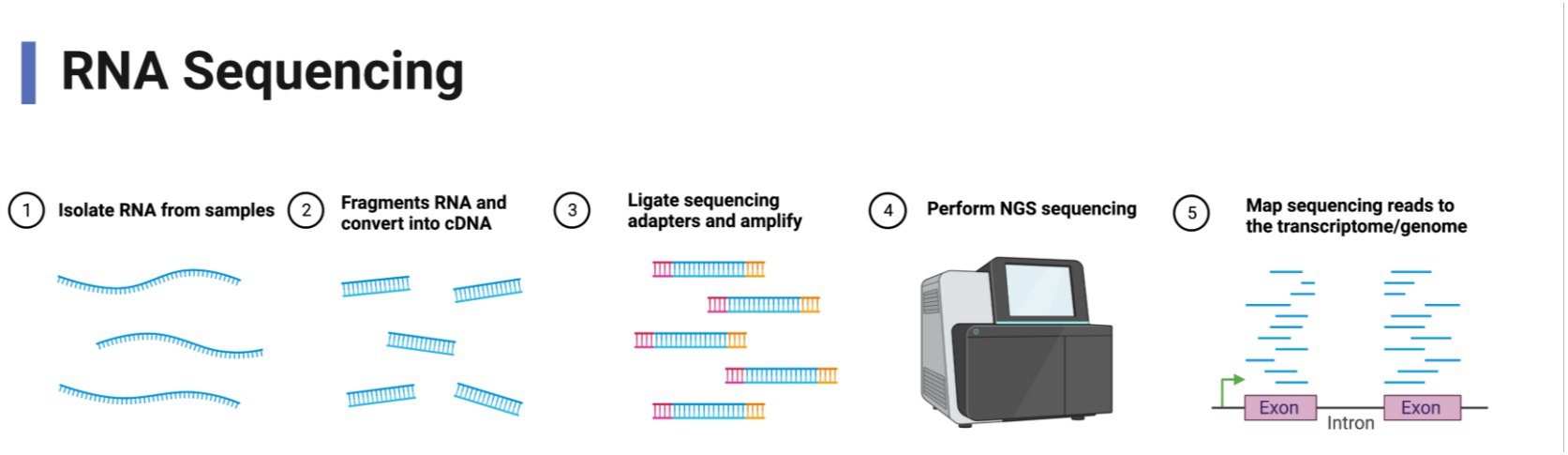
Experimental Step:
Library preparation and Sequencing

Analytical Step:
Assembly/Alignment and Quantification



Steps	Tools
Quality control	FASTQ NGSQC/RNA-SeQC
Pre-processing	Trimmomatic/PRINSEQ/Soapnuke
Read alignment	Tophat2/HISAT2/STAR/BWA/Bowtie
Transcript assembly	Cufflinks/StringTie/Trinity/ SOAPdenovoTrans/Trans-ABySS
Expression quantification	FeatureCount/HTSeq-count /Cufflinks/eXpress/RSEM/ DEXSeq/Kallisto/Sailfish/Salmon
Differential expression analysis	DESeq2/edgeR

ILLUMINA *SHORT-READ* SEQUENCING



PROs

- The first "unbiased" characterization of RNA species
- Quantitative and very sensitive (ultra low input protocols up to <1ng RNA)
- Coding and Non-coding species
- polyA-based or Total RNA
- Strand-specific reactions
- Pair-end mode helps isoform alignments

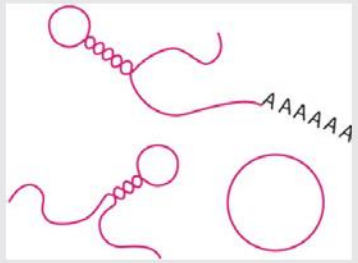
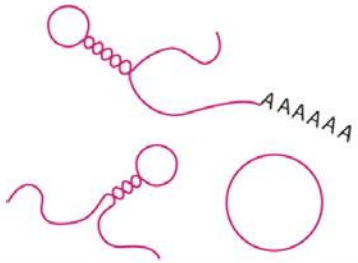
CONs

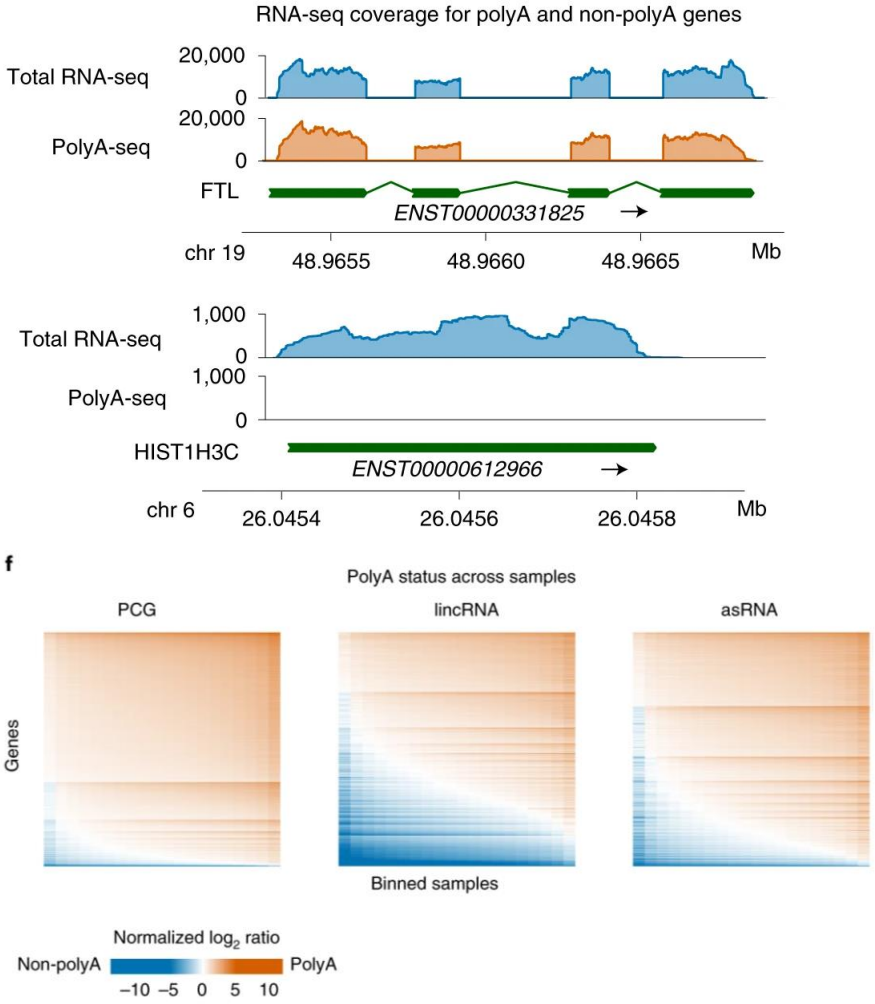
- ***Small RNA species*:** some small RNA species, (tRNAs or microRNAs), requires a different *ad hoc* protocol
- Repetitive elements are hard to analyse (***multi-mapping***)
- **cDNA** based (Artifacts may be introduced upon cDNA and amplification steps)

RNA EXPRESSION BY *SHORT-READ* SEQUENCING

polyA vs non-polyA

TABLE 2 RNA-seq technologies

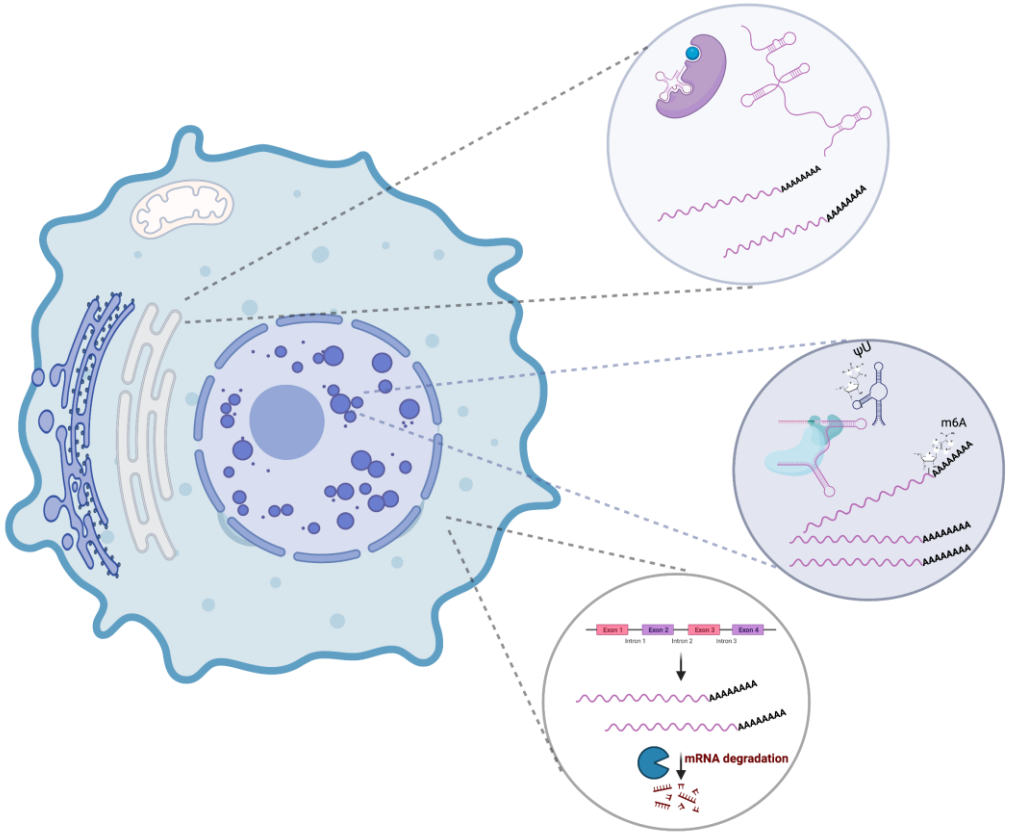
RNA-seq technology	lncRNA	Strength/weakness
polyA+		+ High coverage for polyadenylated transcripts + Limited sequencing depth required –Misses out on non-polyadenylated/ circular transcripts
Total RNA		+ Any lncRNA +/–High percentage of intronic reads –High sequencing depth required
Capture seq		+ Any lncRNA + Focus reads on genes of interest +Useful for the profiling of low-abundant RNAs in circulation –Custom probe design and cost



(P. Mestdagh lab) Lorenzi L, et al. «Genes Chromosomes Cancer 2019
Lorenzi et a. The RNA atlas – Nat Biotech 2021 https://doi.org/10.1038/s41587-021-00936-1

SHORT-READ SEQUENCING METHODOLOGIES

short read sequencing is extremely versatile and allowed the implementation of specific applications to characterize the transcriptome in its details



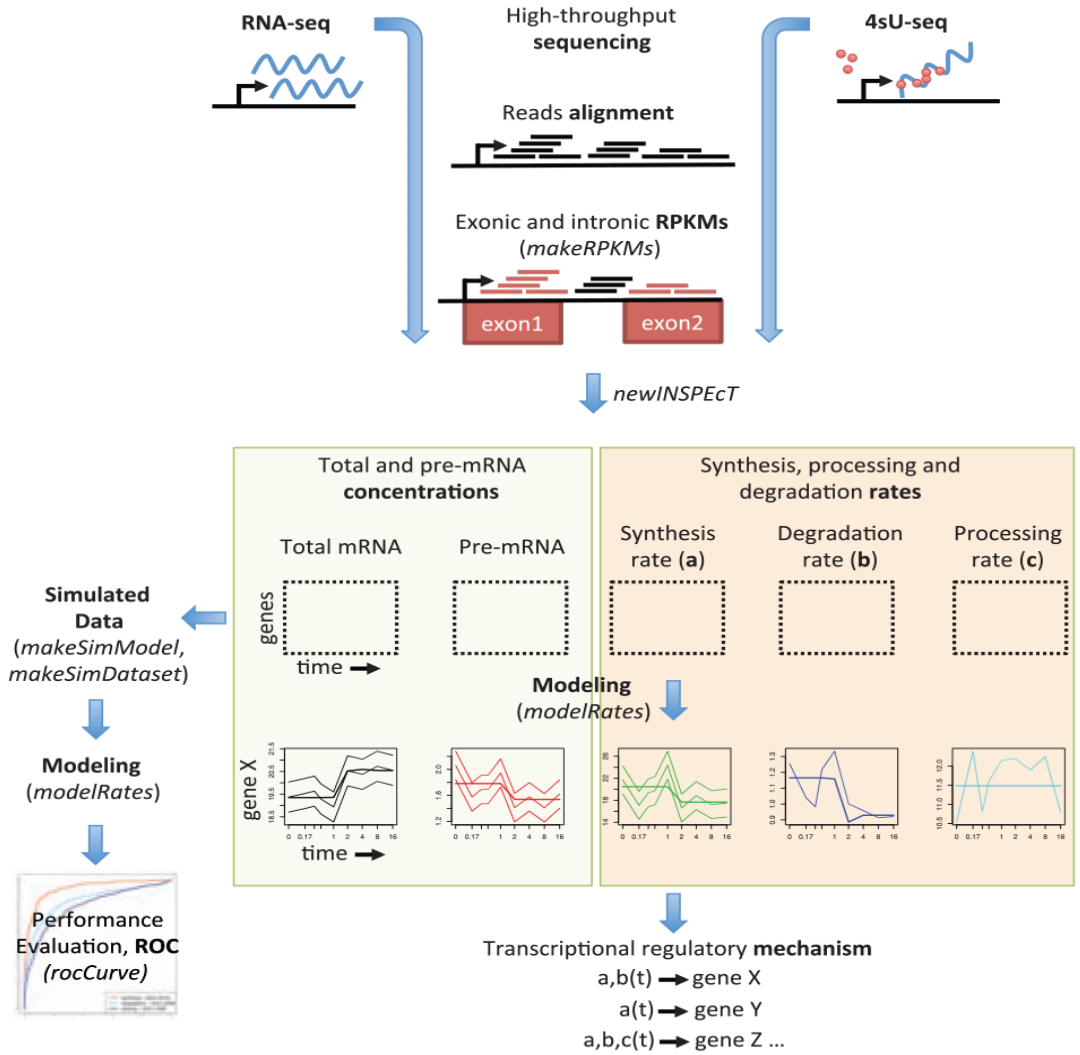
- **RNA localization** (*subcellular localization*)
  - *Fractionation protocols >> sequencing*
  - *Proximity ligation >> APEX-sequencing*

- **RNA modifications** and **RNA structure**
  - *Biochemical protocols*
    - *AB-based (miCLIP2)*
    - *Chemical modification (GLORI,SHAPE/C-SHAPE)*

- **RNA dynamics** (*synthesis, maturation, decay*)
  - *5'UTR and TSS (CAGE, NET-CAGE)*
  - *3'UTR (polyA-seq, FLAM-seq)*
  - *Metabolic labelling (4SU or IAA – SLAM-SEQ)*
  - *Global Run-On sequencing (GRO-seq))*
  - *Computational*: intron/exon ratios

SHORT-READ SEQUENCING METHODOLOGIES

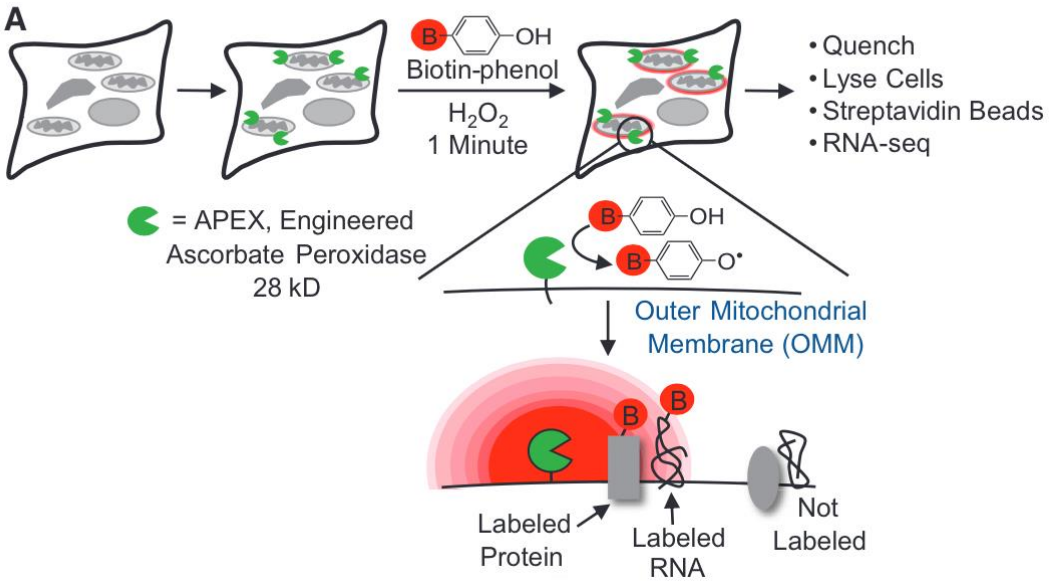
INSPEcT: a computational tool to infer mRNA synthesis, processing and degradation dynamics from RNA- and 4sU-seq time course experiments

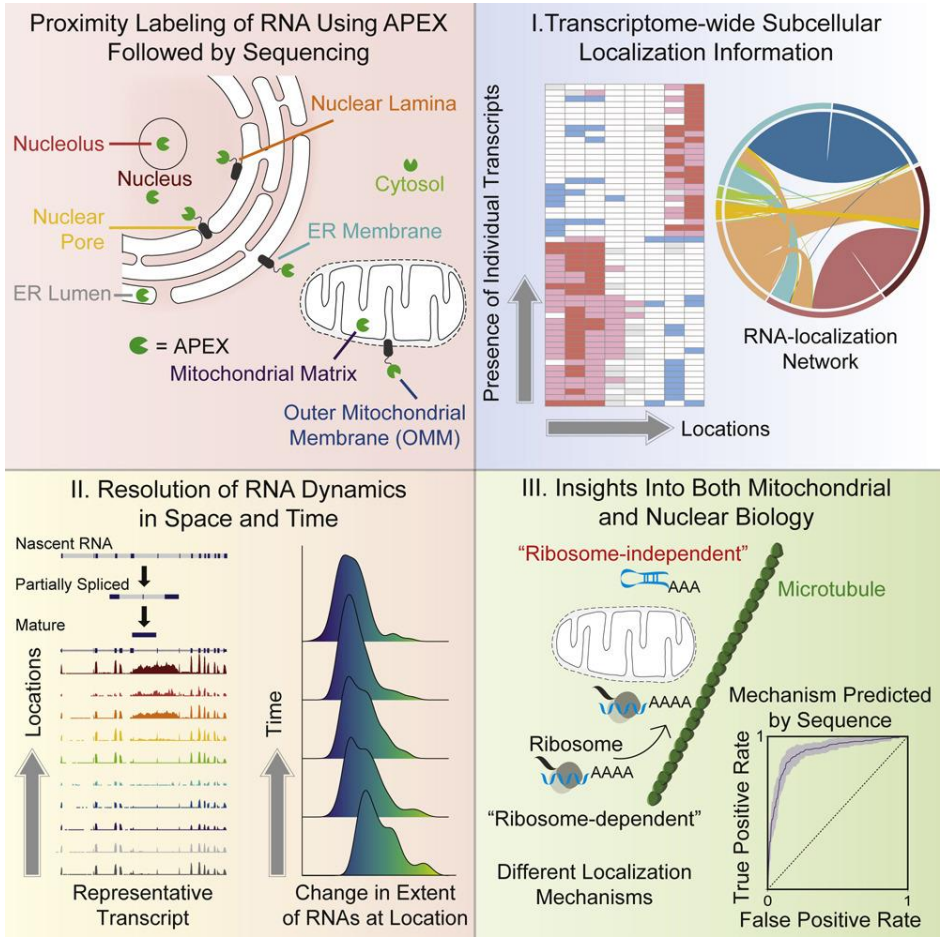


(M.Pelizzola lab) - De pretis et al. Bioinformatics, 2015, https://doi.org/10.1093/bioinformatics/btv288

SHORT-READ SEQUENCING METHODOLOGIES

APEX-seq: a method for RNA sequencing based on direct proximity labelling of RNA using the peroxidase enzyme APEX2.

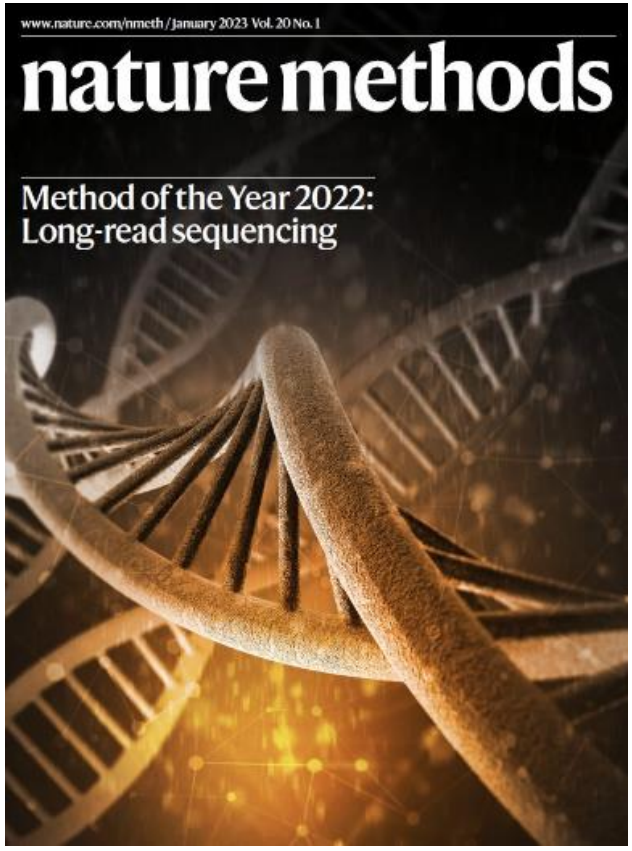




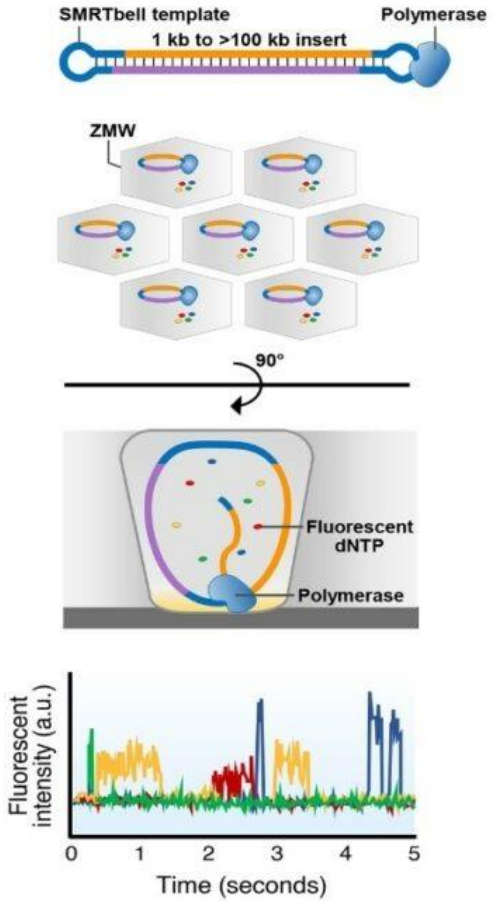
(H.Chang lab) – Fazal et al. Cell, 2019,
https://doi.org/10.1016/j.cell.2019.05.027

THIRD-GENERATION - LONG-READ SEQUENCING

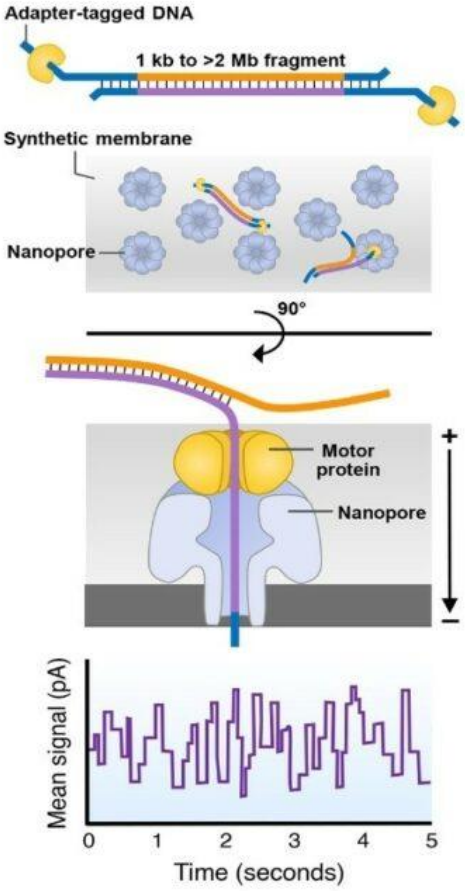
Advent of **Long Read sequencing technologies** shifted the paradigm of sequencing, with <u>single-molecule applications</u>



Pacific Biosciences SMRT sequencing

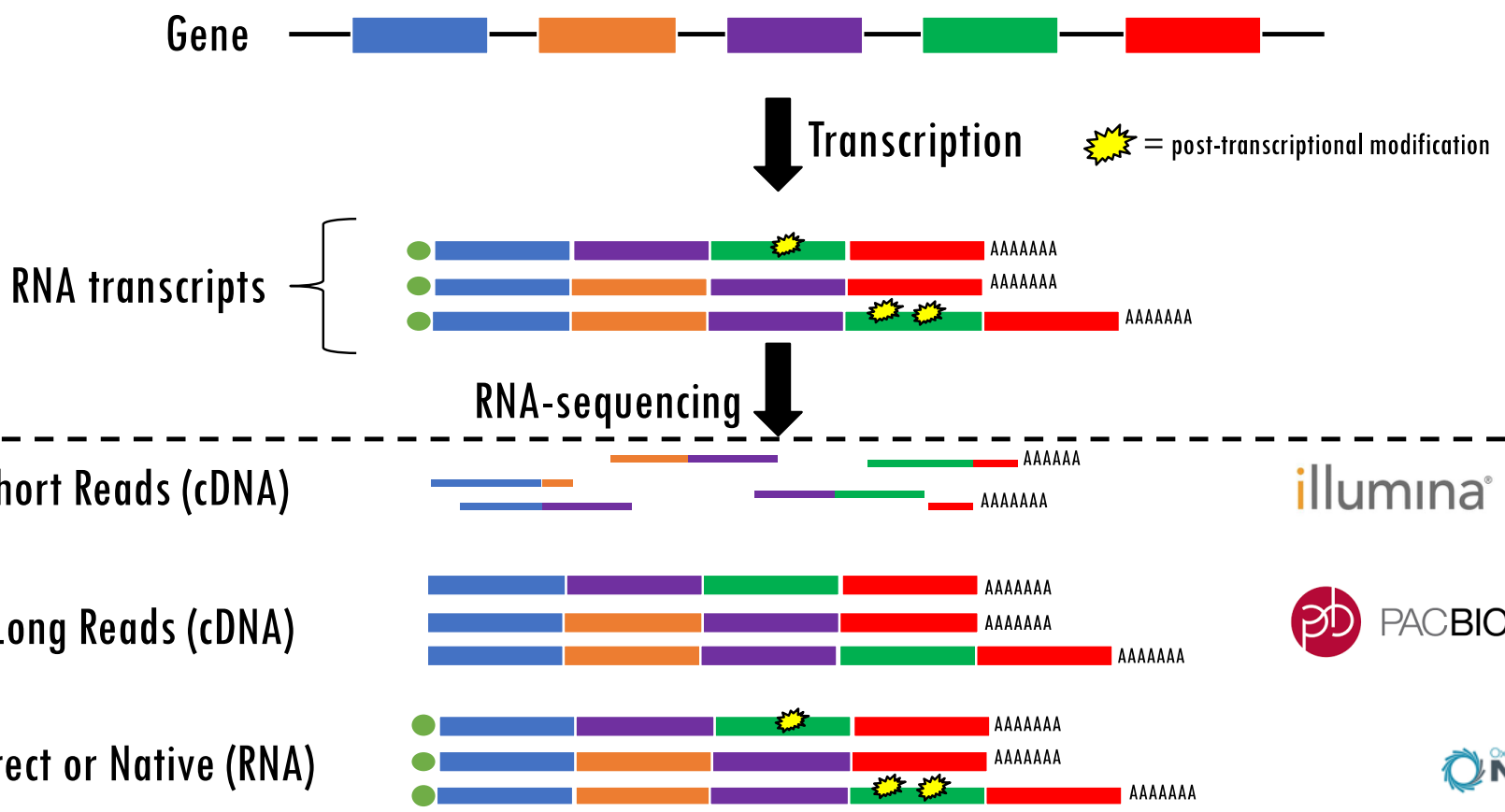


Oxford Nanopore Technologies sequencing

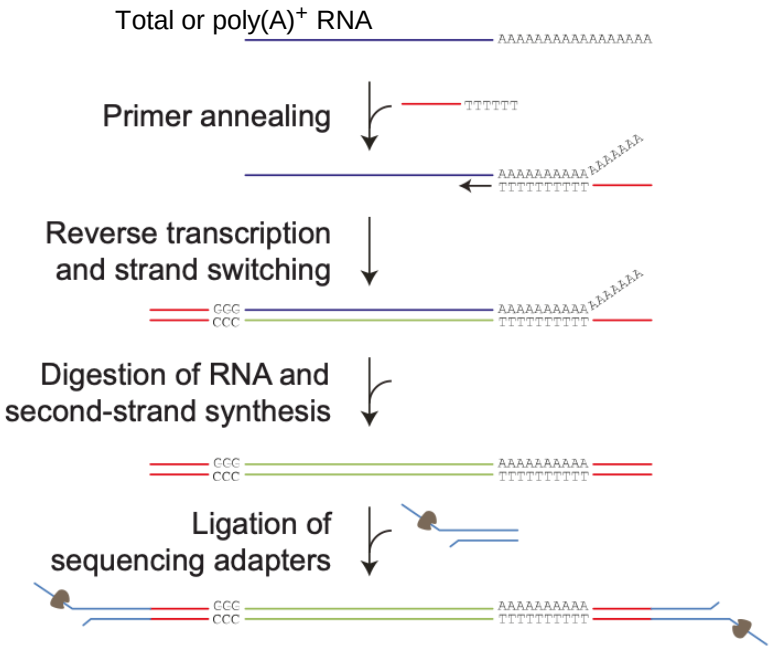


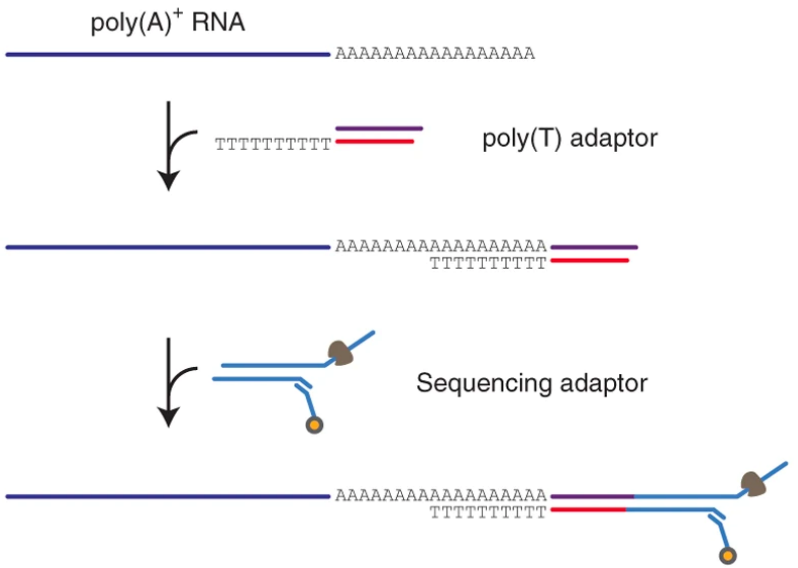
THE *LONG READ* TECHNOLOGIES IN RNA SEQUENCING

Advent of **Long Read sequencing technologies** improve mRNA analysis
longer read, single molecule information and native sequencing



THE *LONG READ* TECHNOLOGIES IN RNA SEQUENCING





cDNA sequencing

- Higher coverage
- Full-length Sequencing
- Sensitivity can be increased with few cycles of cDNA amplification

- RT-bias can be introduced
- Direct identification of modifications is not possible

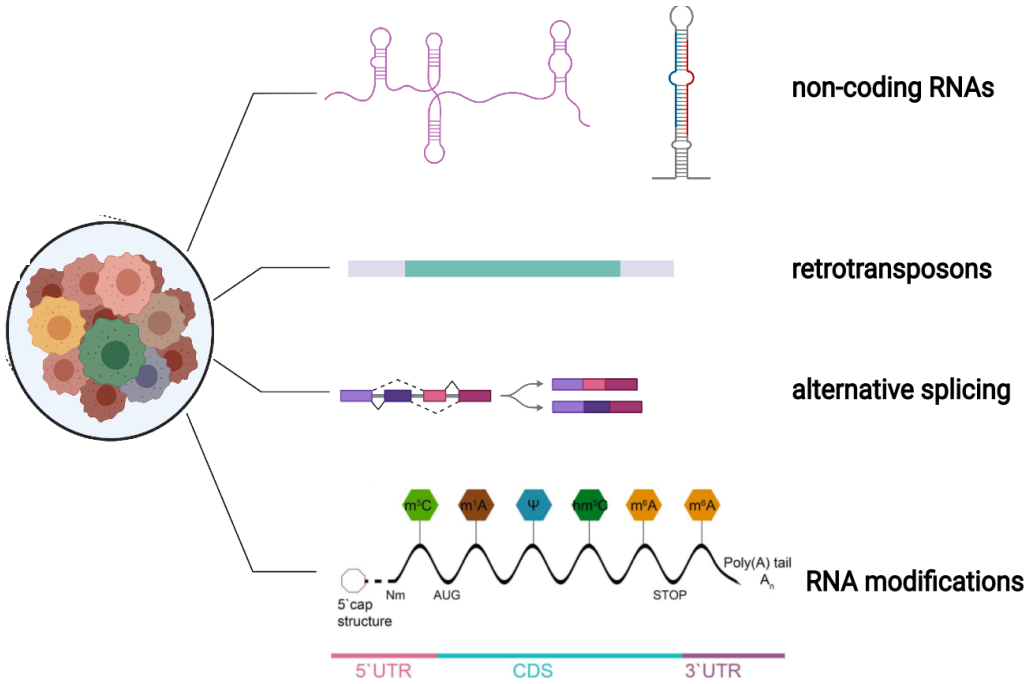
Direct RNA sequencing

- Lower coverage
- Full-length Sequencing is not standard
- Low Sensitivity and high amount of material

- No RT-bias
- Direct identification of modifications

RNA APPLICATIONS OF LONG-READ SEQUENCING

High-resolution and multi-level transcriptional and epitranscriptomics characterisation



Long read RNA Seq applications:

- Viral RNA characterization (es. SARS-Cov2)
- Transposable Element (TE) Characterization
- Full-length RNAs, isoform characterization
- polyA-length analysis
- Multi-level transcriptional analysis upon perturbation
  - Splicing : transcript isoforms
  - Epitranscriptomics: RNA mods
  - PolyA-length
  - Quantification of Repetitive Elements

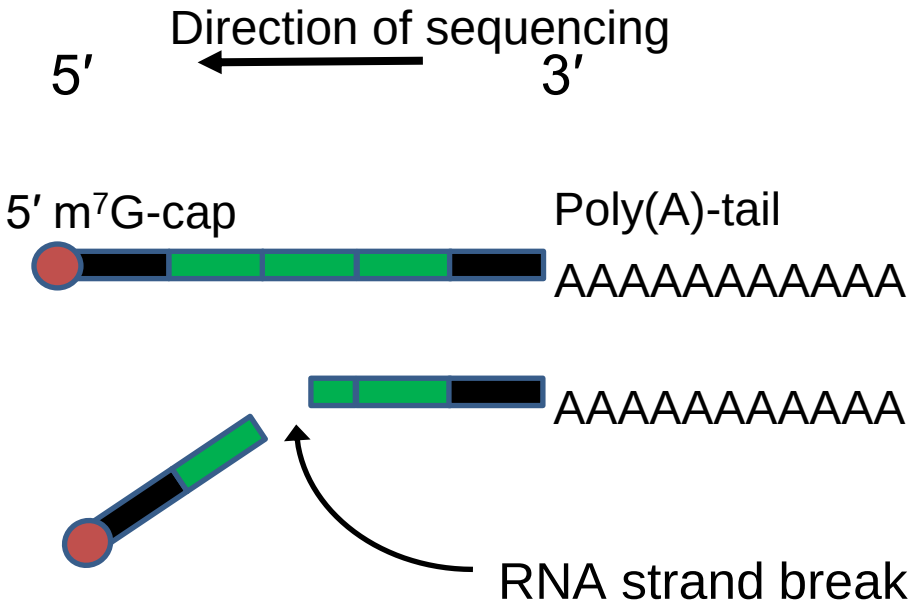
Direct RNA Seq applications:

- Detection of modified bases: epitranscriptomics

NOT ALL *LONG RNA READS* ARE FULL-LENGTH

strand breaks can terminate reads.

How to discriminate for TRUE 5' ends?



NOT ALL *LONG RNA READS* ARE FULL-LENGTH

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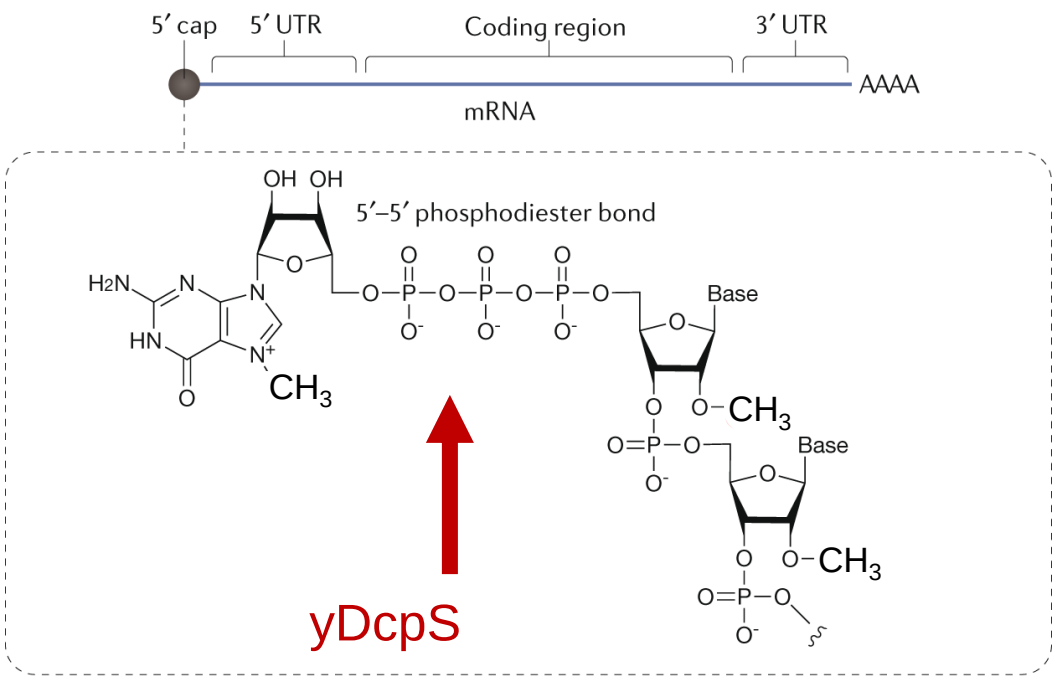
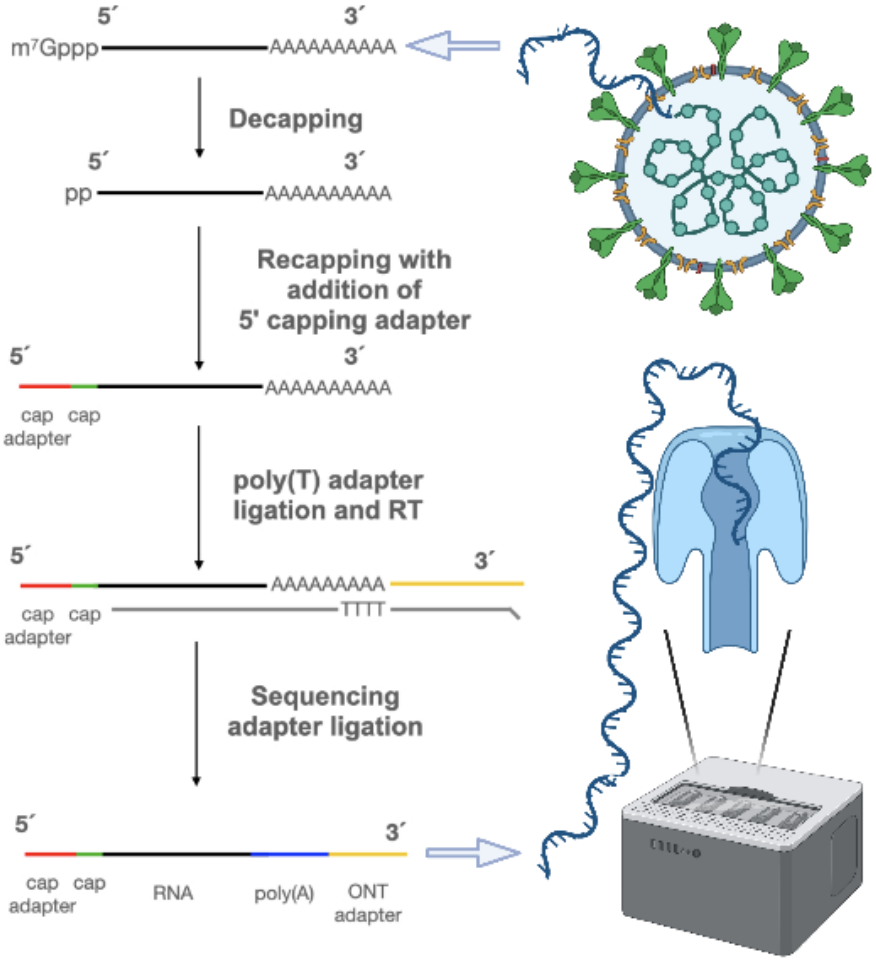


THE MRNA M7G CAP

Mulroney, Wulf et al. **2022** *RNA*

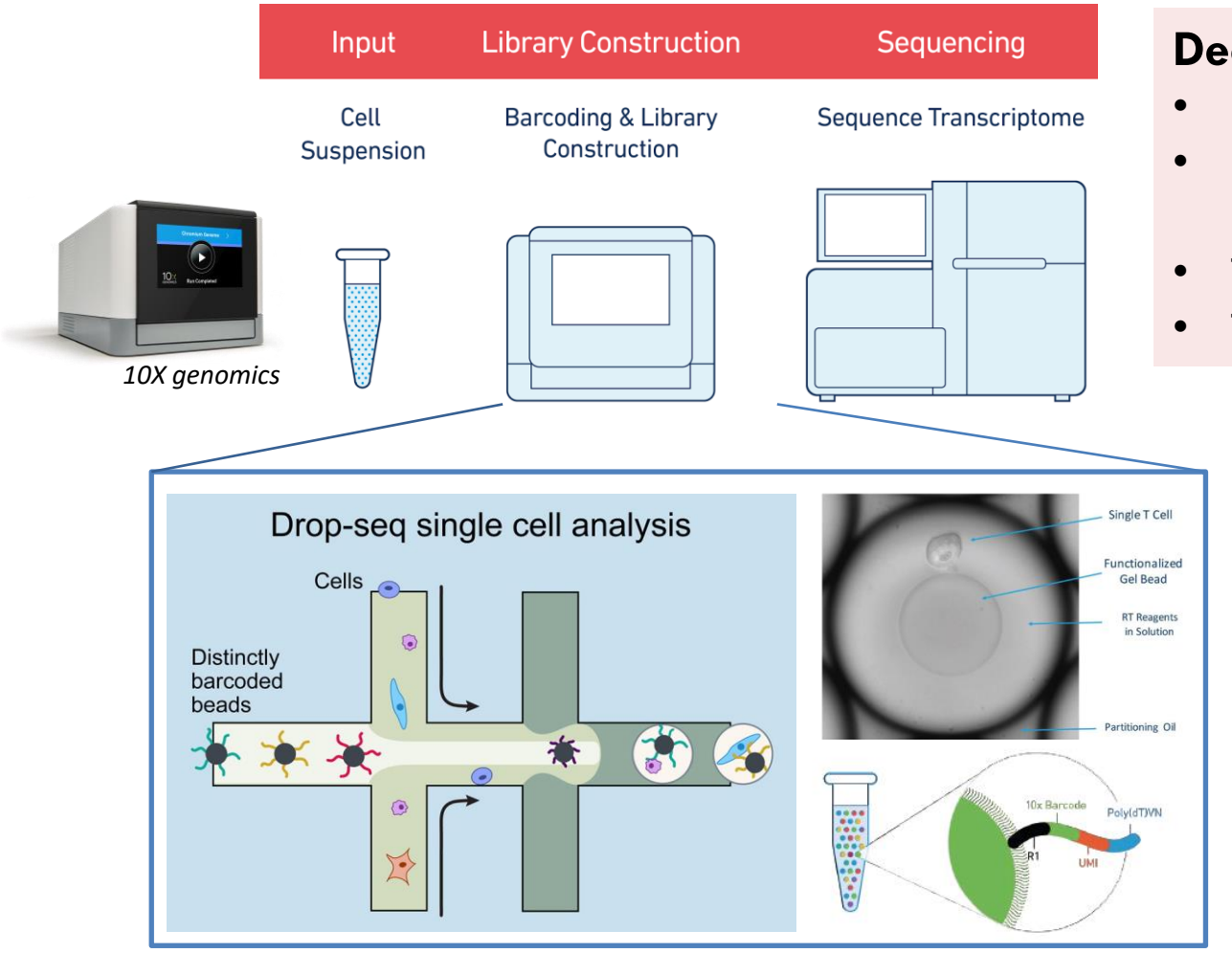


Ugolini et al. **2022** *Nucleic Acid Research*

SINGLE-CELL TECHNOLOGIES IN RNA SEQUENCING

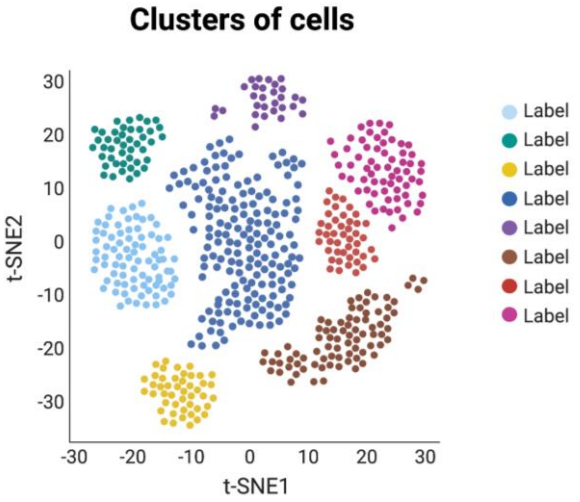
RNA sequencing has been evolved at single cell resolution (different from BULK)

Dropseq (i.e.10XGenomics) vs ***Single-well*** approach (icell8)



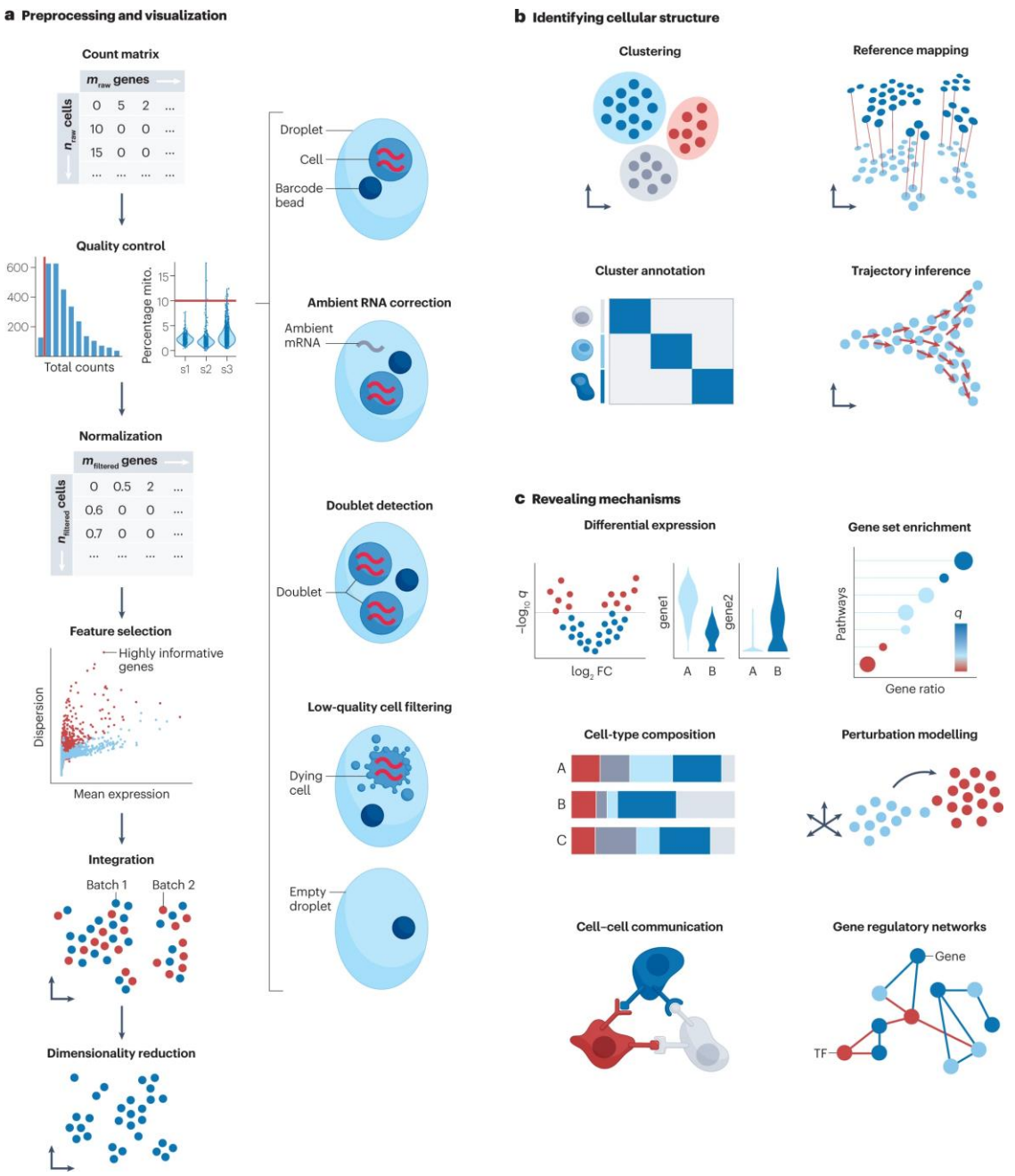
Deconvolution of *Cellular* Heterogeneity

- Diversification of cell identity
- Different cell states within the same population
- Transcriptional markers
- Trajectories (developmental)



SINGLE-CELL TECHNOLOGIES IN RNA SEQUENCING

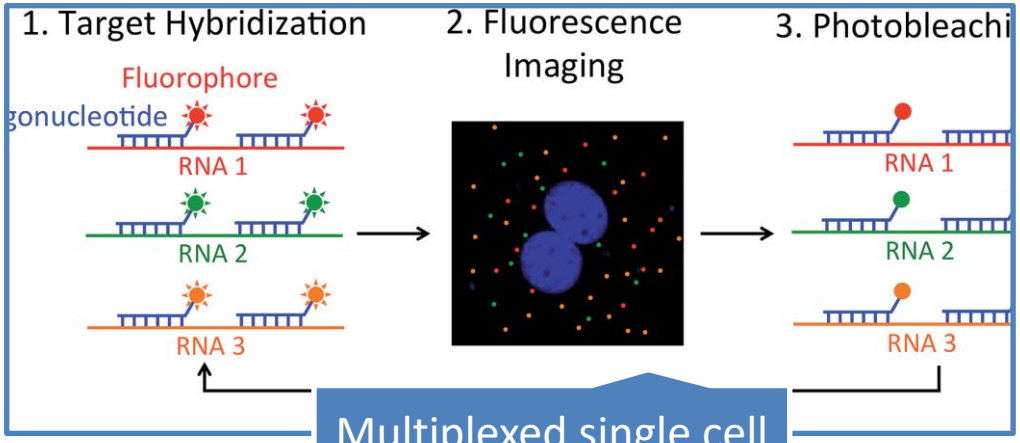
Overview of unimodal analysis steps for scRNA-seq



(F. Theis lab) Heumos, L., Schaar, A.C., Lance, C. *et al.* Best practices for single-cell analysis across modalities. *Nat Rev Genet* (2023). https://doi.org/10.1038/s41576-023-00586-w

SPATIALLY-RESOLVED OMICs IN RNA SEQUENCING

RNA sequencing with spatial information provides new challenges and opportunities for Transcriptome Analysis



Multiplexed single cell in situ RNA analysis

Spatially Resolved OMICS

- Imaging
- Few RNA species but specific (with probes)
- Single Molecule + Single Cell Resolution
- 2D cell culture

- Sequencing
- Many RNA species (>10,000, with probes)
- Few cell resolution
- Clinical oriented - (frozen samples or FFPE) Brain- or Cancer-

COSTS OF THE TECHNOLOGY IS HIGH

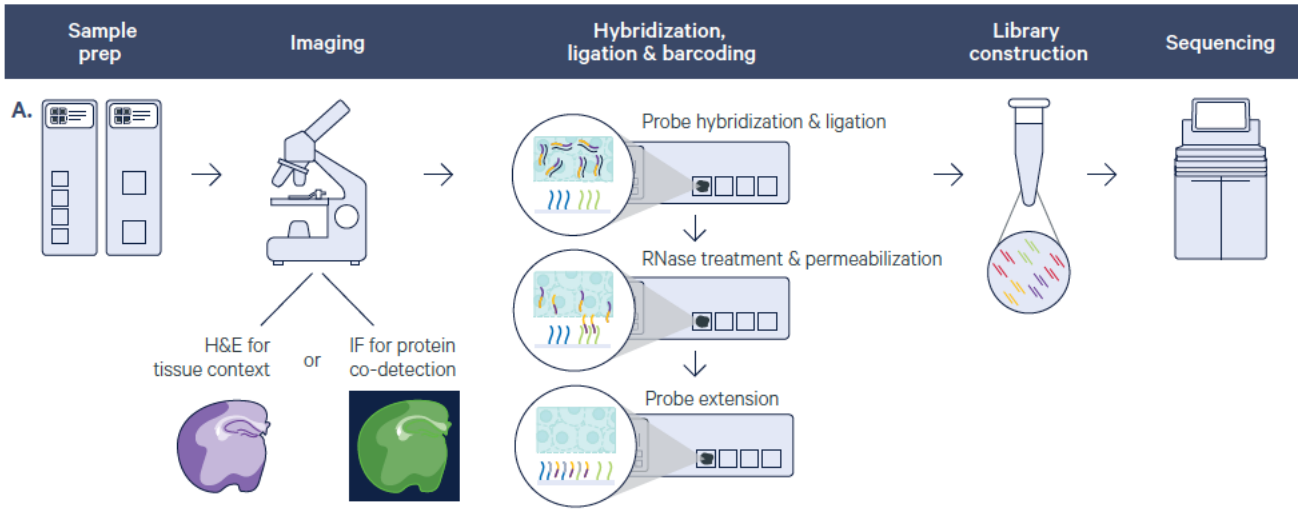
a preliminary assessment of the technology before acquiring the infrastructure is advisable

AD HOC ANALYTICAL TOOLS REQUIRED

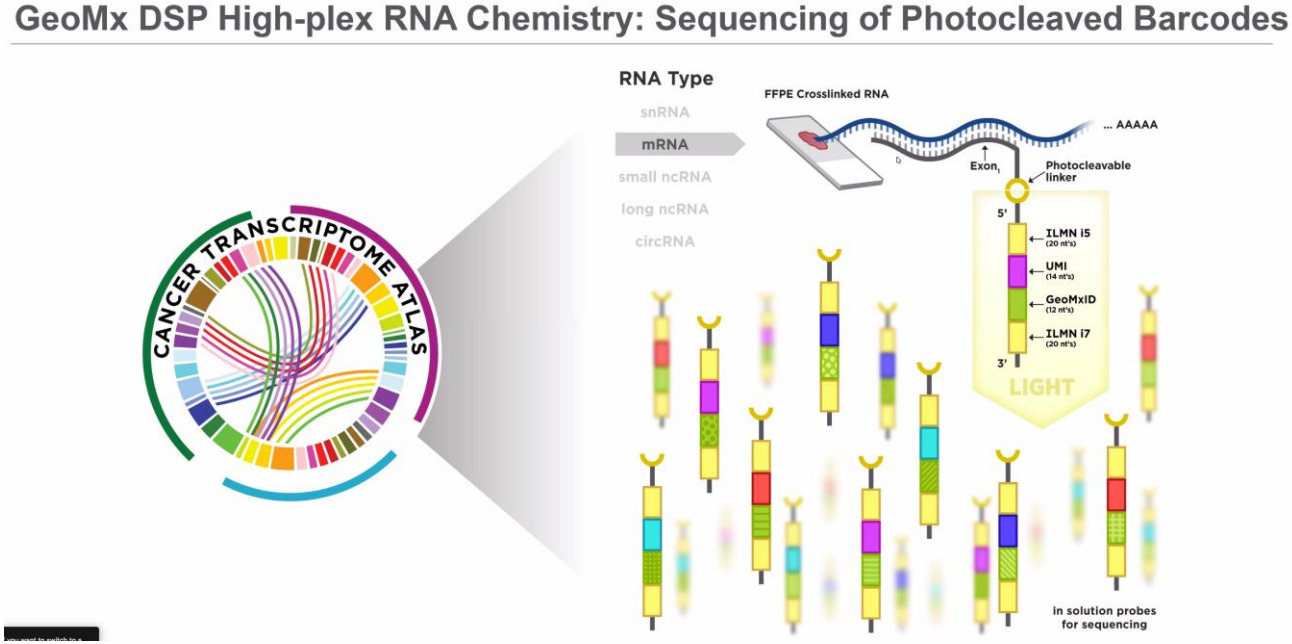
exploitation of a technology depends mostly on the development of proper analytical tools

SPATIALLY-RESOLVED OMICs IN RNA SEQUENCING

Visium
Spatial Gene
Expression
10X
GENOMCIS

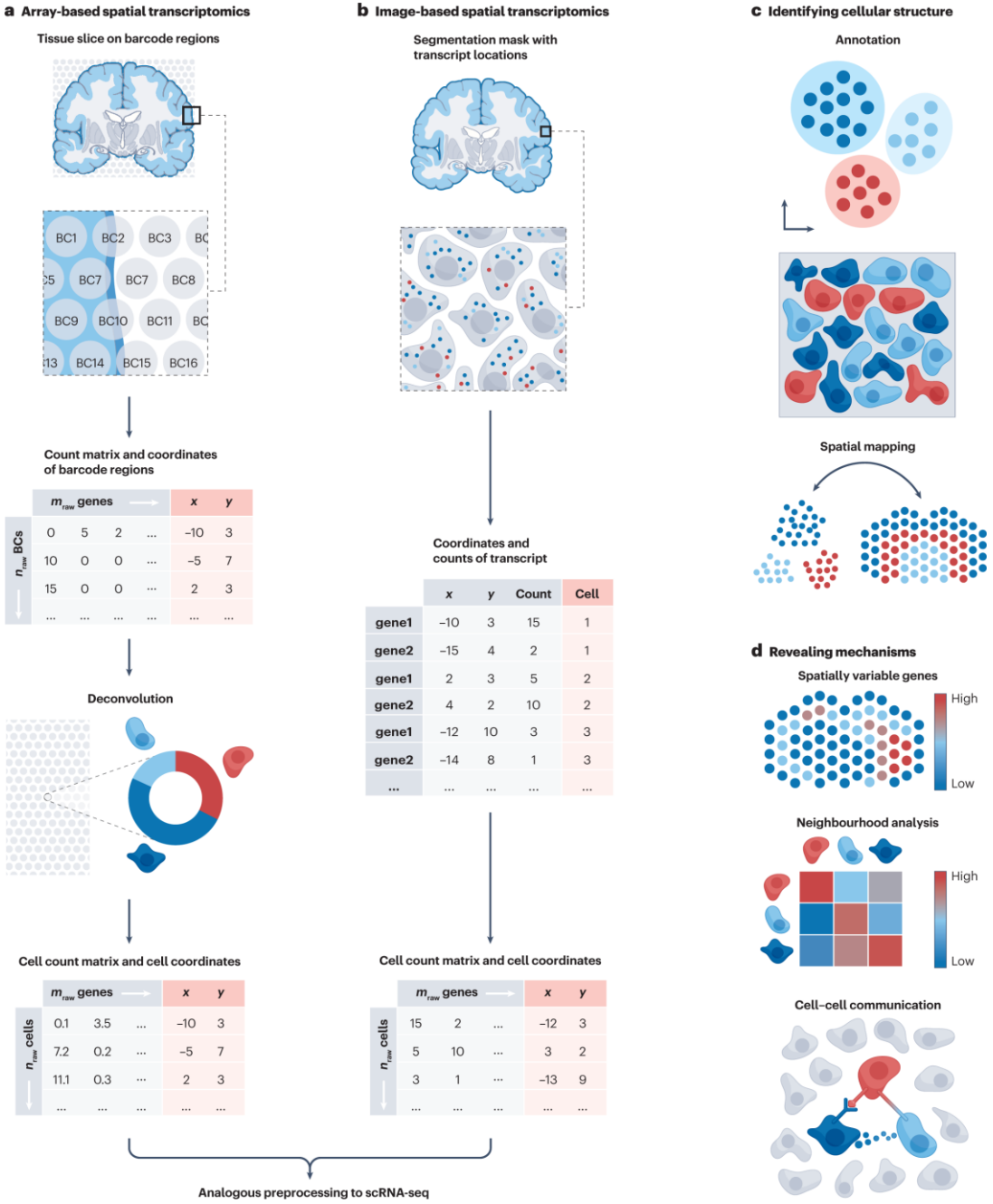


GeoMx
Spatial Omics
NANOSTRING



SPATIALLY-RESOLVED OMICs IN RNA SEQUENCING

Overview of spatial transcriptomics pre-processing and downstream analysis steps



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CONCLUDING REMARKS

RNA

- **It's time to move from a GENE-centered to an RNA-centered approach**
- the functions of the cells, as well as cell identity and properties are specified by regulating the number and the type of transcripts which are generated (a.k.a. the transcriptome)

BIOTYPES

- **The structure and functions of RNA are remarkably complex**
- Several RNA biotypes exist with specific functions

TECHNOLOGY

- **Technology can help decipher the complexity**
- Each approach has pros and cons >> nothing is perfect !
- Biological question can be answered by a set of orthogonal approaches, including omics

SCIENTISTS

- **Multidisciplinary approach is mandatory**
- Computational Biology is discipline. Bioinformatics is not just a support for research: it actively participates in all the steps of a research project