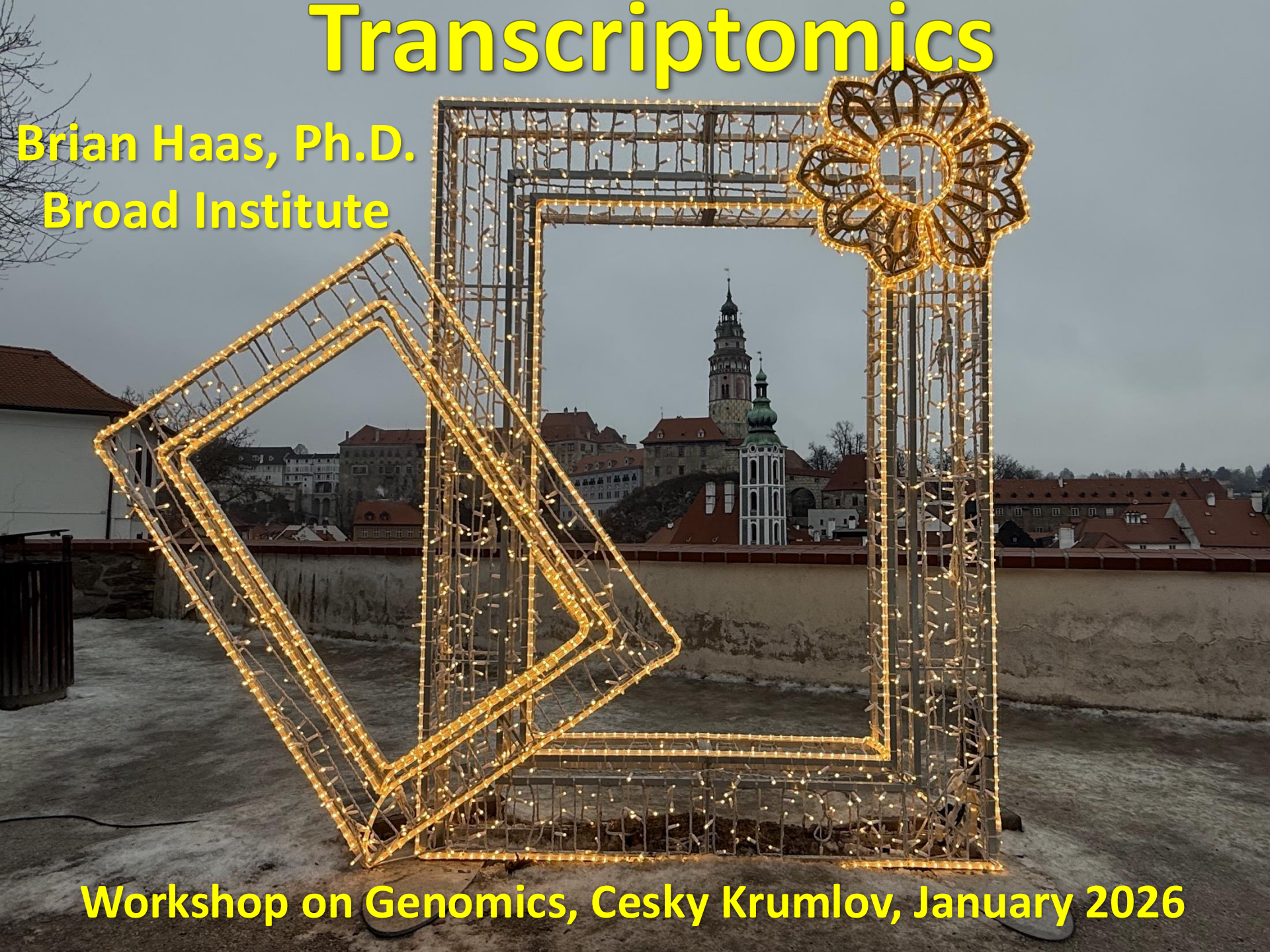
Transcriptomics

Brian Haas, Ph.D.
Broad Institute



Workshop on Genomics, Cesky Krumlov, January 2026

Intro to Brian Haas

Education and Career History

BS,MS Molecular Bio
DNA Repair
SUNY Albany, New York

1991-1999

The Institute for Genomic Research
Rockville, Maryland, USA
(1999-2007)

Bioinformatics Analyst & Engineer

MS. Computer Science / Johns Hopkins

BROAD INSTITUTE

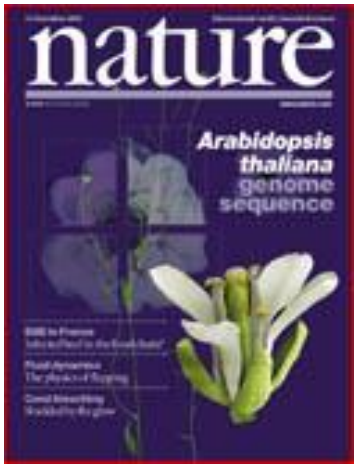
Cambridge, Massachusetts, USA

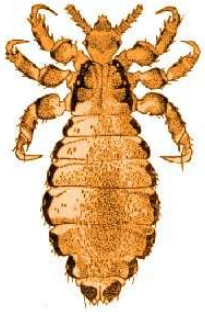
2007-current

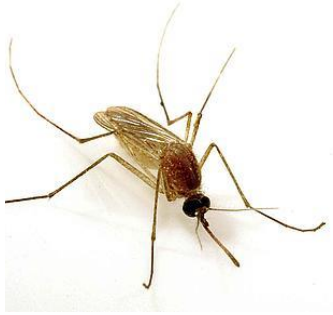
Computational Biologist / Manager /
Principal Computational Scientist

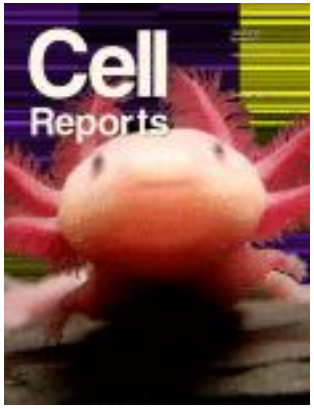
Ph.D. Bioinformatics / Boston University

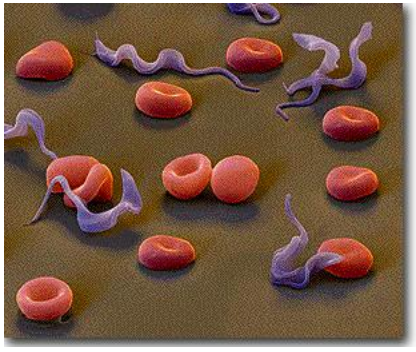
Annotation and Analysis for Diverse Genomes and Transcriptomes

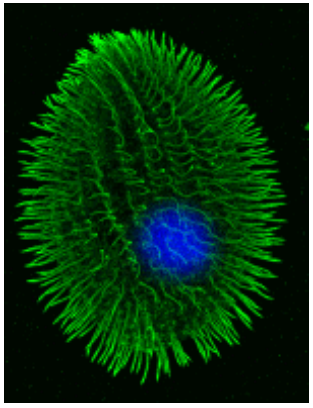


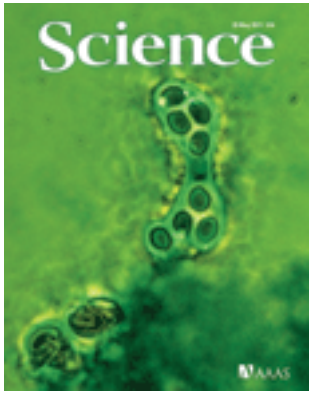


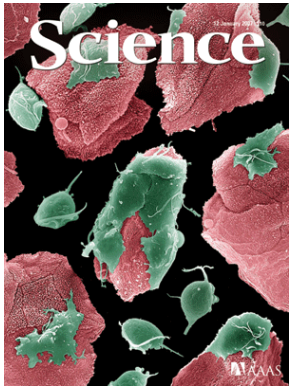












BROAD INSTITUTE

My Favorite Activity – Bioinformatics Tool Development and Applications

BROAD INSTITUTE

PASA

NAR, 2003

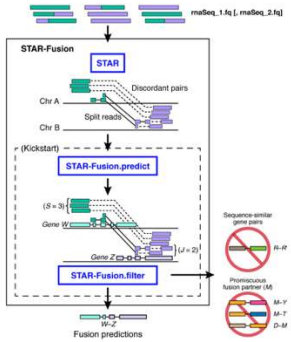
Bioinformatics, 2004

EVM

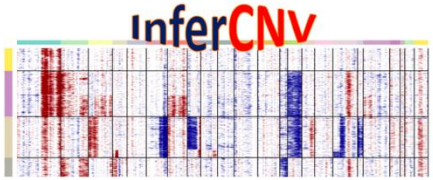
EVidenceModeler
Genome Biology, 2008

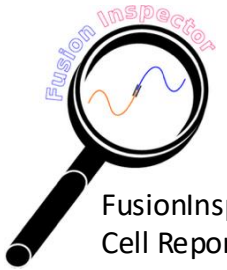
Chimera Slayer
Genome Research, 2011

Nature Biotech, 2011
Nature Protocols, 2013



STAR-Fusion
Genome Biology, 2019





FusionInspector
Cell Reports Methods, 2023

My Favorite Activity – Bioinformatics Tool Development and Application

PASA

NAR, 2003

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BROAD INSTITUTE

Chimera Slayer
Genome Research, 2011

Trinity
Inchworm
Chrysalis
Butterfly

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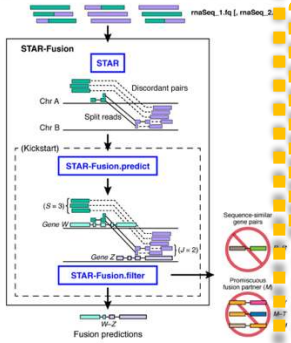


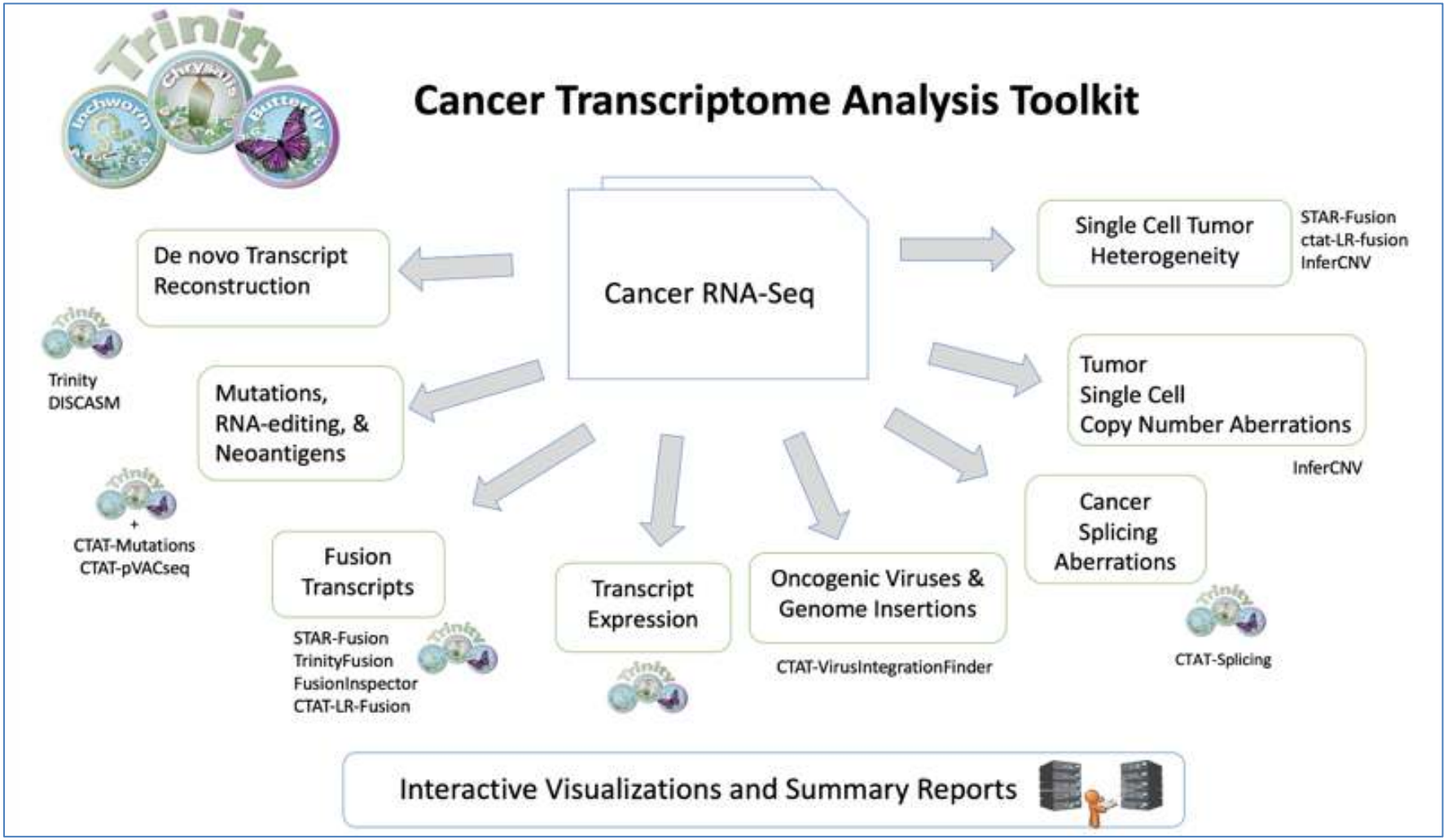
STAR-Fusion
Genome Biology, 2019

InferCNV

FusionInspector
Cell Reports Methods, 2023

Earlier developments focused on cancer transcriptomics:

Years 2013-2023



Overview of Trinity CTAT. Given cancer RNA-seq as input, Trinity CTAT provides modules for exploring characteristics of the cancer transcriptome (and cancer genome) including both genome-guided and genome-free analyses, targeting bulk or single-cell transcriptomes. Interactive visualizations and reports are provided to facilitate downstream analysis and for clinical review.

This certificate attests that

Brian Haas

having completed ten years of faithful service
in the halls, taverns, and computational chambers
of Český Krumlov has this evening been formally knighted
into the Order of the Molekulys and is henceforth known as

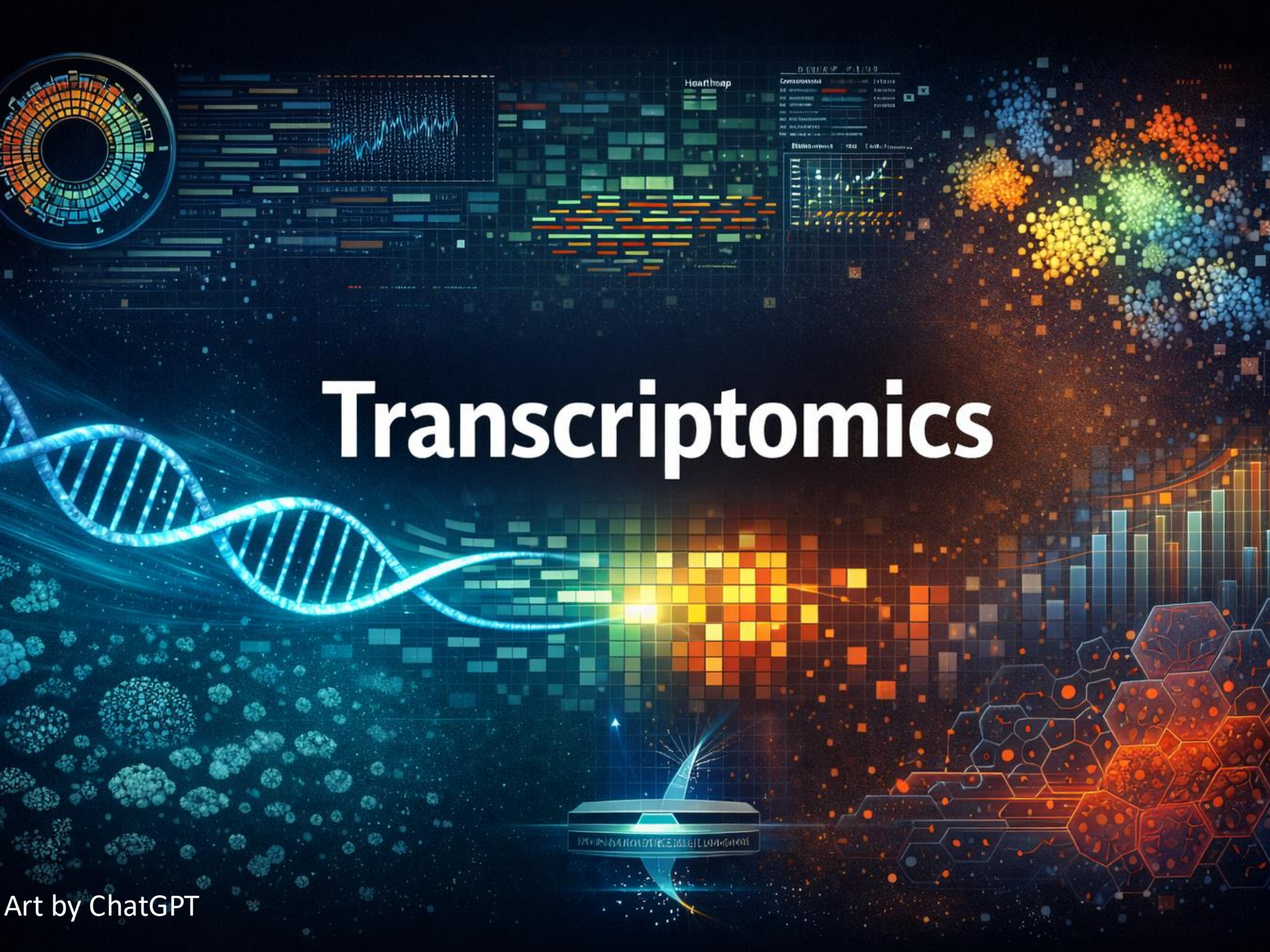
Sir Brian, Master of the Genome Forge

bestowed this day (17th January 2026) by decree of

Lord Scott of the Molekulys
and the assembled Faculty of the Workshop on Genomics

-- Introducing --

Mega Keychain!



Art by ChatGPT

Transcriptomics Lecture Outline

1. Intro to transcriptomics
2. Transcript reconstruction methods
3. Genome-free transcriptomics (eg. for non-model orgs)
4. Expression quantification
5. Differential expression (brief – more details in Rachel's workshop!)
6. Latest advancements in long read isoform sequencing

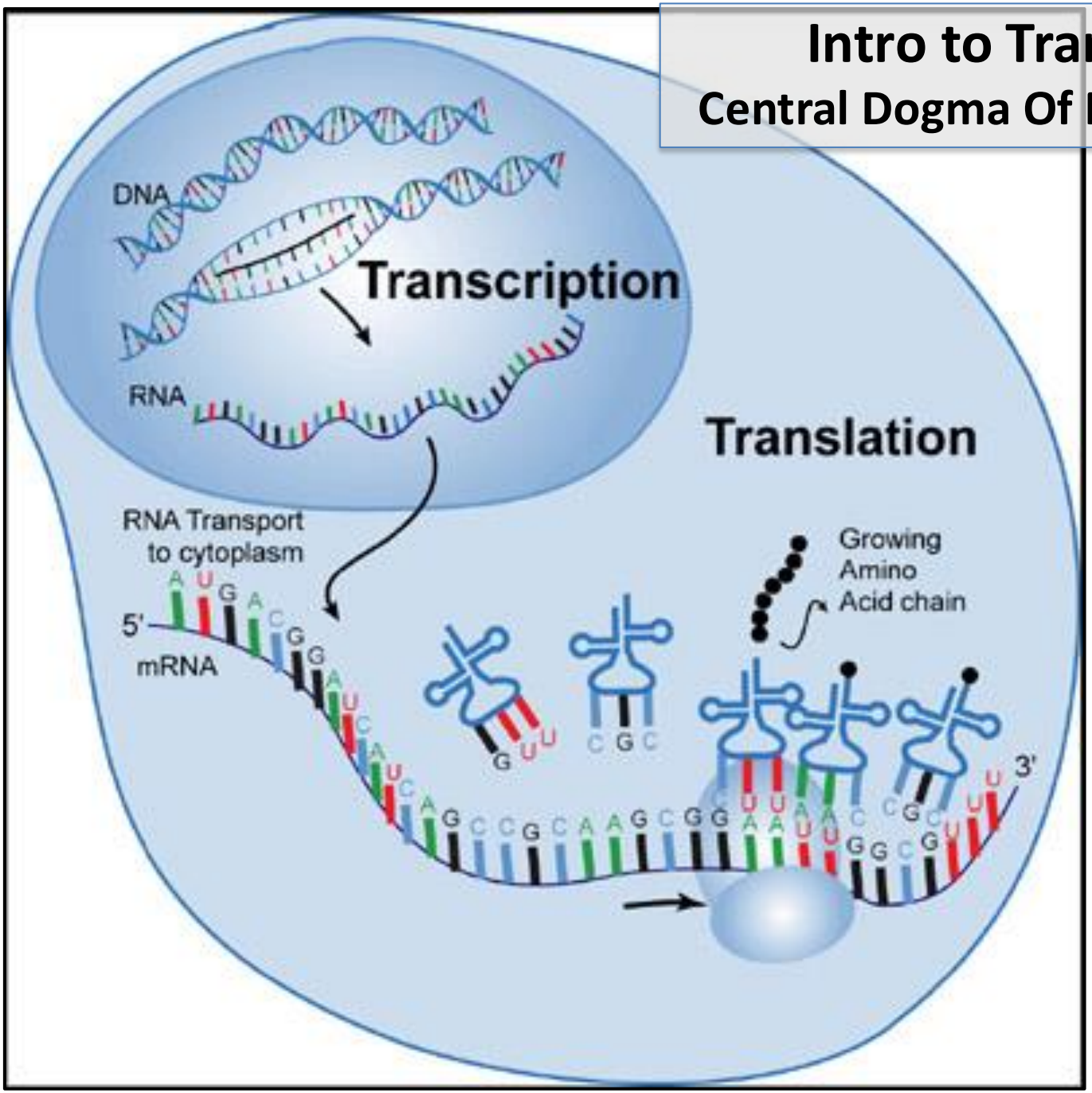
Break?

7. Overview of single cell transcriptomics
8. Overview of spatial transcriptomics
9. Applications in Cancer Transcriptomics

** Followed by comments on how I've recently been using LLMs in my work*

Part 1. Intro to Transcriptomics

Intro to Transcriptomics
Central Dogma Of Molecular Biology



https://www.simply.science/images/content/biology/genetics/molecular_genetics/conceptmap/Central_Dogma_of_Molecular_Biology.html

Primary mRNA molecules Often Undergo Splicing in Eukaryotes

Gene in the Genome

Transcription

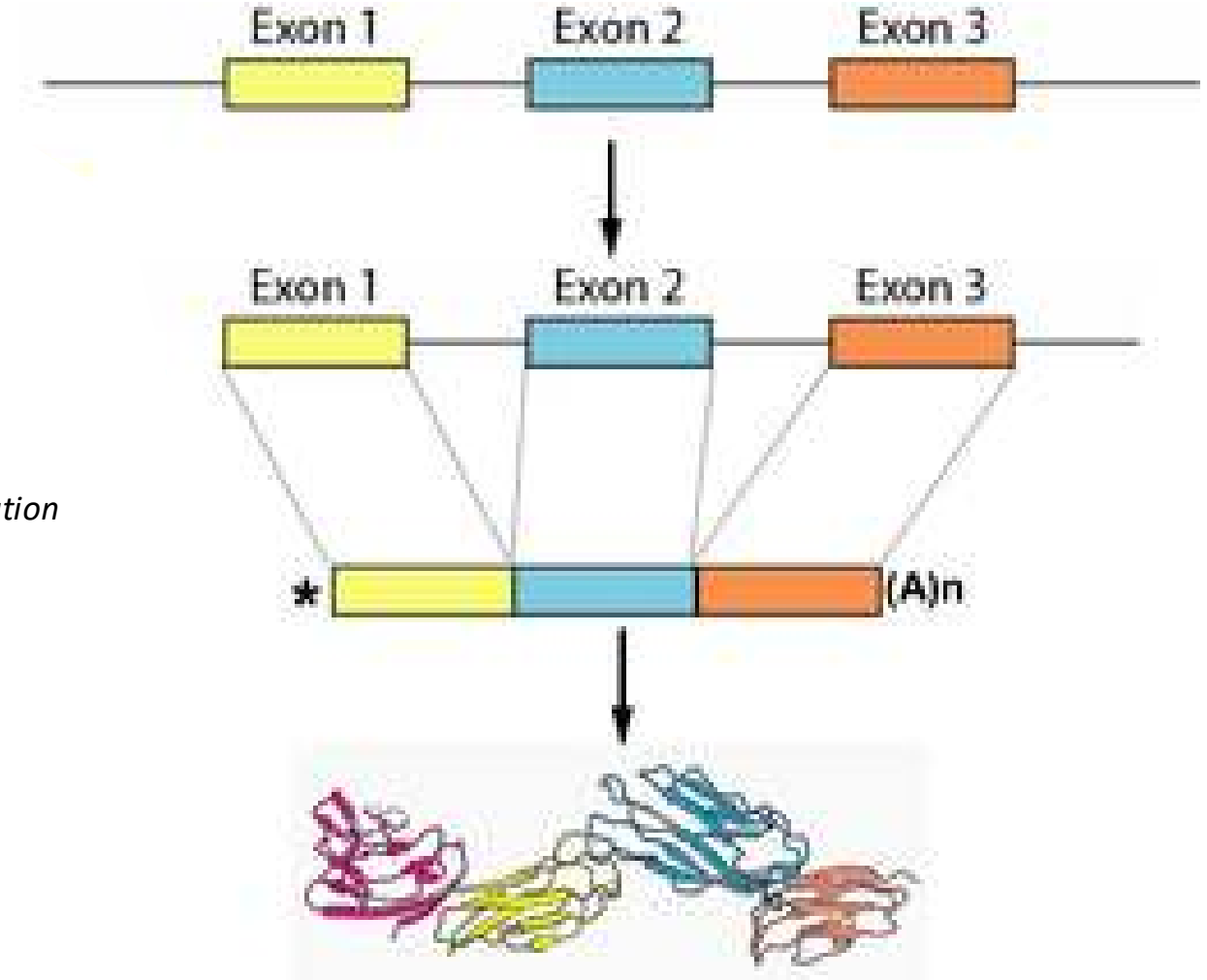
Primary transcript

Intron Splicing,
5' Capping,
and 3' Polyadenylation

Processed RNA

Translation

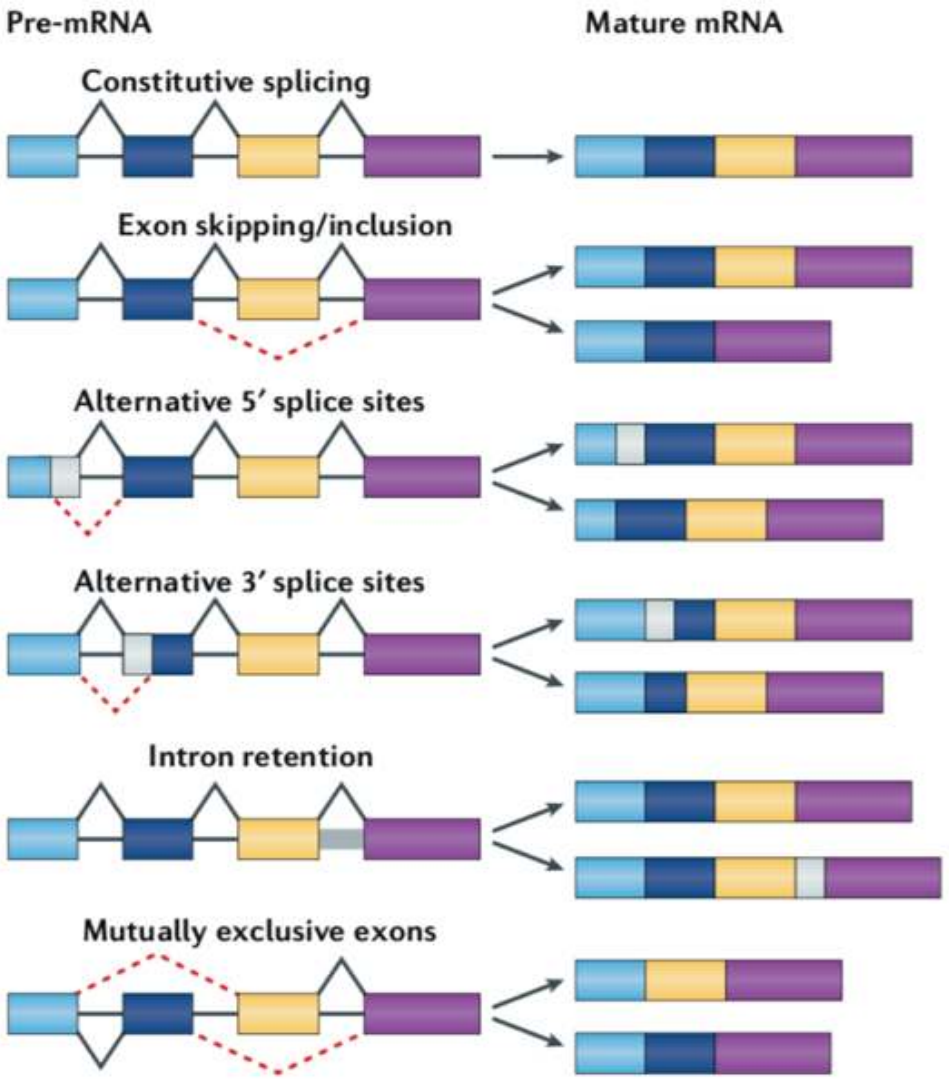
Protein



Adapted from: https://cs.wikipedia.org/wiki/Splicing

Alternative Splicing – Multiple Products from Single Genes

- Core regulatory process – diversifies the function of genes.
- Generates mRNAs that differ in coding sequence and UTRs. Effects:
  - Protein isoforms
  - Translation efficiency
  - Stability
  - Localization
  - Reading frame changes
- Estimated 90-95% of human genes undergo alternative splicing



From Aziz Al'Khafaji, Broad Inst.

Think of genes as protosentences

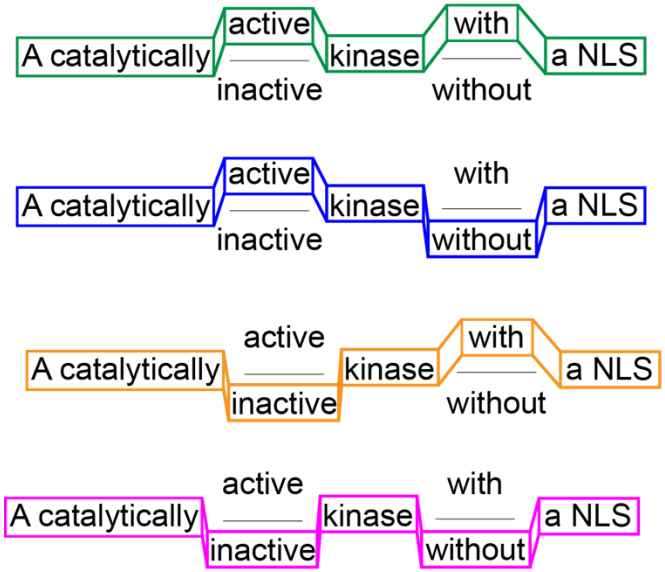
Gene: A catalytically $\frac{\text{active}}{\text{inactive}}$ kinase $\frac{\text{with}}{\text{without}}$ a NLS

From Aziz Al'Khafaji, Broad Inst.

Think of genes as protosentences

Gene: A catalytically active/inactive kinase with/without a NLS

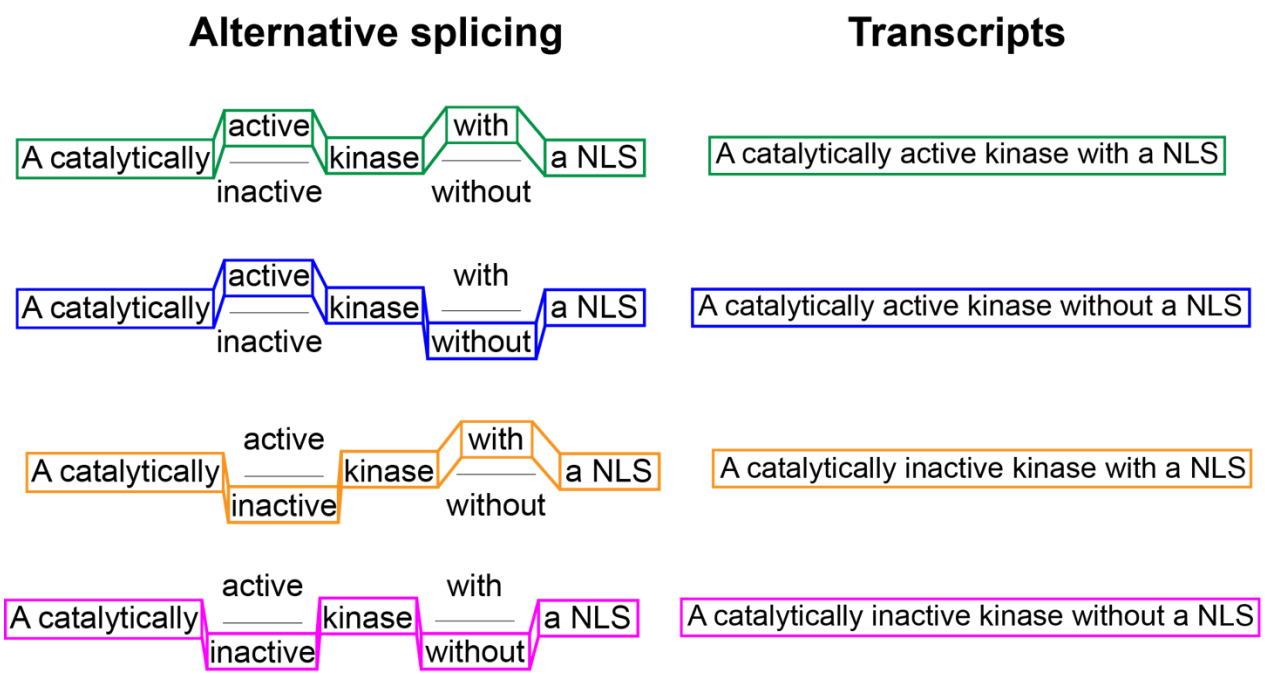
Alternative splicing



From Aziz Al'Khafaji, Broad Inst.

Fully formed sentences ≈ mature mRNA

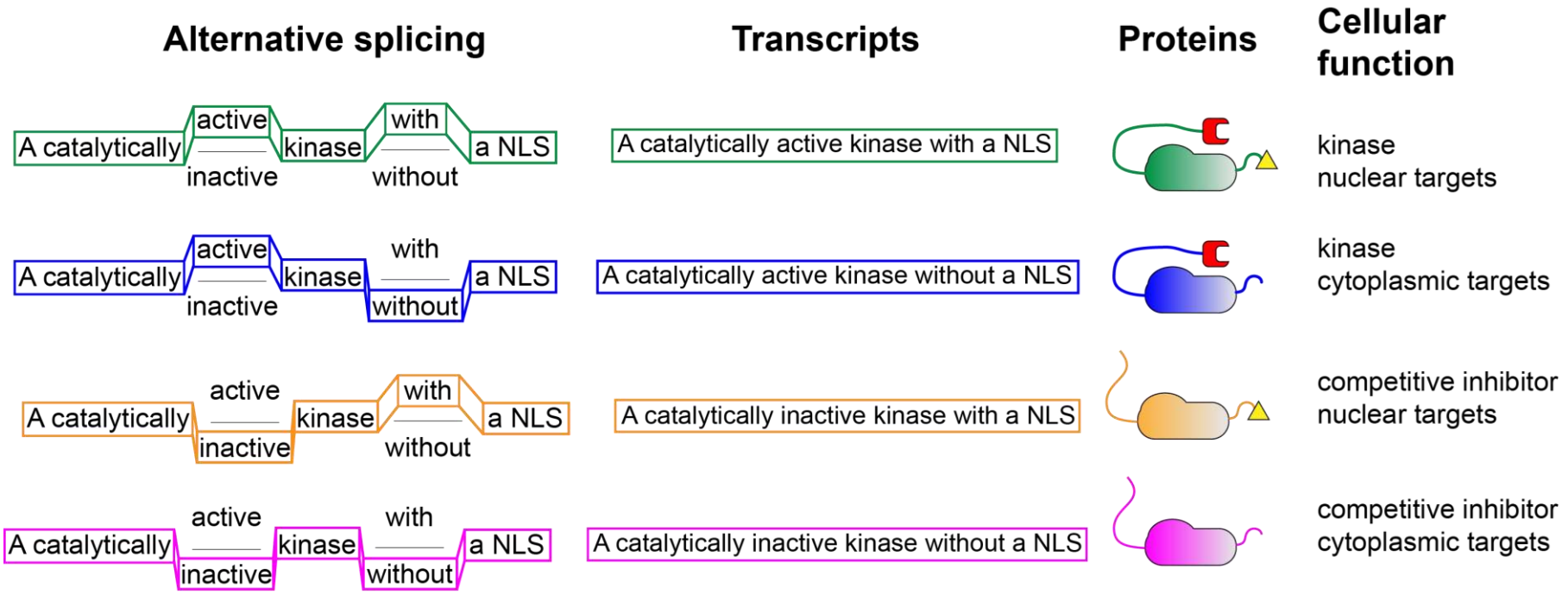
Gene: A catalytically active / inactive kinase with / without a NLS



From Aziz Al'Khafaji, Broad Inst.

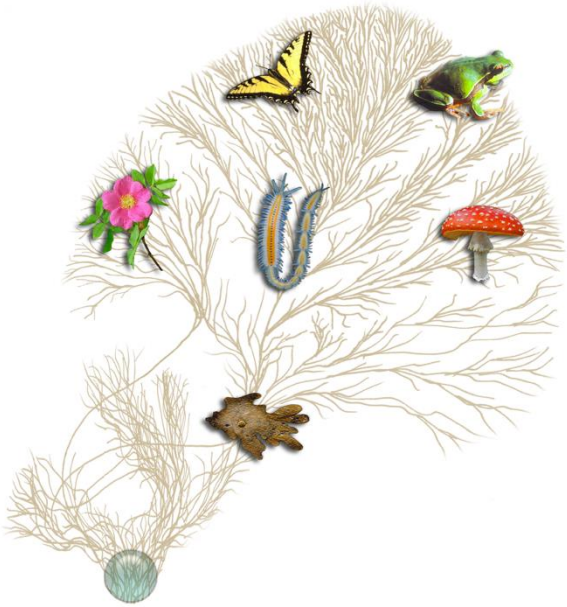
RNA isoform sequencing provides structural insight

Gene: A catalytically active/inactive kinase with/without a NLS

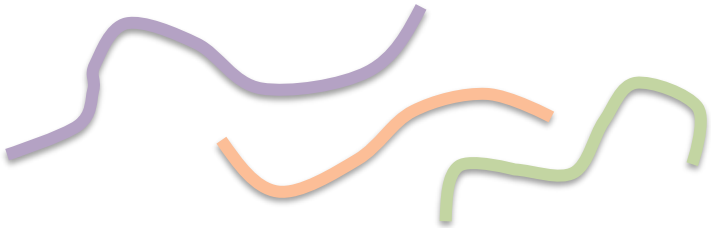


From Aziz Al'Khafaji, Broad Inst.

Biological Investigations Empowered by Transcriptomics

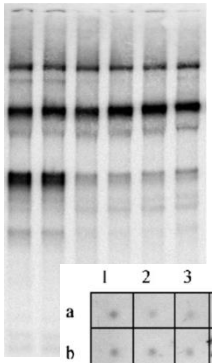


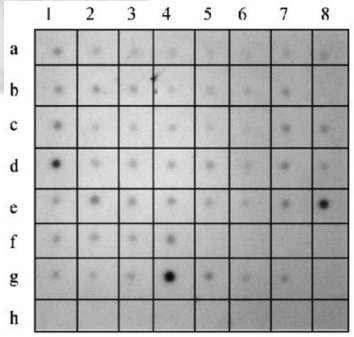
Extract RNA,
… some protocol for processing, …



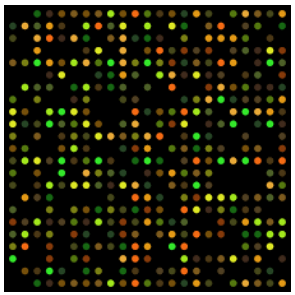
Analysis Method
(pick your favorite)

Northern

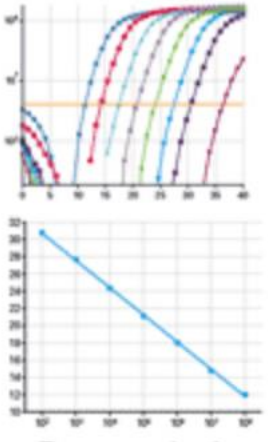




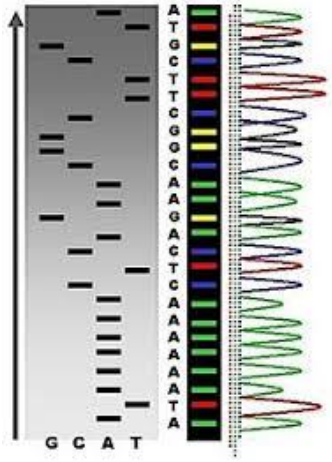
Dot Blot



Microarray



qRT-PCR



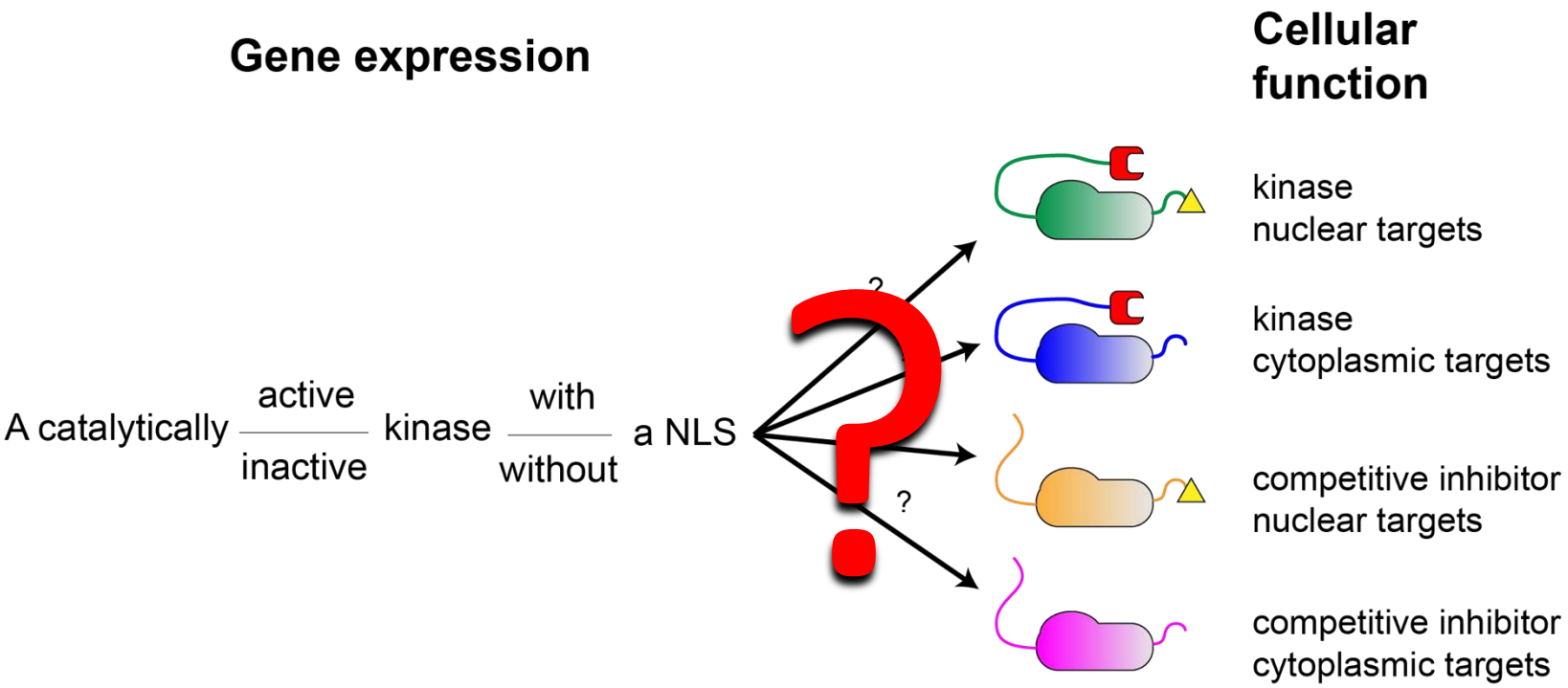
Sanger Sequencing

Other…

Minion

MinION

Gene expression analyses ignore isoform variation



Need to resolve isoforms for deeper insights into cellular functions

From Aziz Al'Khafaji, Broad Inst.

Historical Timeline to Modern Transcriptomics (from 1970)

Reverse Transcription (1970)

Northern Blot
Sanger Sequencing
(1977)

Expressed Sequence Tags (1992)

cDNA microarrays (1995)

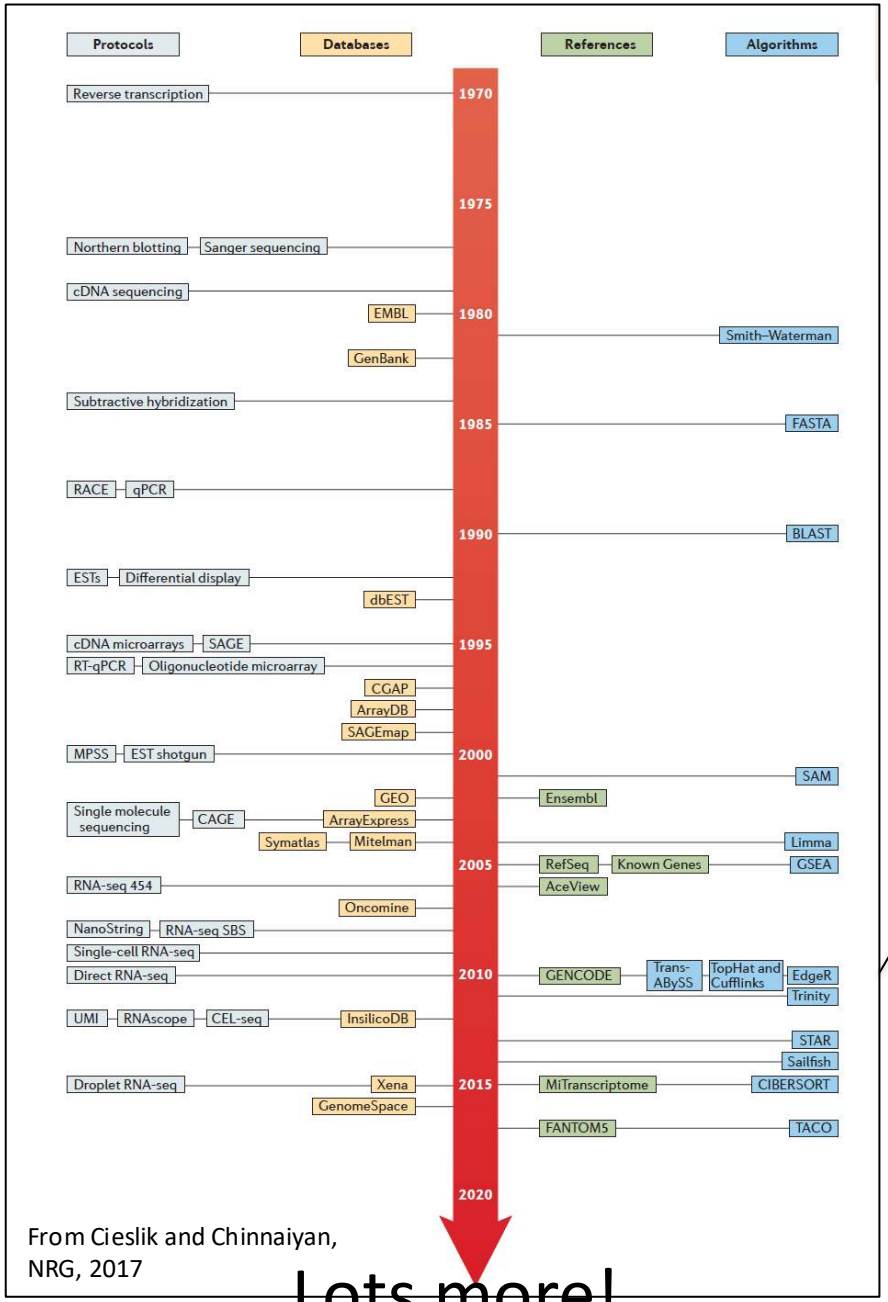
RNA-Seq (2006-2008)

PacBio IsoSeq (2014)

Droplet single cell RNA-Seq (2015)

Direct RNA Seq Nanopore (2018)

SlideSeq-v2 (2021)



From Cieslik and Chinnaiyan, NRG, 2017

Lots more!

Note: Just a small sampling of what's available.

Smith Waterman (1981)

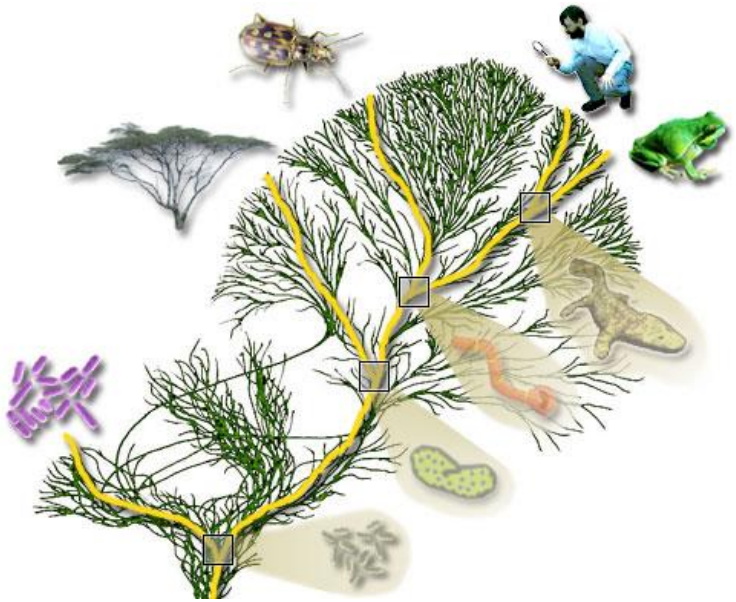
BLAST (1990)

SAMtools (2009)
Tophat/Cufflinks (2010)

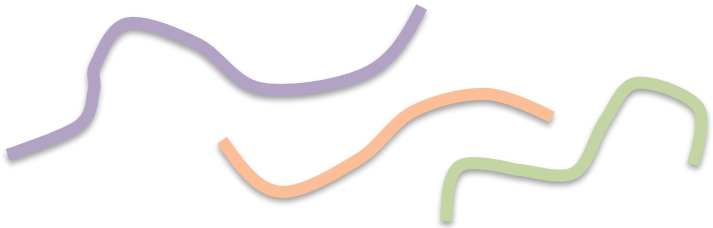
RSEM (2011)

STAR (2013)
StringTie (2015)
Kallisto (2016)
Salmon (2017)
minimap2 (2018)
Seurat-v2 (2021)

Modern Transcriptome Studies Empowered by RNA-seq



Extract RNA, convert to cDNA



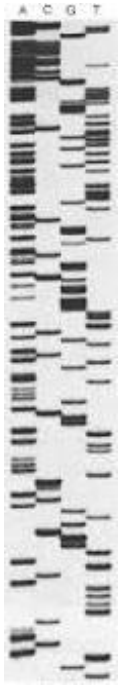
Next-gen Sequencer
(pick your favorite)

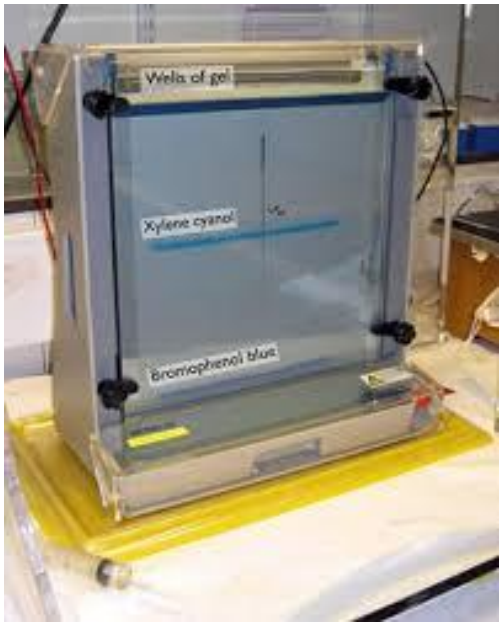
Millions to Billions of Reads

RNA-seq

Personal Reflections...

Circa 1995





Generating RNA-Seq: *How to Choose?*

Platform	iSeq Project Firefly 2018	MiniSeq	MiSeq	Next Seq 550	HiSeq 2500 RR	Hiseq 2500 V3	HiSeq 2500 V4	HiSeq 4000	HiSeq X	Nova Seq S1 2018	Nova Seq S2	Nova Seq S4	5500 XL	318 HiQ 520	Ion 530	Ion Proton P1	PGM HiQ 540	RS P6-C4	Sequel	R&D end 2018	Smidg ION RnD	Mini ION R9.5	Grid ION X5	Prome thION RnD	Prome thION theor etical	QiaGen Gene Reader	BGI SEQ 500	BGI SEQ 50	#
Reads: (M)	4	25	25	400	600	3000	4000	5000	6000	3300	6600	20000	1400	3-5	15-20	165	60-80	5.5	38.5	--	--	--	--	--	--	400	1600	1600	--
Read length: (paired-end*)	150*	150*	300*	150*	100*	100*	125*	150*	150*	150*	150*	150*	60	200 400	200 400	200	200	15K	12K	32K	--	--	--	--	--	--	100*	50	--
Run time: (d)	0.54	1	2	1.2	1.125	11	6	3.5	3	1.66	1.66	1.66	7	0.37	0.16	--	0.16	4.3	--	--	--	2	2	2	--	--	1	0.4	--
Yield: (Gb)	1	7.5	15	120	120	600	1000	1500	1800	1000	2000	6000	180	1.5	7	10	12	12	5	150	4	8	40	2400	11000	80	200	8	--
Rate: (Gb/d)	1.85	7.5	7.5	100	106.6	55	166	400	600	600	1200	3600	30	5.5	50	--	93.75	2.8	--	--	--	4	20	1200	5500	--	200	20	--
Reagents: ($K)	0.1	1.75	1	5	6.145	23.47	29.9	--	--	--	--	--	10.5	0.6	--	1	1.2	2.4	--	1	--	0.5	1.5	--	--	0.5	--	--	--
per-Gb: ($)	100	233	66	50	51.2	39.1	31.7	20.5	7.08	18	15	5.8	58.33	--	--	100	--	200	80	6.6	--	62.5	37.5	20	4.3	--	--	--	--
hg-30x: ($)	12000	28000	8000	5000	6144	4692	3804	2460	849.6	1800	1564	700	7000	--	--	12000	--	24000	9600	1000	--	7500	4500	2400	500	--	600	--	
Machine: ($)	30K	49.5K	99K	250K	740K	690K	690K	900K	1M	999K	999K	999K	595K	50K	65K	243K	242K	695K	350K	350K	--	--	125K	75K	75K	--	200K	--	--
#Page maintained by http://twitter.com/albertvilella http://tinyurl.com/ngslytics #Editable version: http://tinyurl.com/ngsspecsshared																													
#curl "https://docs.google.com/spreadsheets/d/1GMMfhyLK0-q8XkIo3YxlWaZA5vVMuhU1kg41g4xLkXc/export?gid=4&format=csv" | grep -v '^#' | grep -v '^"' | column -t -s\, | less -S																													

Stats circa 2018

For current, see: **https://tinyurl.com/wbgcs65**

Illumina PacBio ONT

*Not all shown at scale

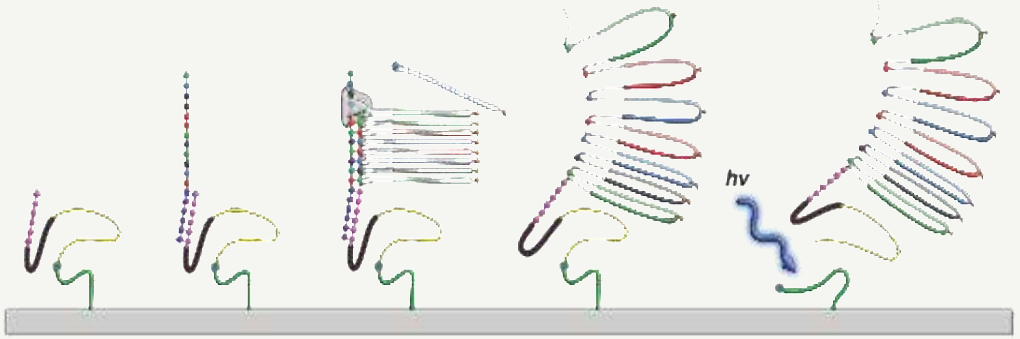
Maybe something fast and portable?

Oxford Nanopore Technology (ONT) Minion

Sequencing by Expansion (SBX) Technology

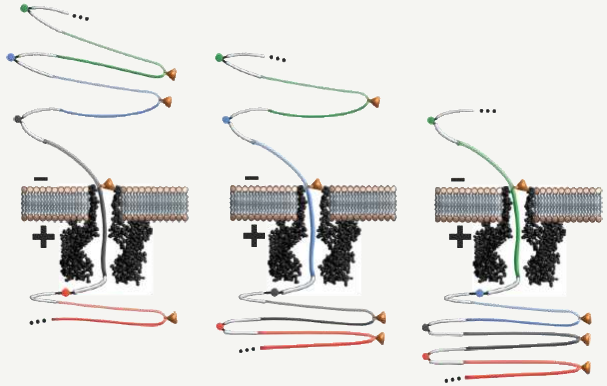
X-NTPs

Xpandomer Synthesis

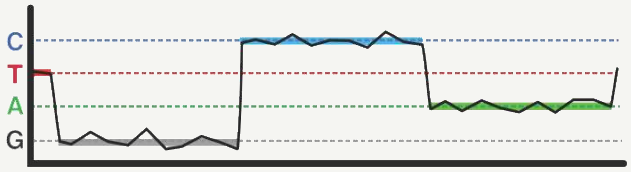


Backbone Cleavage and
Xpandomer Expansion

Xpandomers Travel Through Nanopore Detectors



Xpandomer Signals Detected



https://sequencing.roche.com/us/en/article-listing/sequencing-platform-technologies.html

Lab Sets Guinness World Record for DNA Sequencing Speed

Oct 17, 2025 | Company News | ★★★★★

Teams complete whole human genome sequencing and analysis in under four hours, demonstrating potential for same-day NICU workflows.

Broad Clinical Labs, in collaboration with Roche Sequencing Solutions and Boston Children's Hospital, has achieved official recognition from Guinness World Records for the fastest DNA sequencing technique, completing sequencing and analysis of a whole human genome in less than four hours.

https://clpmag.com/lab-management/company-news/lab-collaboration-sets-guinness-world-record-dna-sequencing-speed/

A Plethora of Biological Sequence Analyses Enabled by RNA-Seq

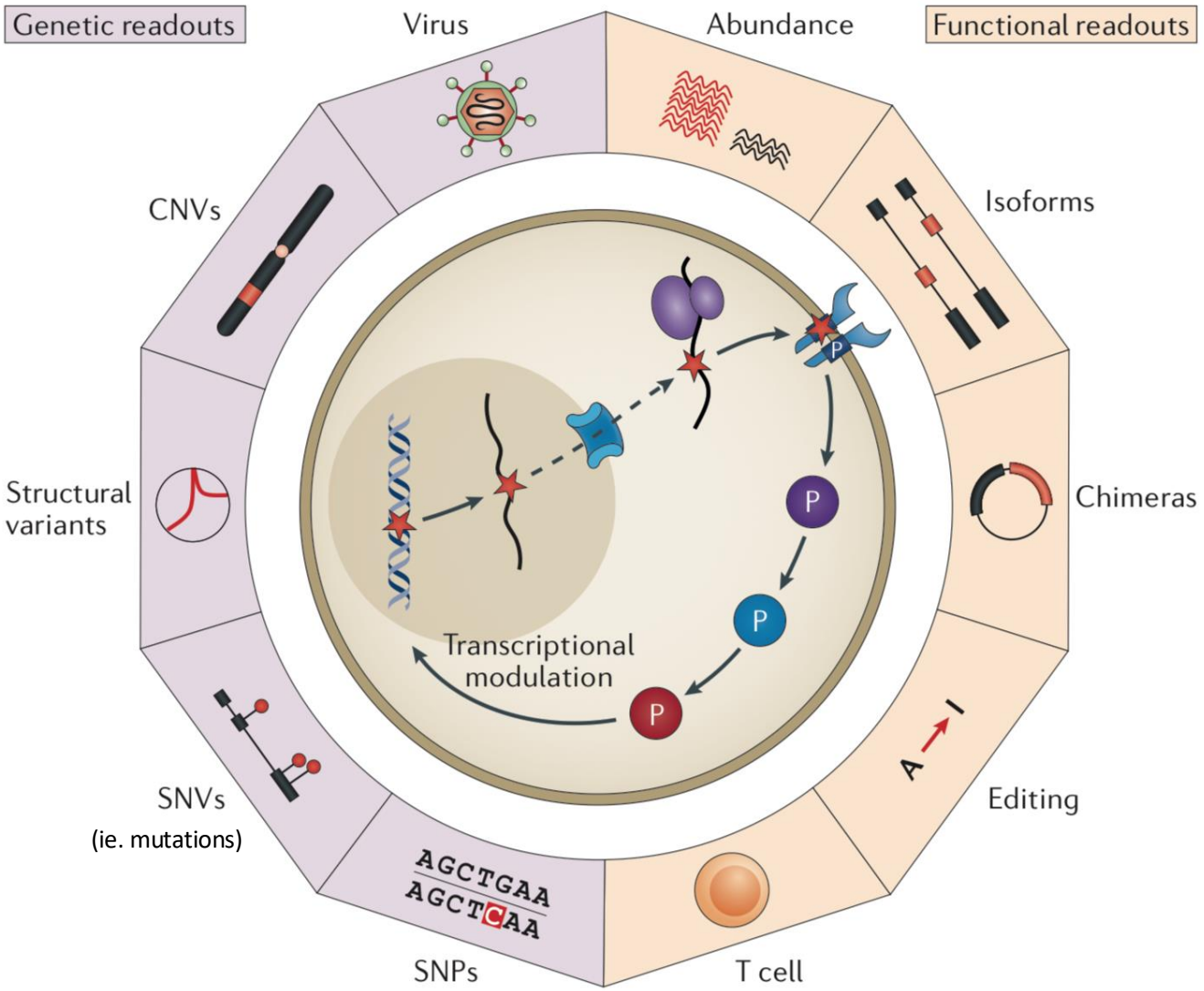
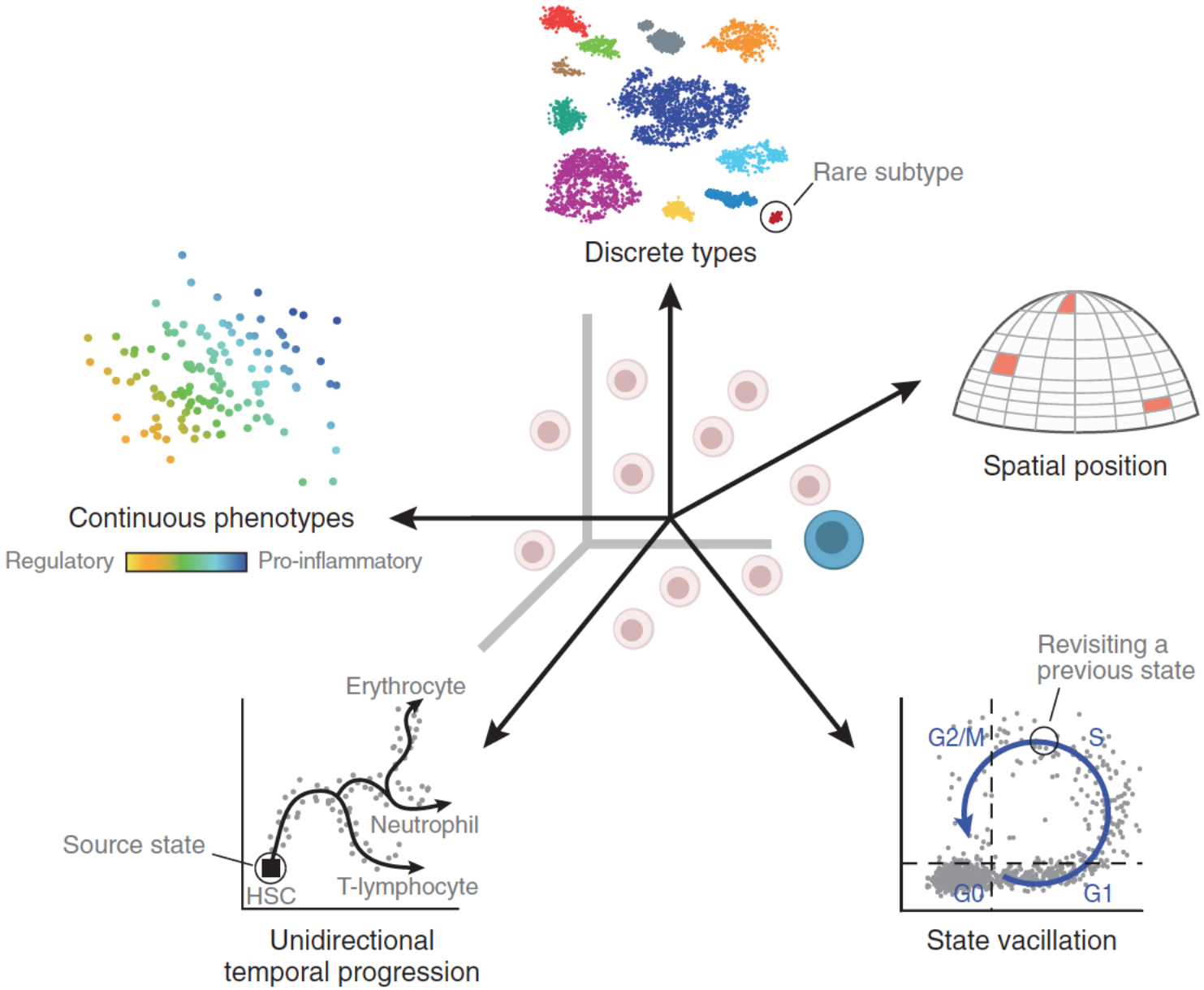


Figure 2 | **Transcriptome profiling for genetic causes and functional phenotypic readouts.**

From Cieslik and Chinnaiyan, NRG, 2017

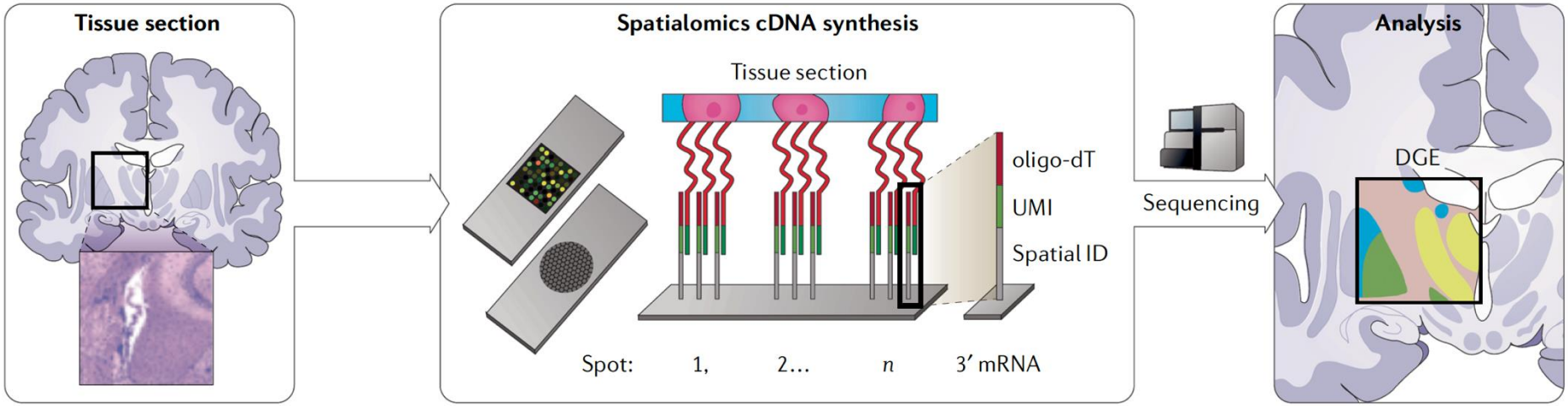
RNA-Seq is Empowering Discovery at Single Cell Resolution



Wagner, Regev, and Yosef. NBT 2016

Spatial Transcriptomics

Spatial Encoding



From “RNA sequencing: the teenage years”
Rory Stark, Marta Grzelak & James Hadfield
Nature Reviews Genetics volume 20, pages631–656(2019)

A Myriad of Other Specialized RNA-seq -based Applications

RNA-Sequencing as your lens towards biological discovery

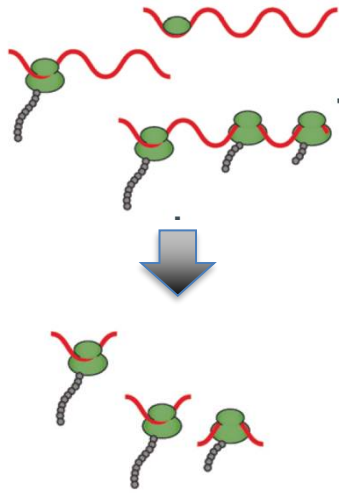
UV crosslink Biotin

RNase V1 (digests dsRNA) RNase S1 (digests ssRNA)

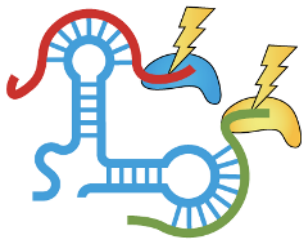
Adapted from "RNA sequencing: the teenage years"
Rory Stark, Marta Grzelak & James Hadfield
Nature Reviews Genetics volume 20, pages631–656(2019)

A Myriad of Other Specialized RNA-seq -based Applications

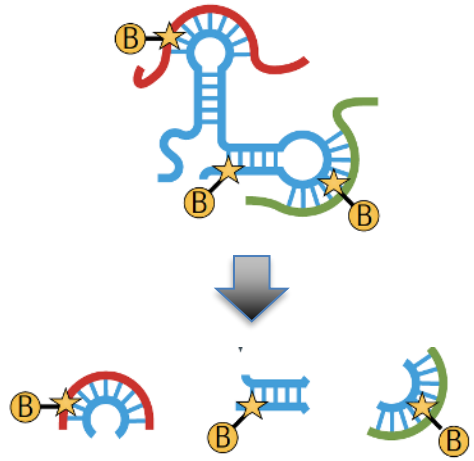
Ribosomal profiling



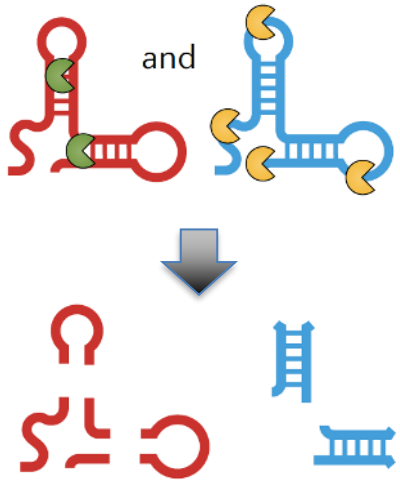
RNA-Protein Interactions

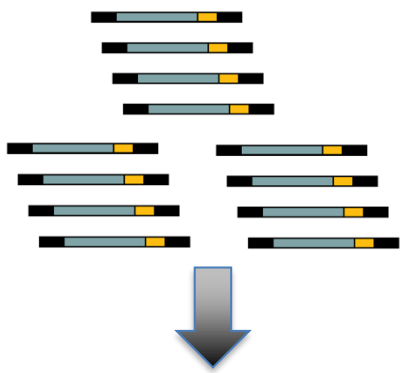


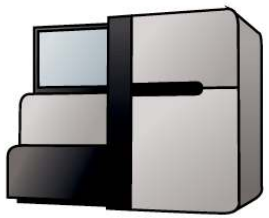
RNA-RNA interactions



RNA Structuromics







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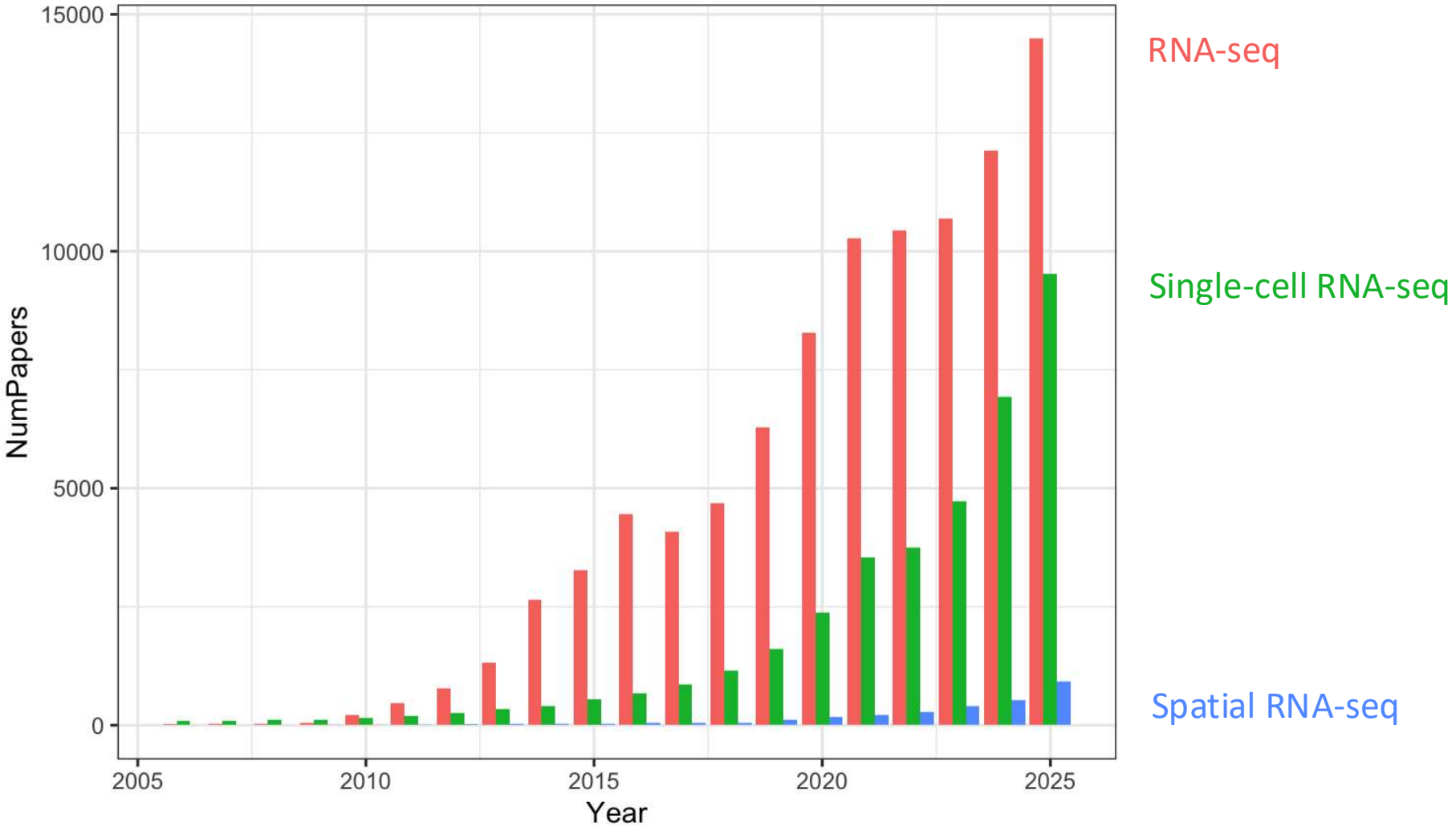
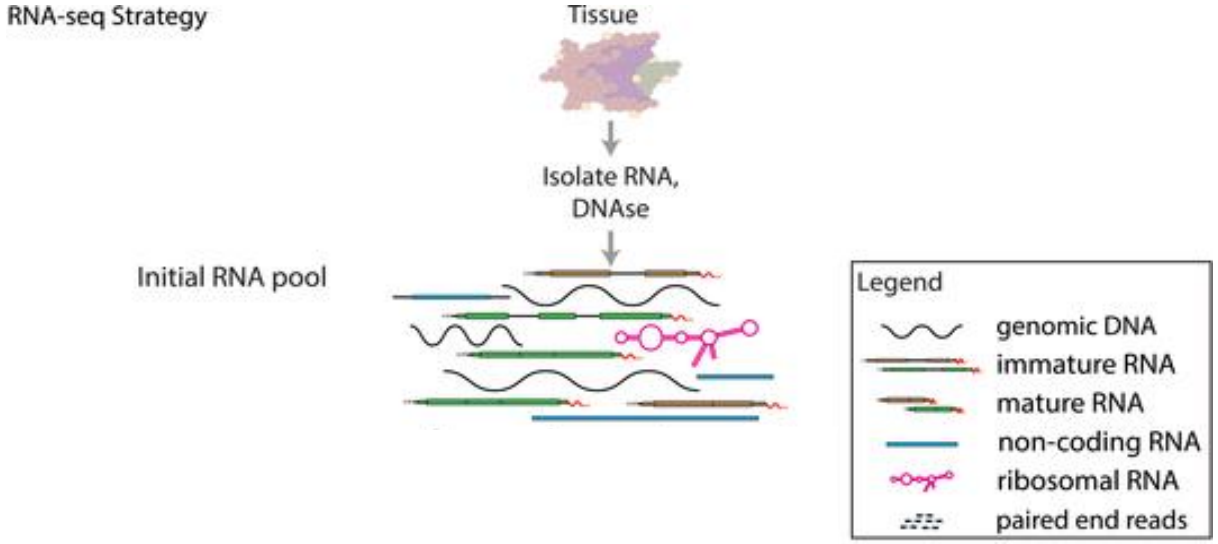
RNA-seq Publication Trend

Paper Counts from PubMed



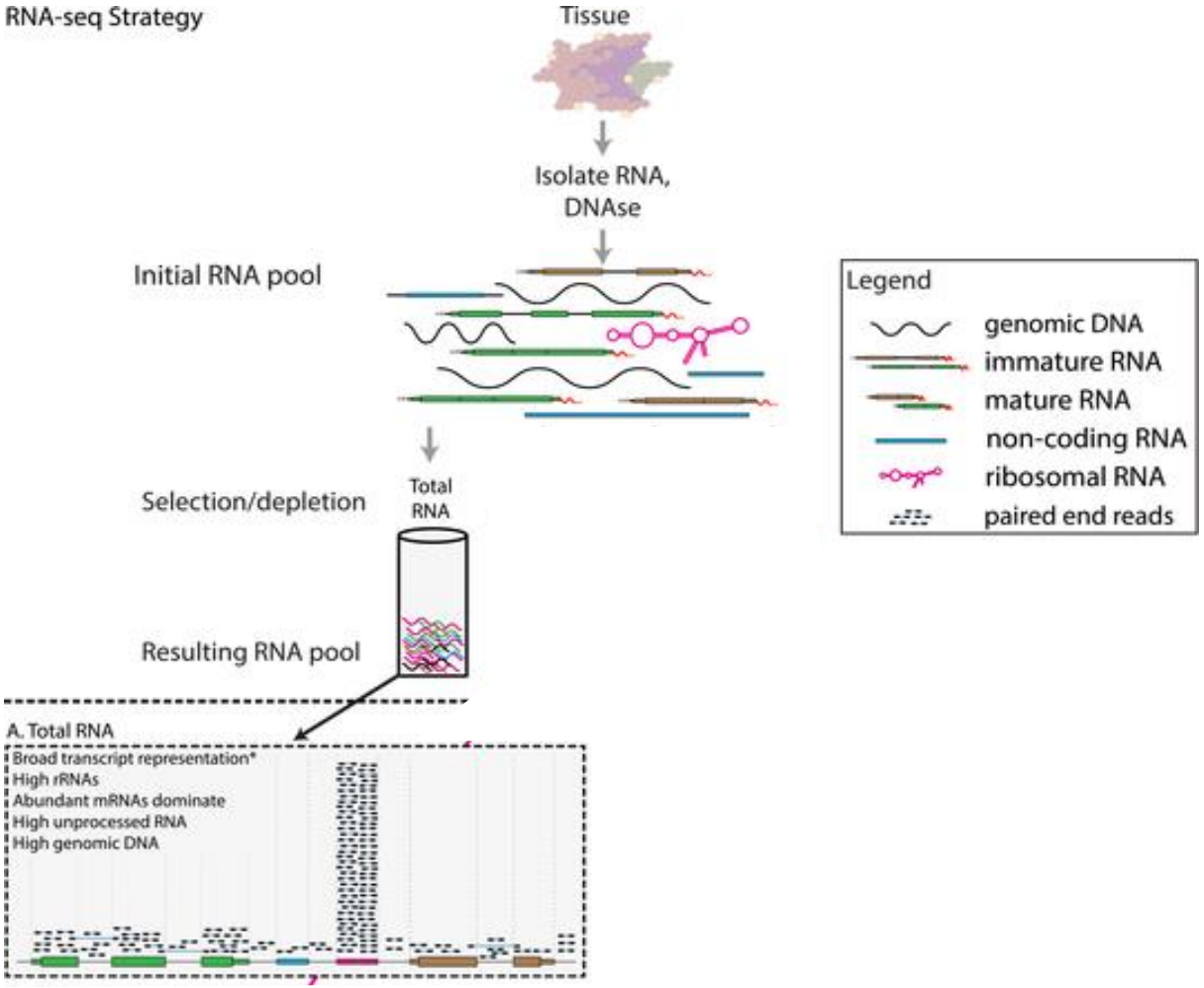
RNA-seq library enrichment strategies that influence interpretation and analysis.



http://journals.plos.org/ploscompbiol/article?id=10.1371/journal.pcbi.1004393

PLOS | COMPUTATIONAL BIOLOGY

RNA-seq library enrichment strategies that influence interpretation and analysis.



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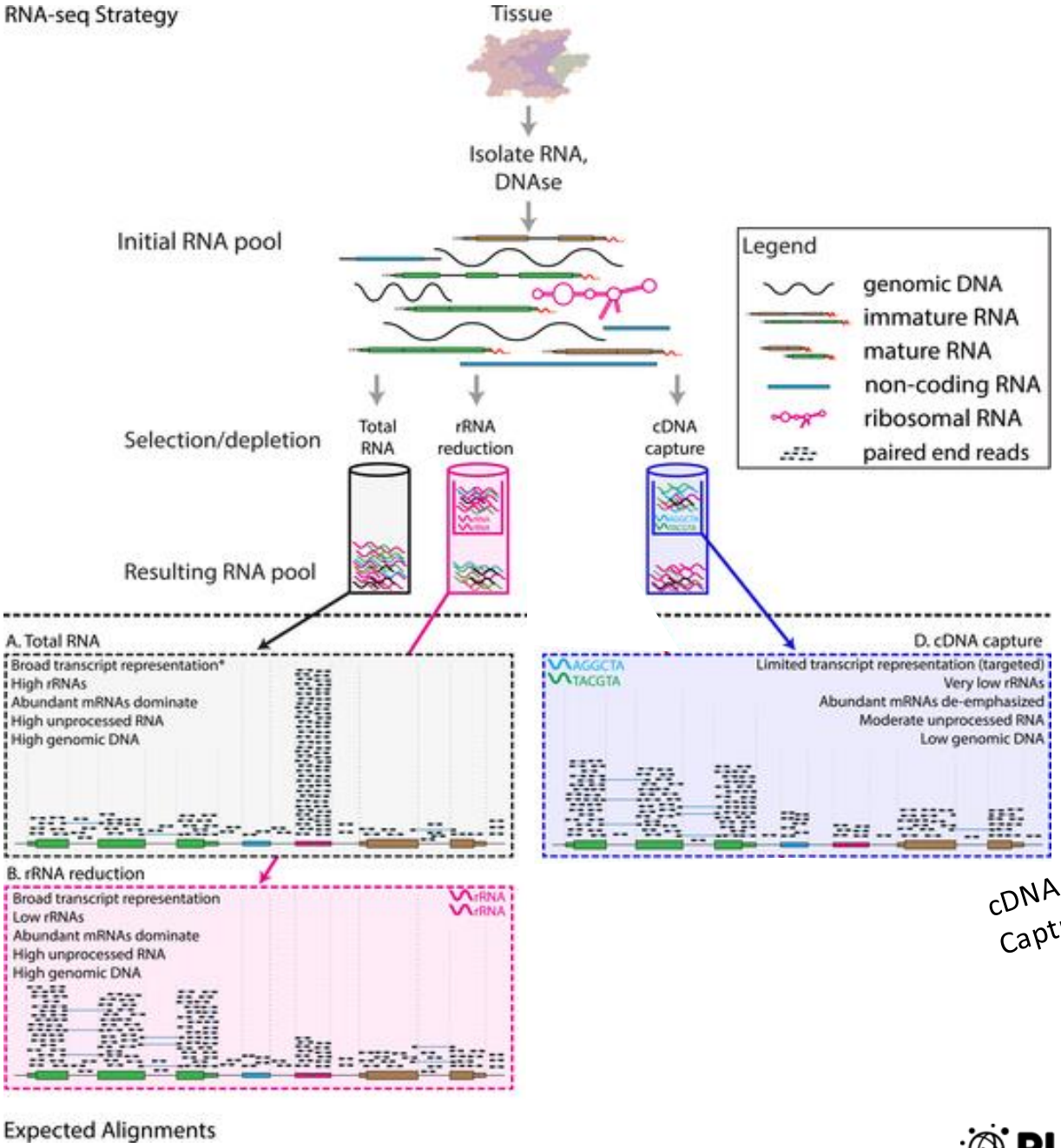

ex. Ribo-Zero

cDNA Exome Capture Kit

http://journals.plos.org/ploscompbiol/article?id=10.1371/journal.pcbi.1004393

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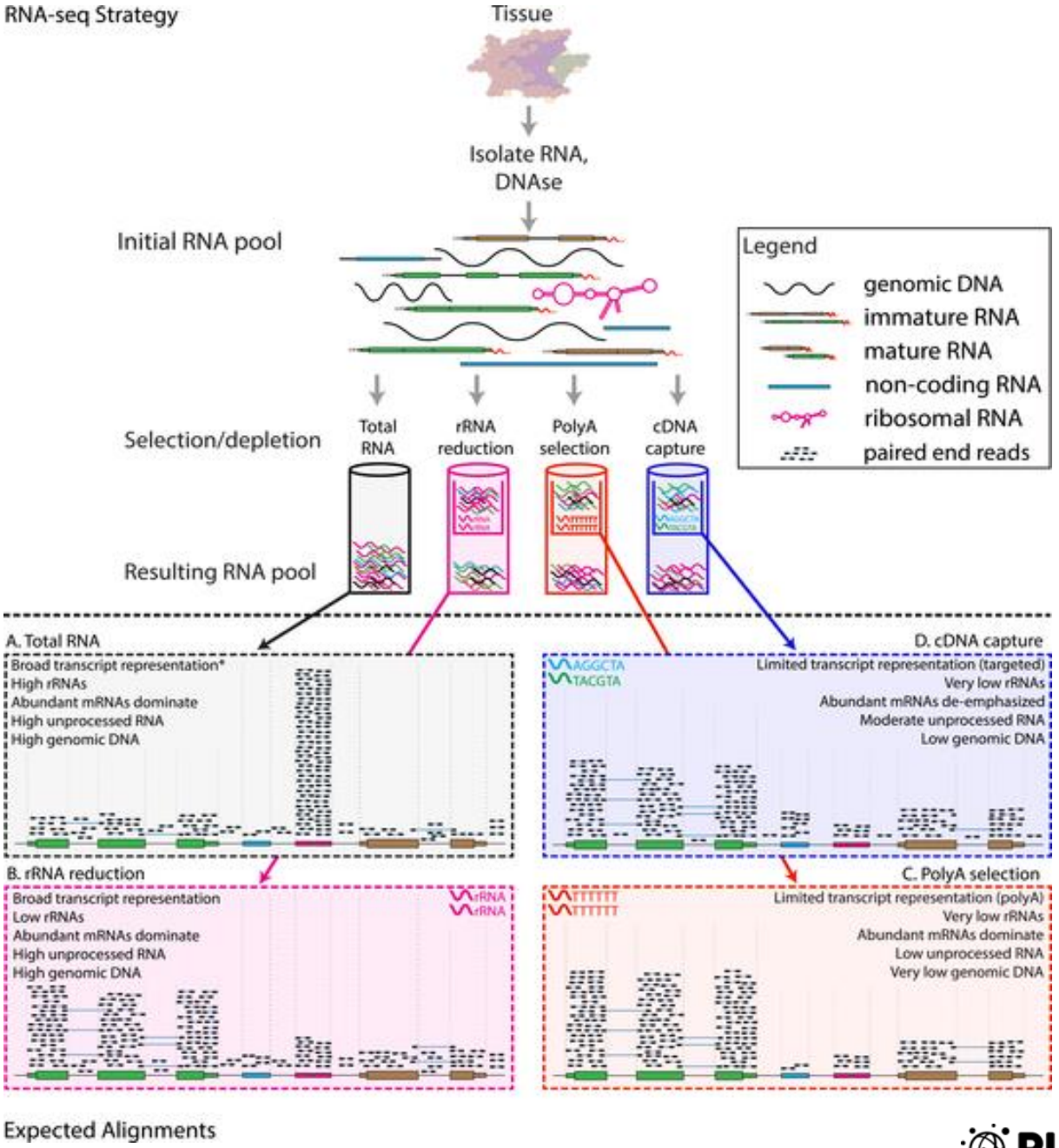
RNA-seq library enrichment strategies that influence interpretation and analysis.



http://journals.plos.org/ploscompbiol/article?id=10.1371/journal.pcbi.1004393

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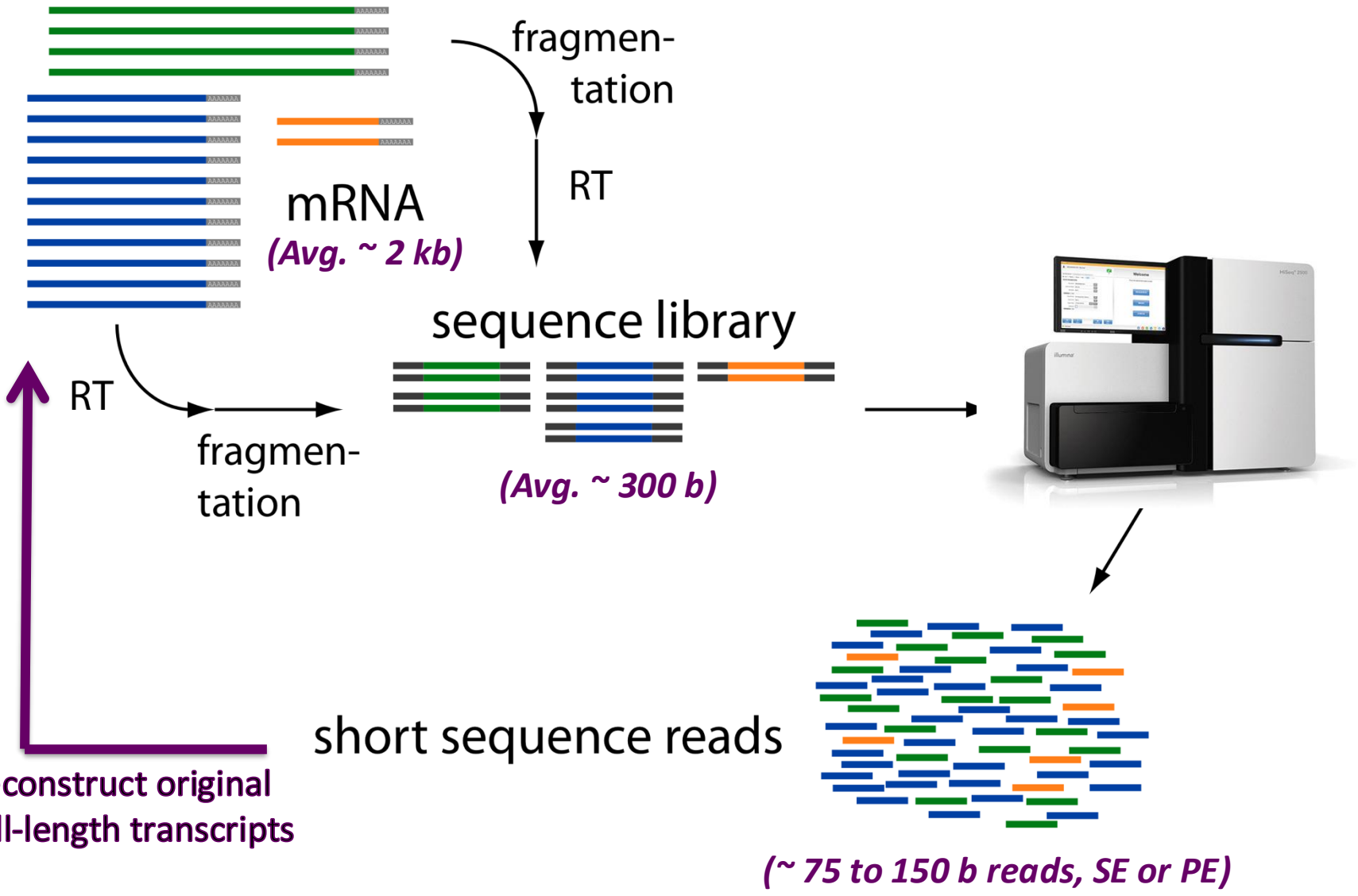
Part 2. Transcript Reconstruction Methods

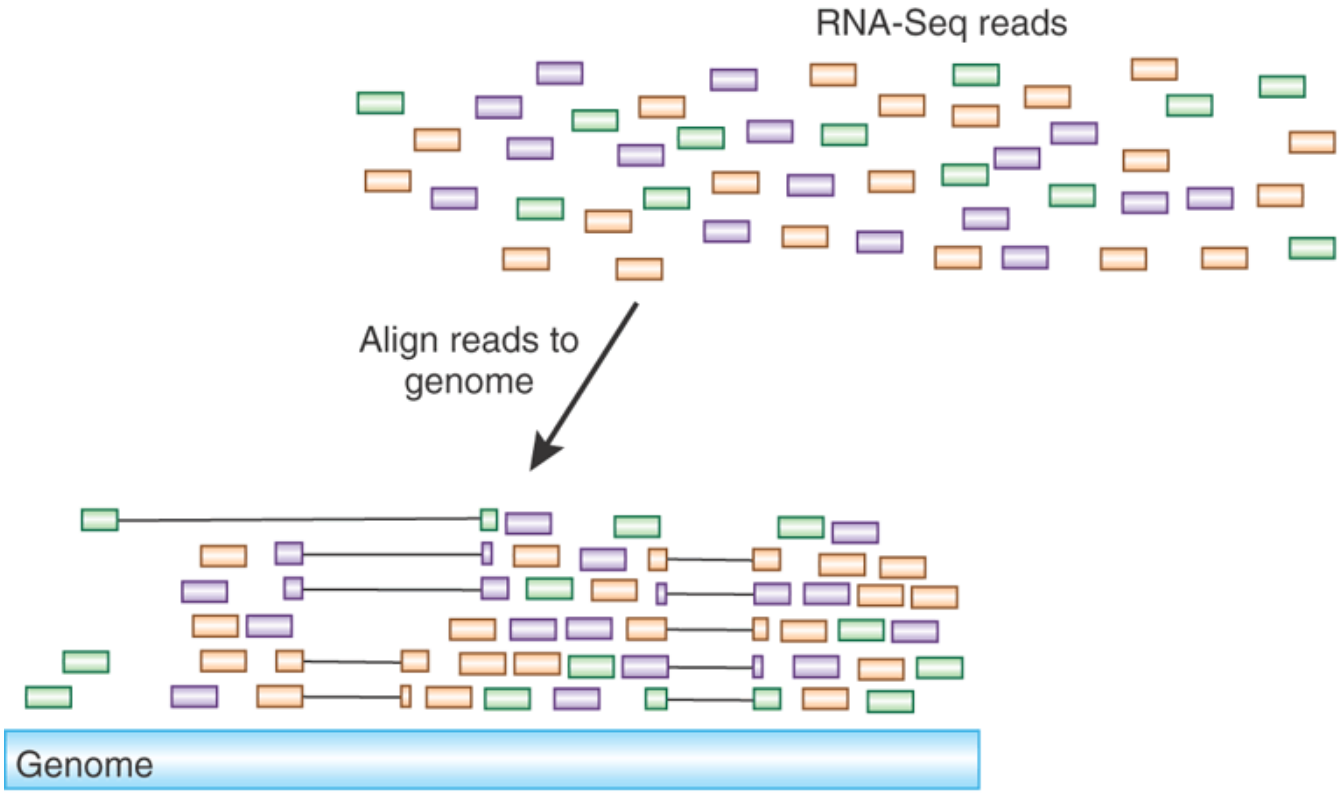
RNA-Seq Challenge: Transcript Reconstruction



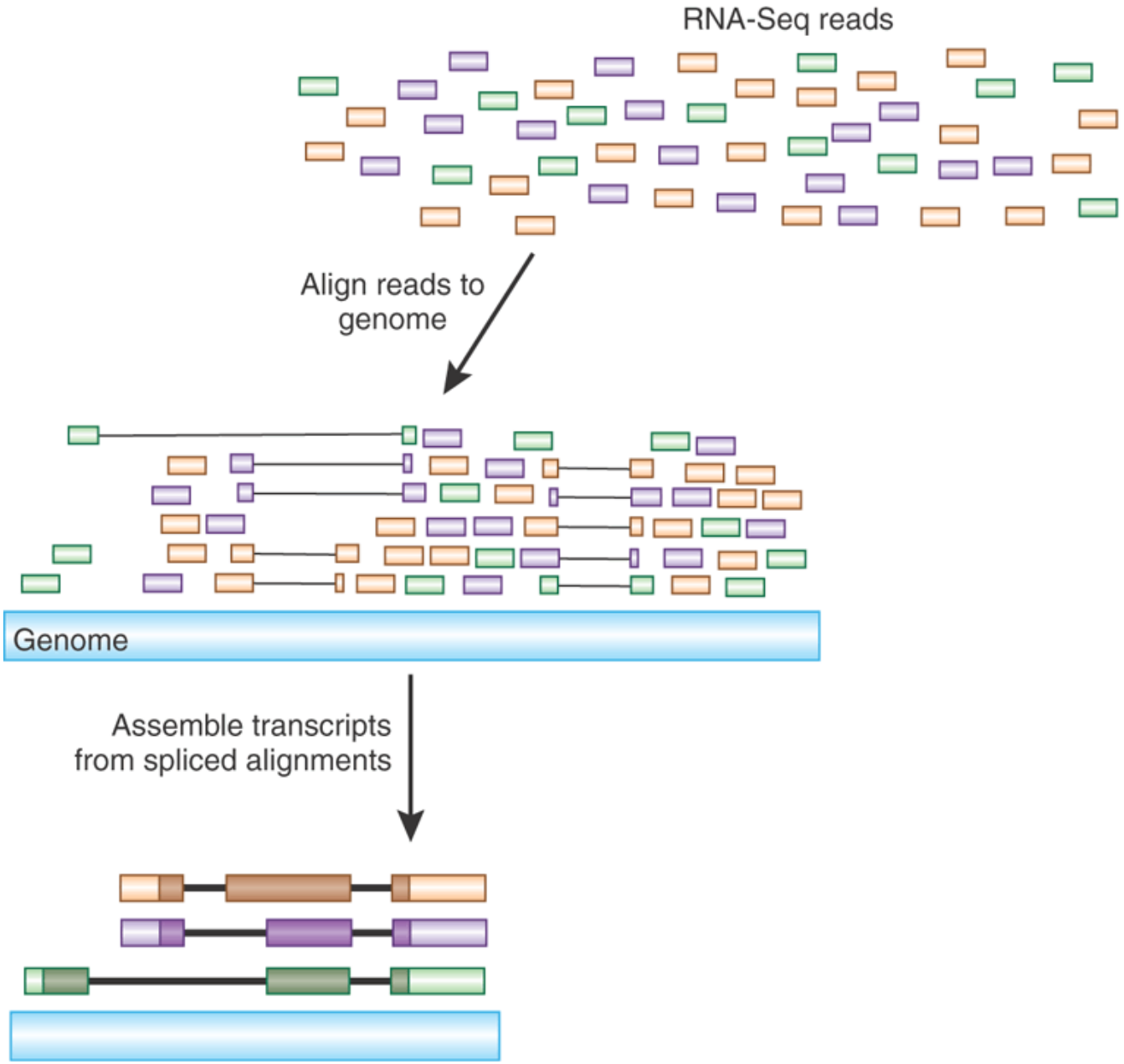
Adapted from: http://www2.fml.tuebingen.mpg.de/raetsch/members/research/transcriptomics.html

Transcript Reconstruction from (short) RNA-Seq Reads

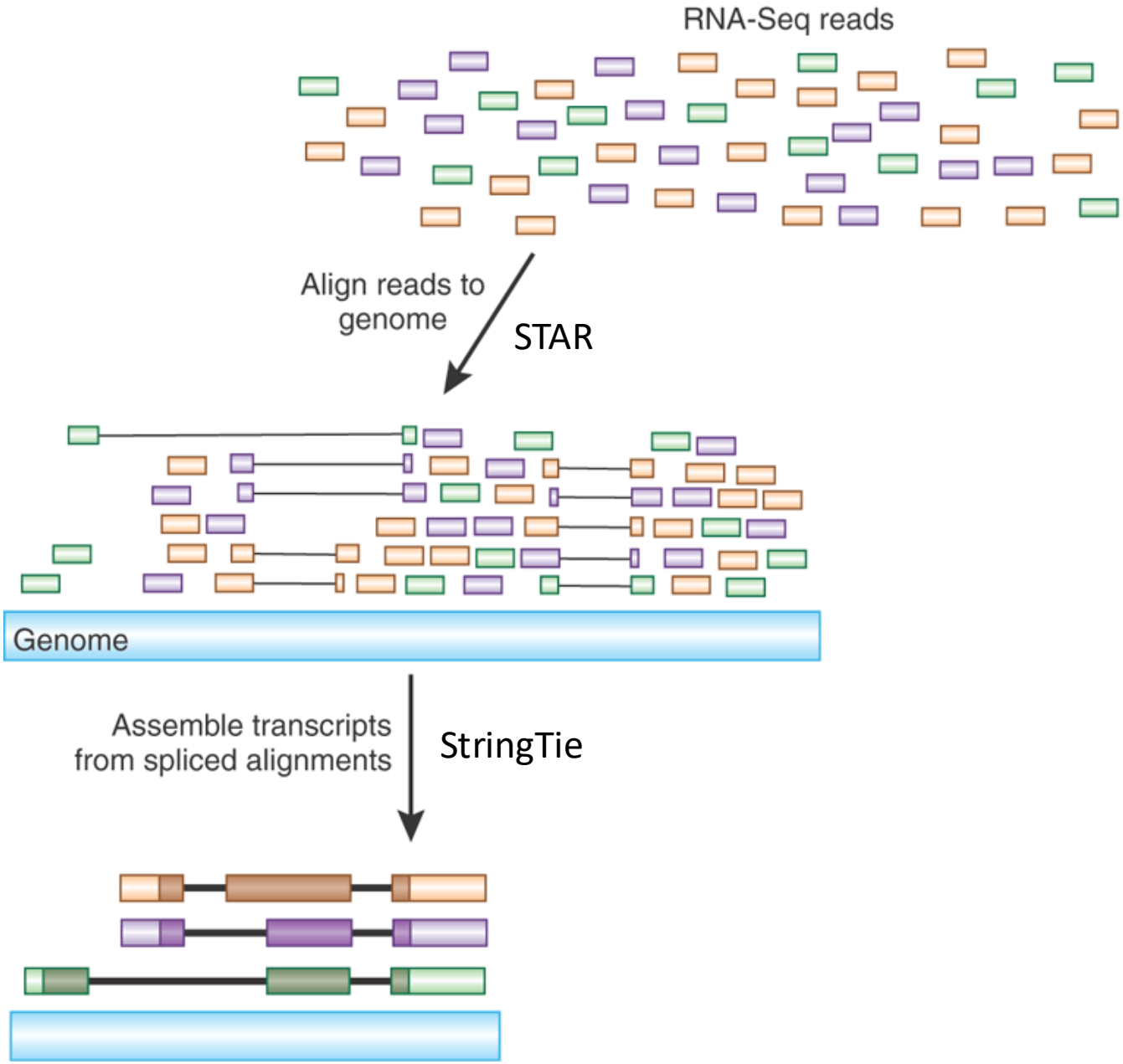
Transcript Reconstruction from (short) RNA-Seq Reads



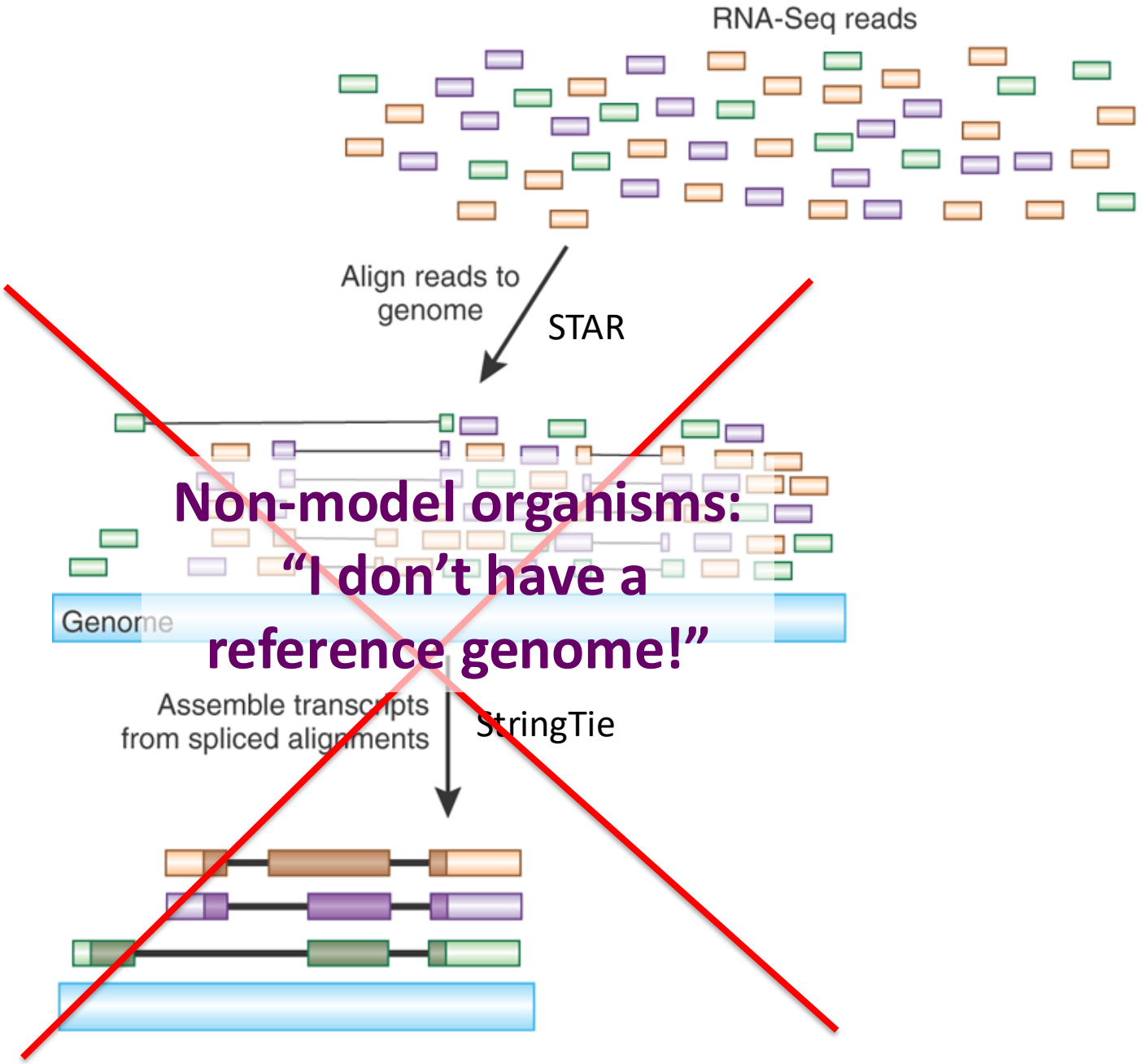
Transcript Reconstruction from (short) RNA-Seq Reads



Transcript Reconstruction from (short) RNA-Seq Reads

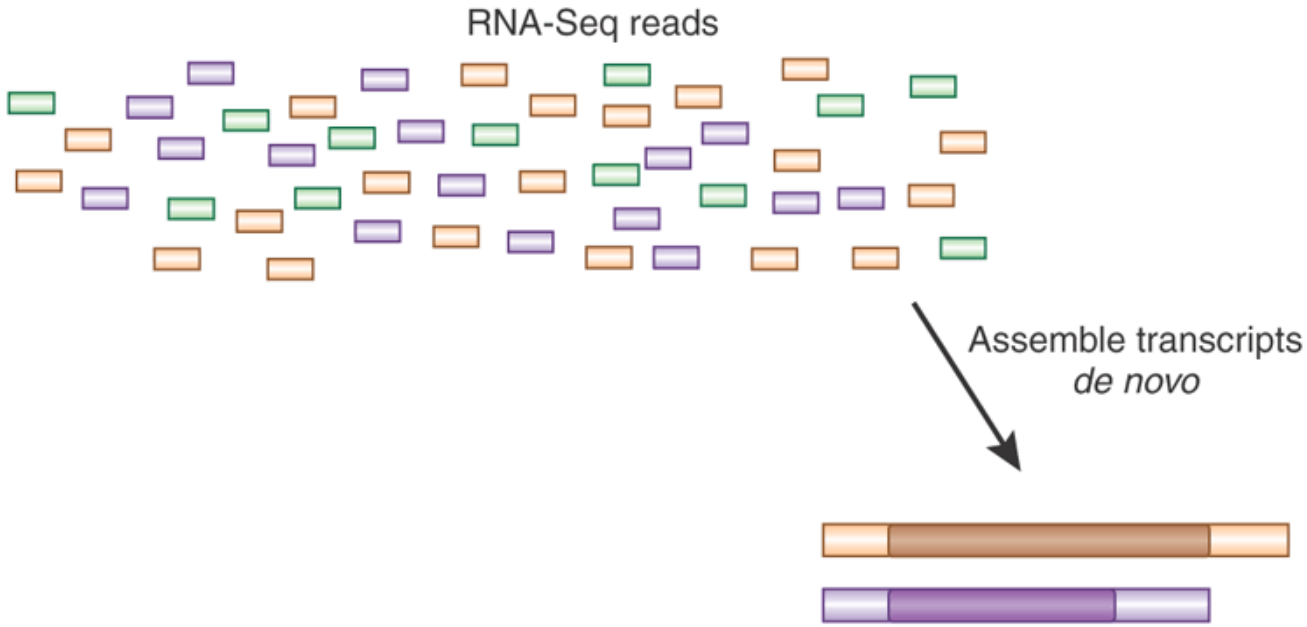


Transcript Reconstruction from (short) RNA-Seq Reads



Non-model organisms: "I don't have a reference genome!"

Transcript Reconstruction from (short) RNA-Seq Reads



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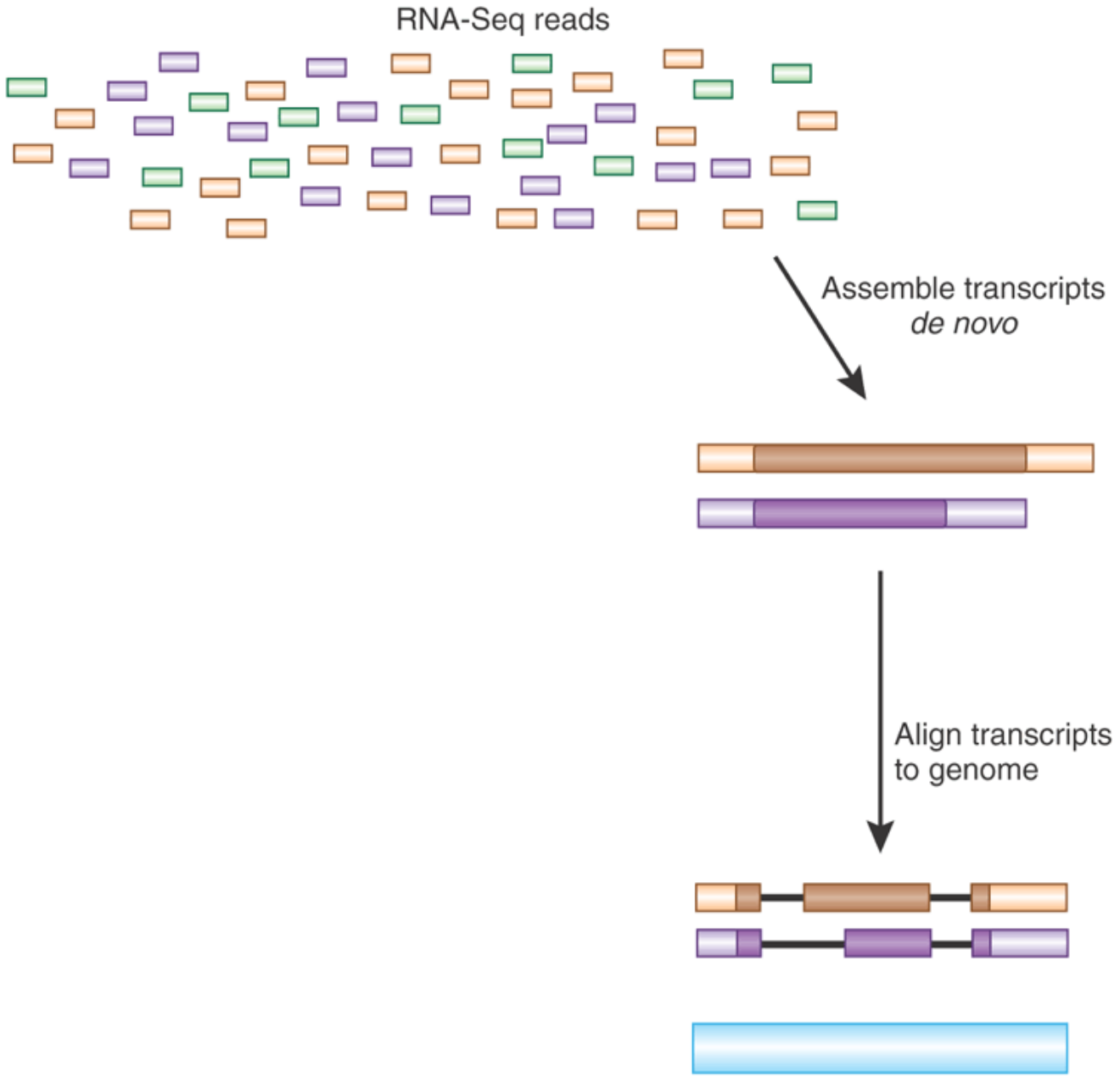


Transcript Reconstruction from (short) RNA-Seq Reads

End-to-end **Transcriptome**-based
RNA-Seq Analysis
Software Package

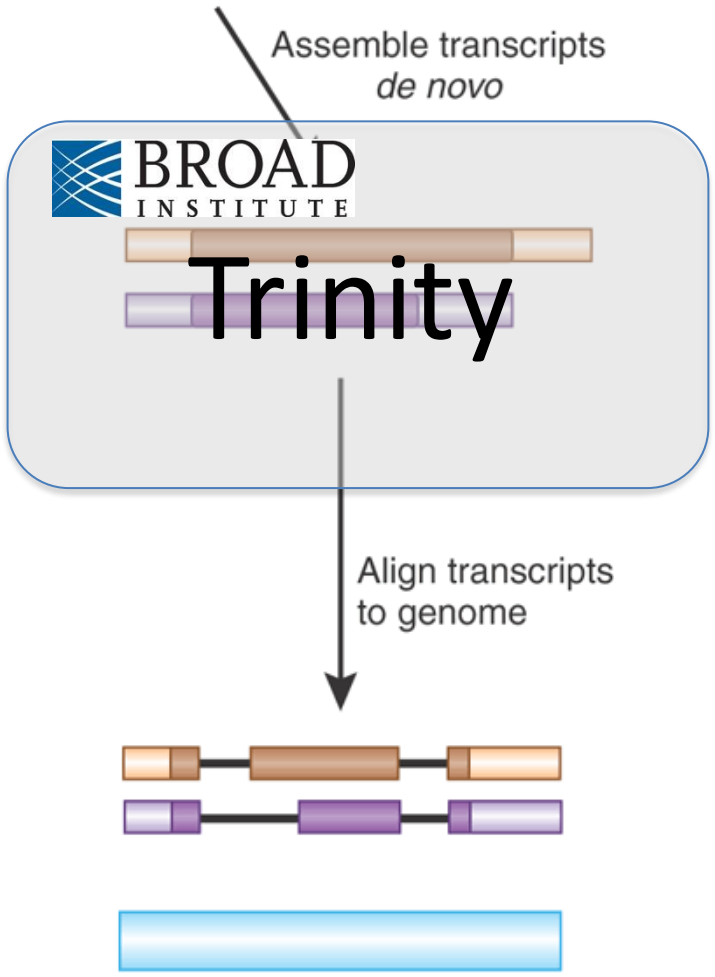
NATURE PROTOCOLS | PROTOCOL

De novo transcript sequence reconstruction from RNA-seq using the Trinity platform for reference generation and analysis

Brian J Haas, Alexie Papanicolaou, Moran Yassour, Manfred Grabherr, Philip D Blood, Joshua Bowden, Matthew Brian Couger, David Eccles, Bo Li, Matthias Lieber, Matthew D MacManes, Michael Ott, Joshua Orvis, Nathalie Pochet, Francesco Strozzi, Nathan Weeks, Rick Westerman, Thomas William, Colin N Dewey, Robert Henschel, Richard D LeDuc, Nir Friedman & Aviv Regev

Affiliations | Contributions | Corresponding authors

Nature Protocols **8**, 1494–1512 (2013) | doi:10.1038/nprot.2013.084
Published online 11 July 2013



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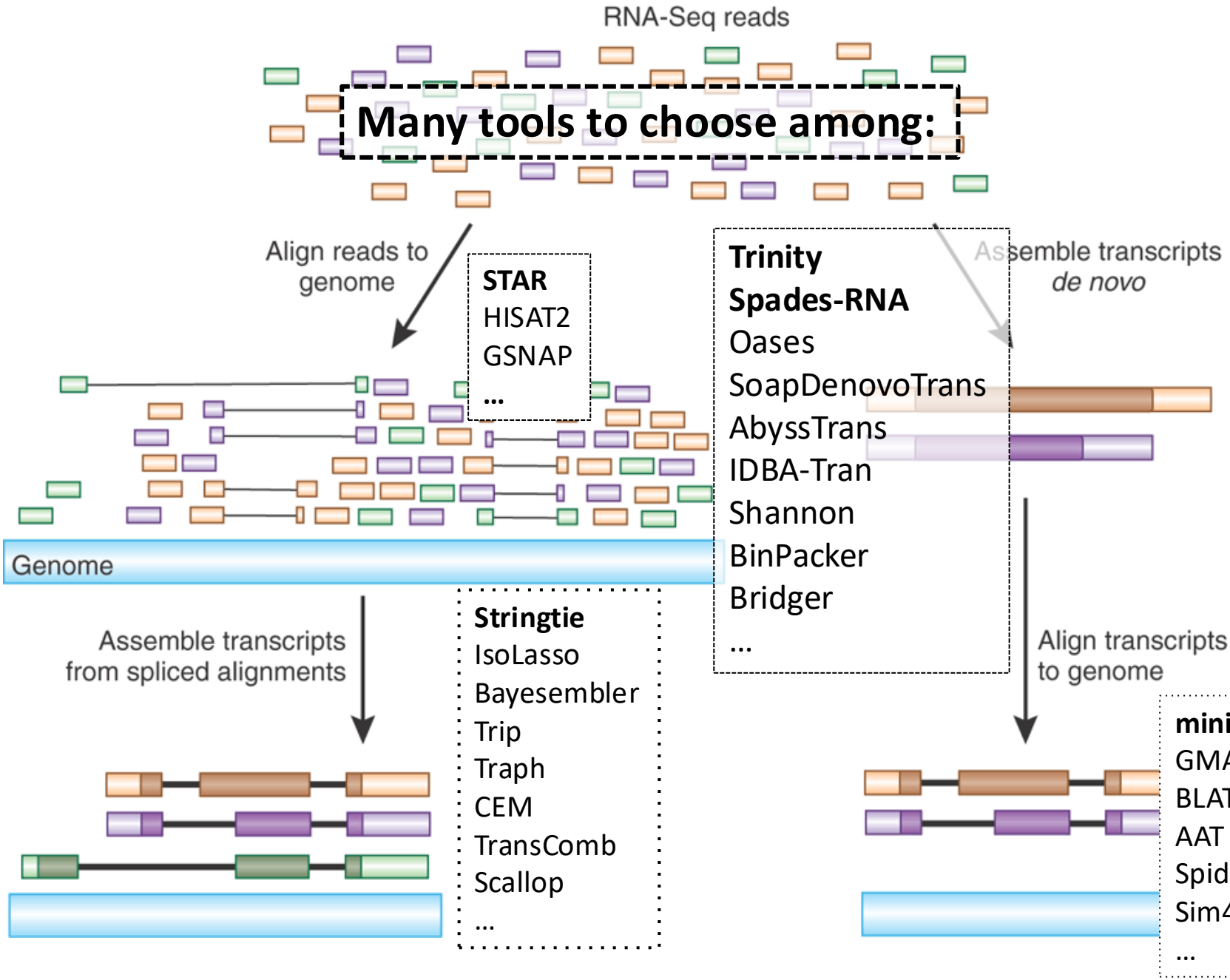
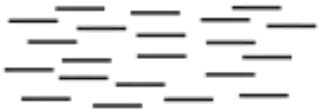
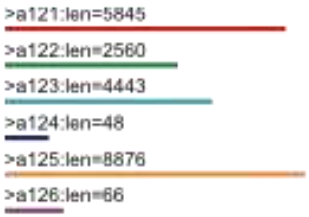


Part 3. Trinity for Genome-free transcriptomics (eg. for non-model orgs)

Trinity – How it works:

RNA-Seq reads → **Linear contigs** → **de-Bruijn graphs** → **Transcripts + Isoforms**





...CTTCGCAA...TGATCGGAT...
...ATTCGCAA...TCATCGGAT...

Thousands of disjoint graphs

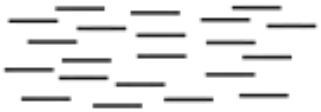
Trinity – How it works:

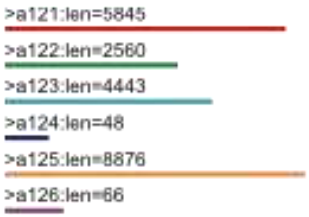
Younger me

Manfred Grabherr

Moran Yassour

RNA-Seq reads → **Linear contigs** → **de-Bruijn graphs** → **Transcripts + Isoforms**





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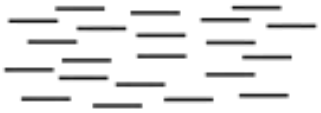

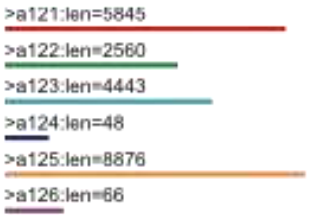

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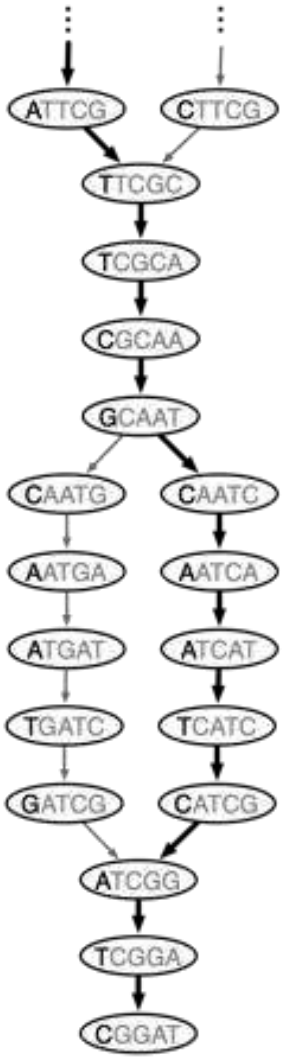
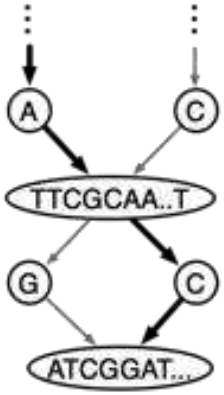
Thousands of disjoint graphs

Butterfly



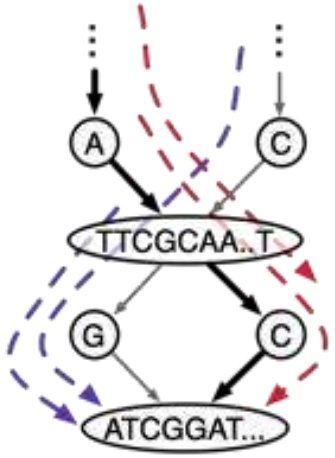
de Bruijn graph

compacting



compact graph

finding paths



compact graph with reads

extracting sequences

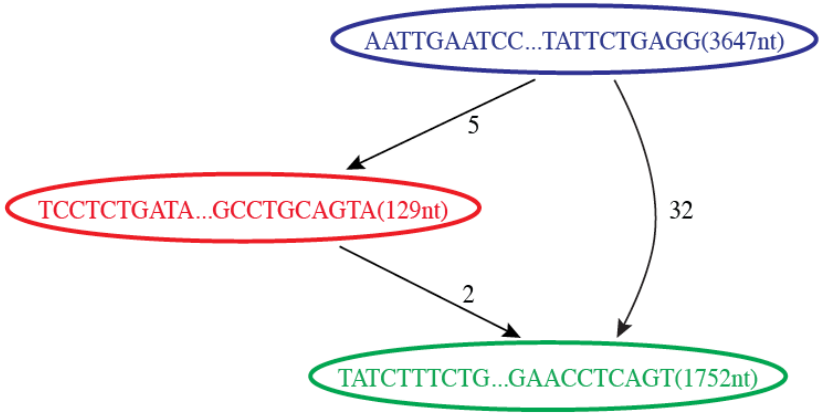
..CTTCGCAA..TGATCGGAT...

..ATTCGCAA..TCATCGGAT...

sequences (isoforms and paralogs)

Butterfly Example 1:
Reconstruction of Alternatively Spliced Transcripts

Butterfly's Compacted Sequence Graph



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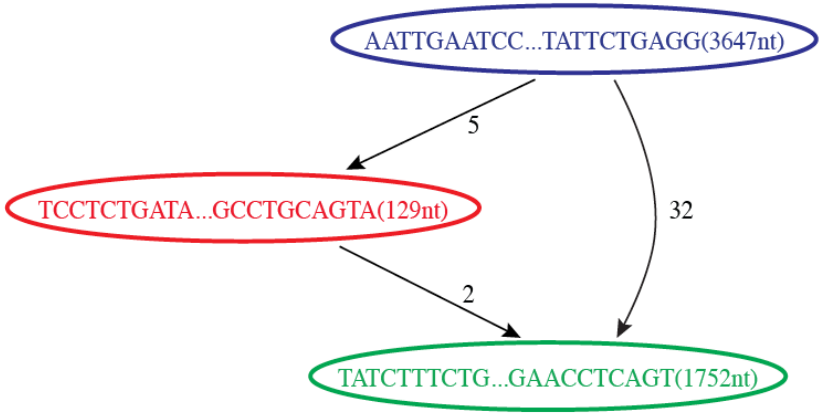

Reconstructed Transcripts

Reconstruction of Alternatively Spliced Transcripts

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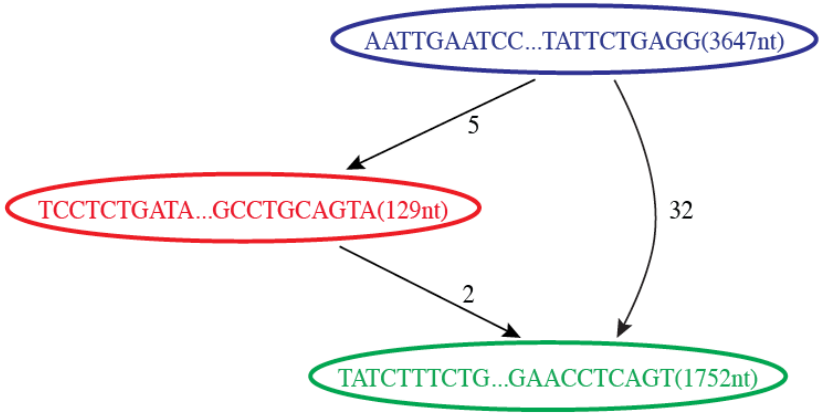

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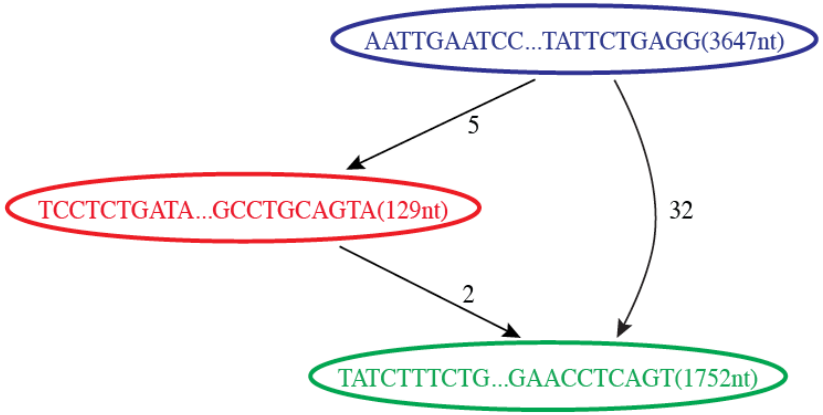
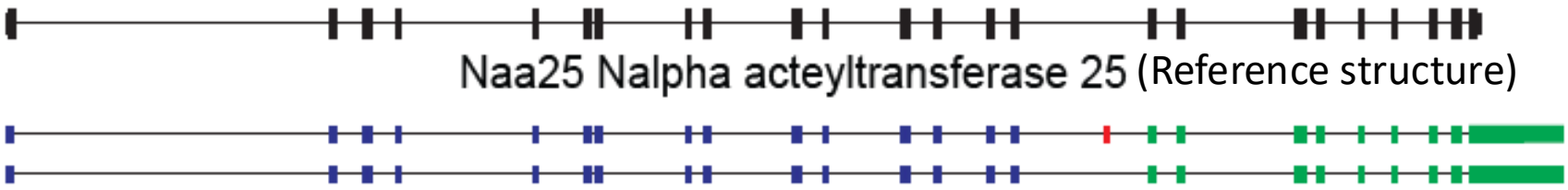
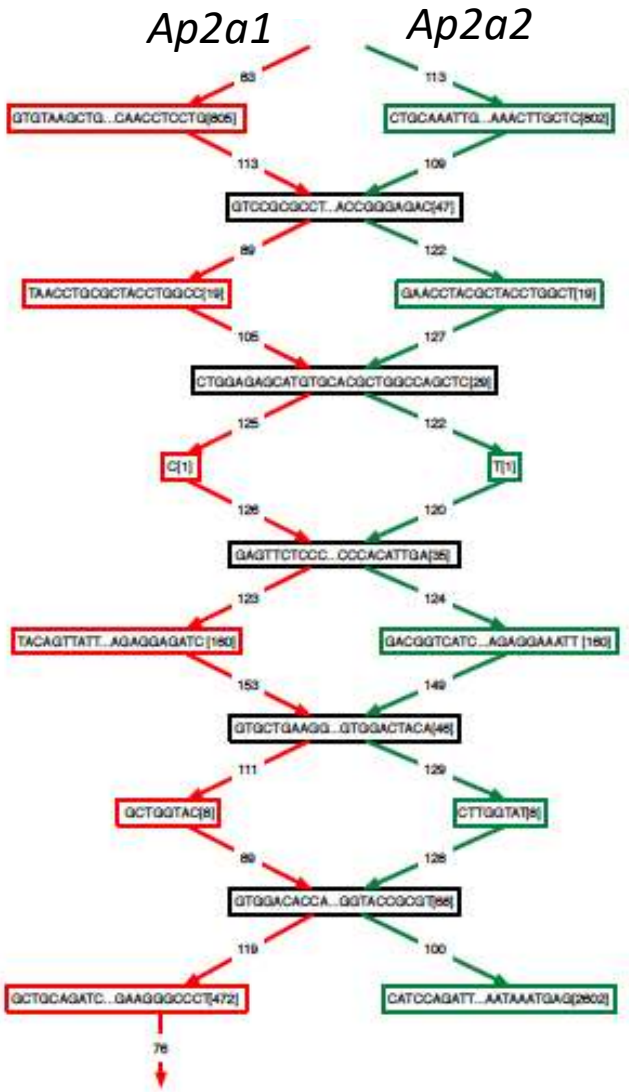


Reconstructed Transcripts

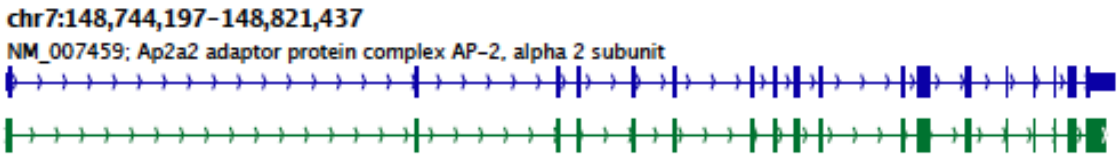
Aligned to Mouse Genome

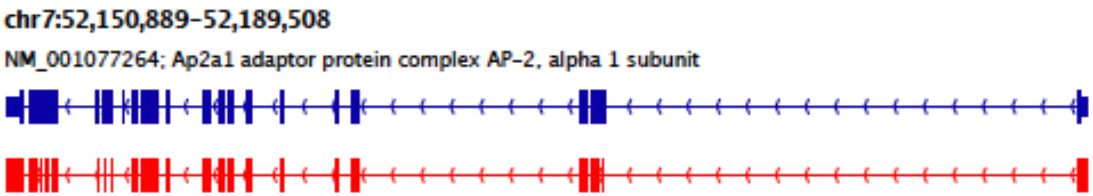


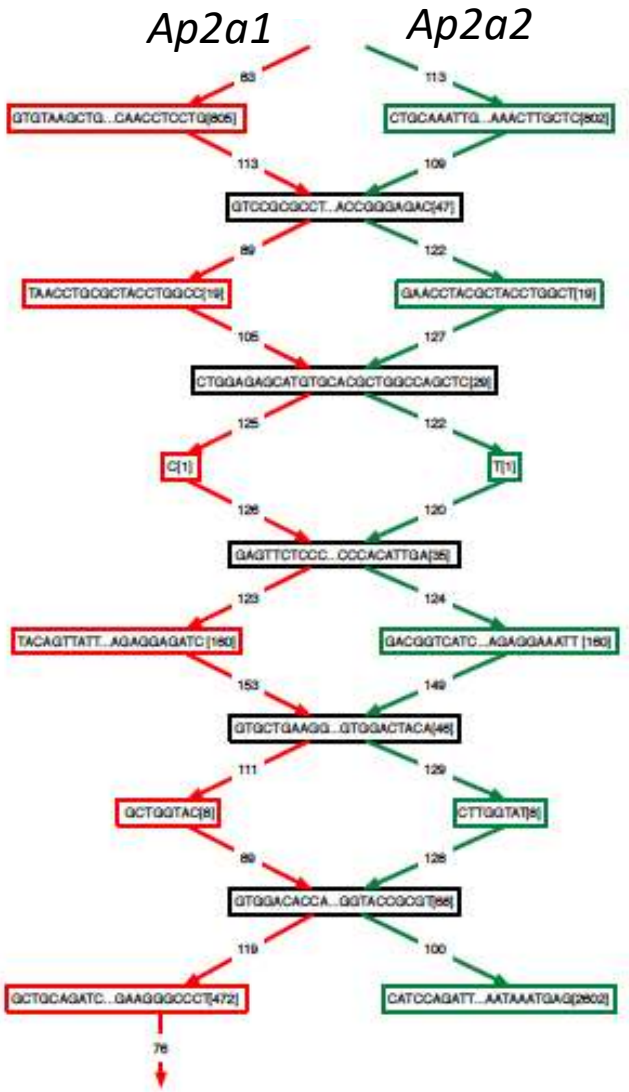
Butterfly Example 2:
Teasing Apart Transcripts of Paralogous Genes



Teasing Apart Transcripts of Paralogous Genes







Strand-specific RNA-Seq is Preferred

Computationally: fewer confounding graph structures in de novo assembly:

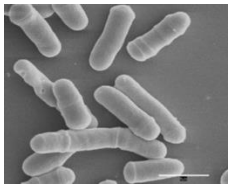
ex. Forward != reverse complement

(GGAA != TTCC)

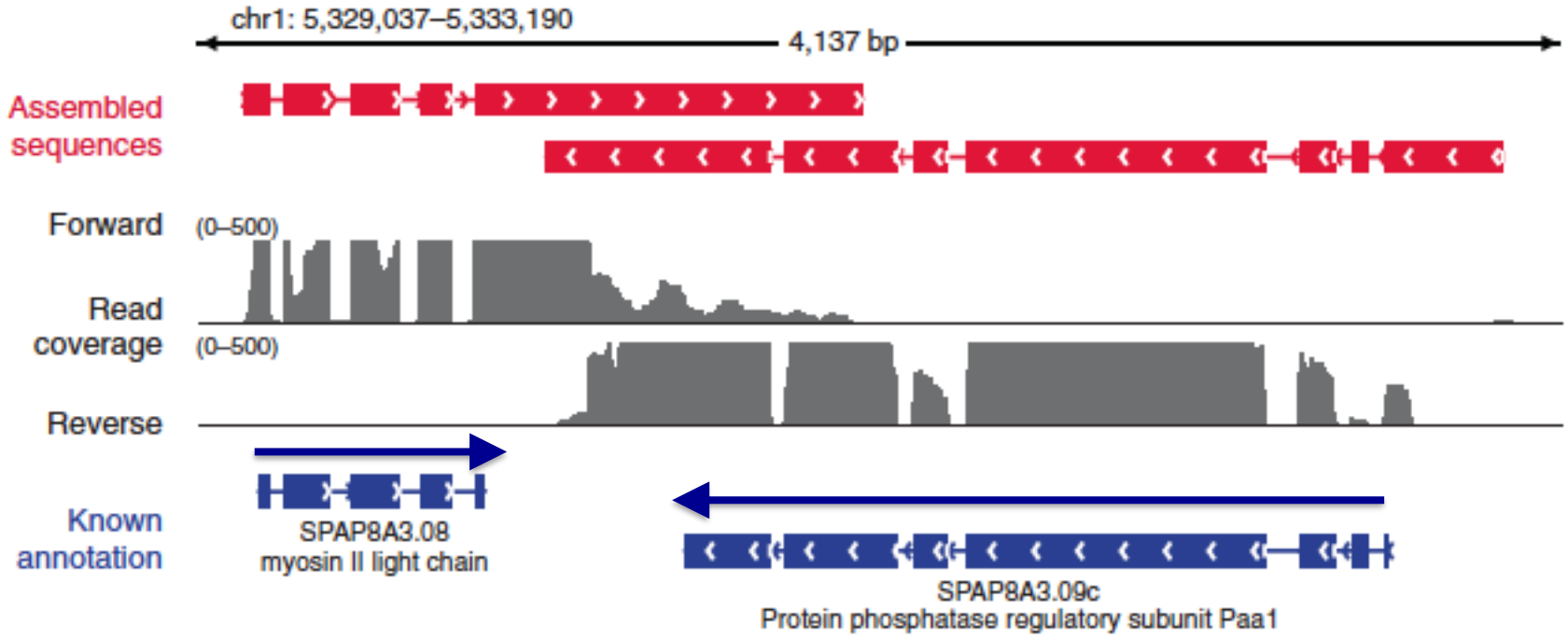
Biologically: separate sense vs. antisense transcription

Illumina TruSeq Stranded mRNA Kit:

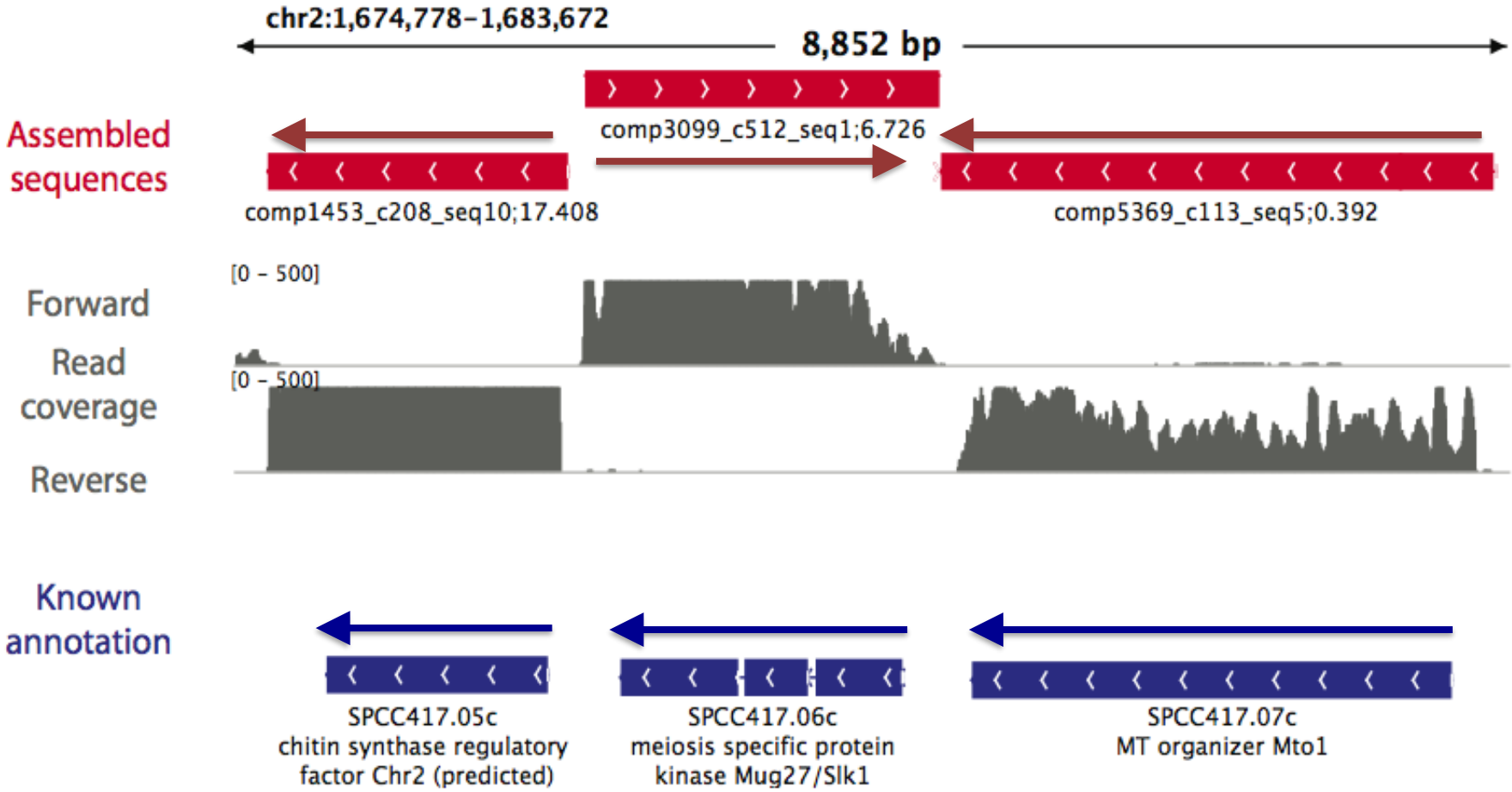
Overlapping UTRs from Opposite Strands

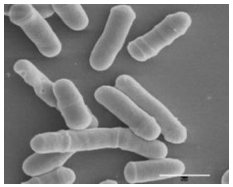


Schizosacharomyces pombe
(fission yeast)



Antisense-dominated Transcription





Trinity is a Highly Effective and Popular RNA-Seq Assembler

Nature Biotechnology, 2011

Thousands of routine users.

~20k literature citations

Freely Available, Well-supported, Open Source Software

GitHub

http://trinityrnaseq.github.io

nature protocols

Transcriptome Assembly is Just the End of the Beginning…

NATURE PROTOCOLS | PROTOCOL

De novo transcript sequence reconstruction from RNA-seq using the Trinity platform for reference generation and analysis

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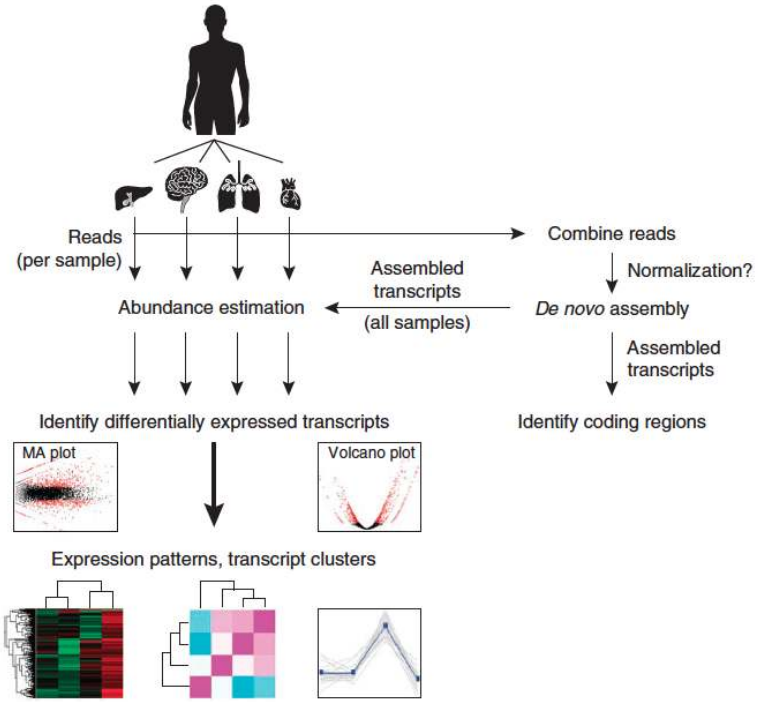
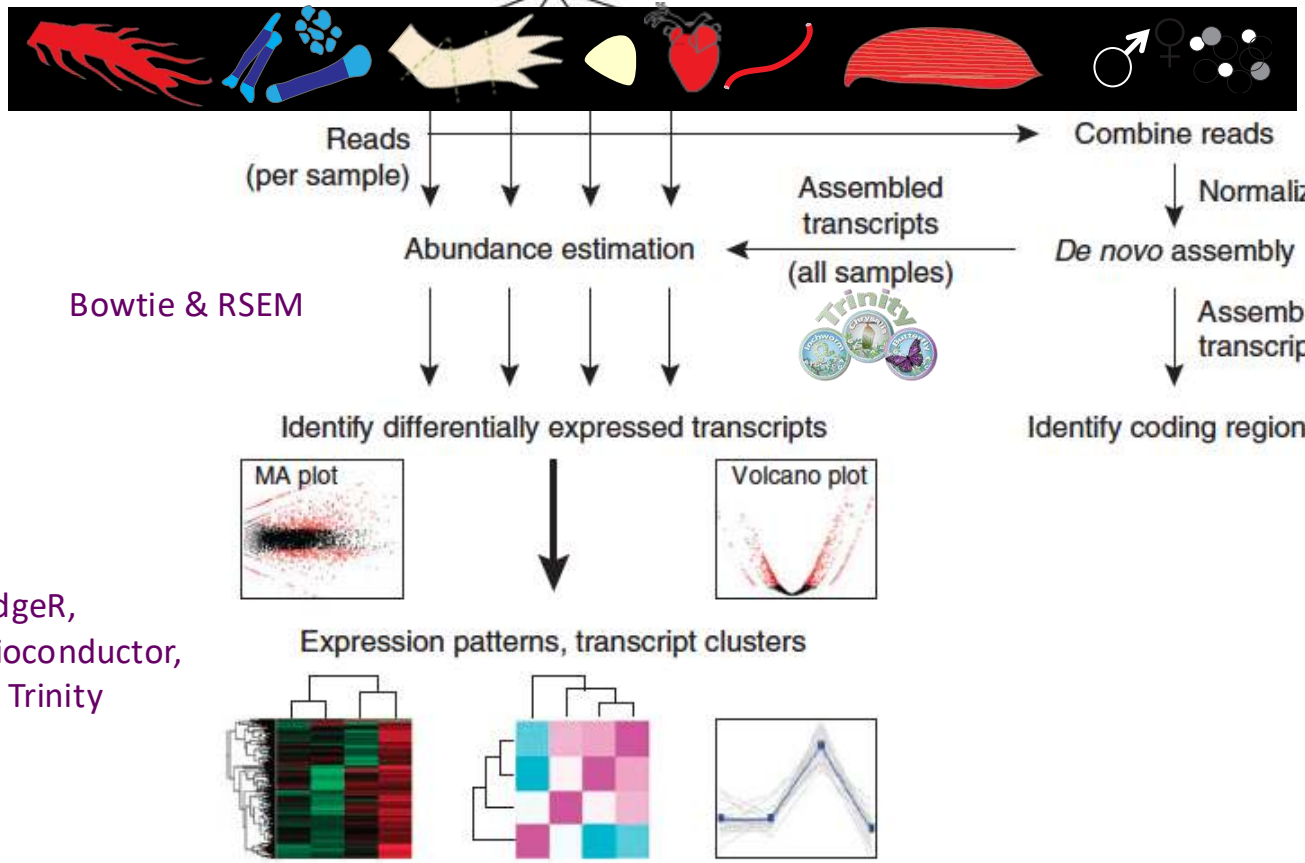


~9k Literature Citations

Framework for De novo Transcriptome Assembly and Analysis

Applied to Axolotl for studies of limb regeneration

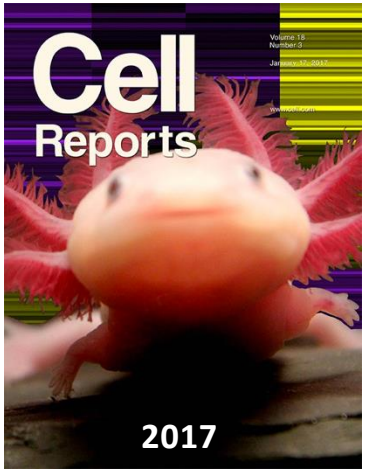


Bowtie & RSEM

EdgeR,
Bioconductor,
& Trinity

1.3 Billion
Total Reads

86 Million
Normalized Reads



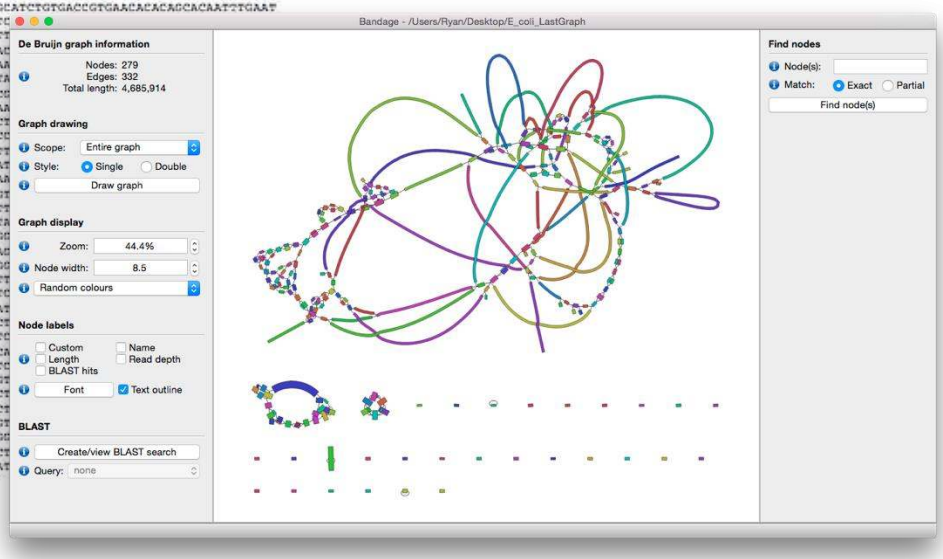
Trinity output: A multi-fasta file

>comp0_c0_seq1 len=5528 path=[1:0-3646 10775:3647-3775 3648:3776-5527]

>comp0_c0_seq2 len=5399 path=[1:0-3646 3648:3647-5398]

Can visualize using Bandage

https://rrwick.github.io/Bandage/



IGV

www.broadinstitute.org/igv/

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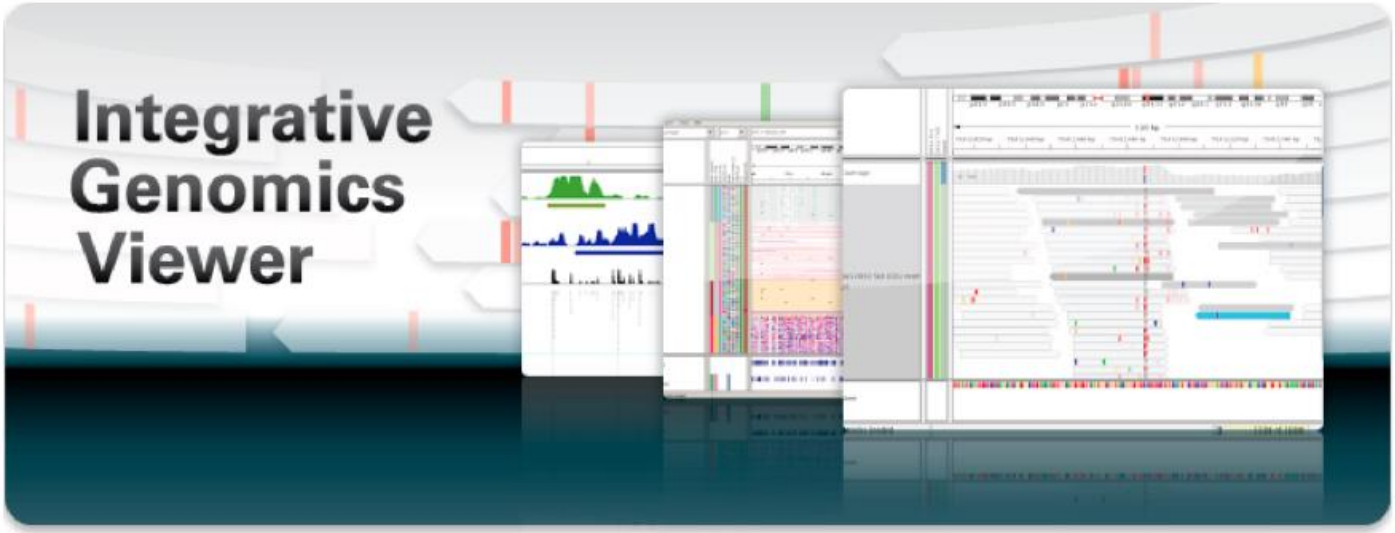


What's New

July 3, 2012. Soybean (Glycine max) and Rat (rn5) genomes have been updated.

April 20, 2012. IGV 2.1 has been released. See the release notes for more details.

April 19, 2012. See our new IGV paper in Briefings in Bioinformatics.

Overview

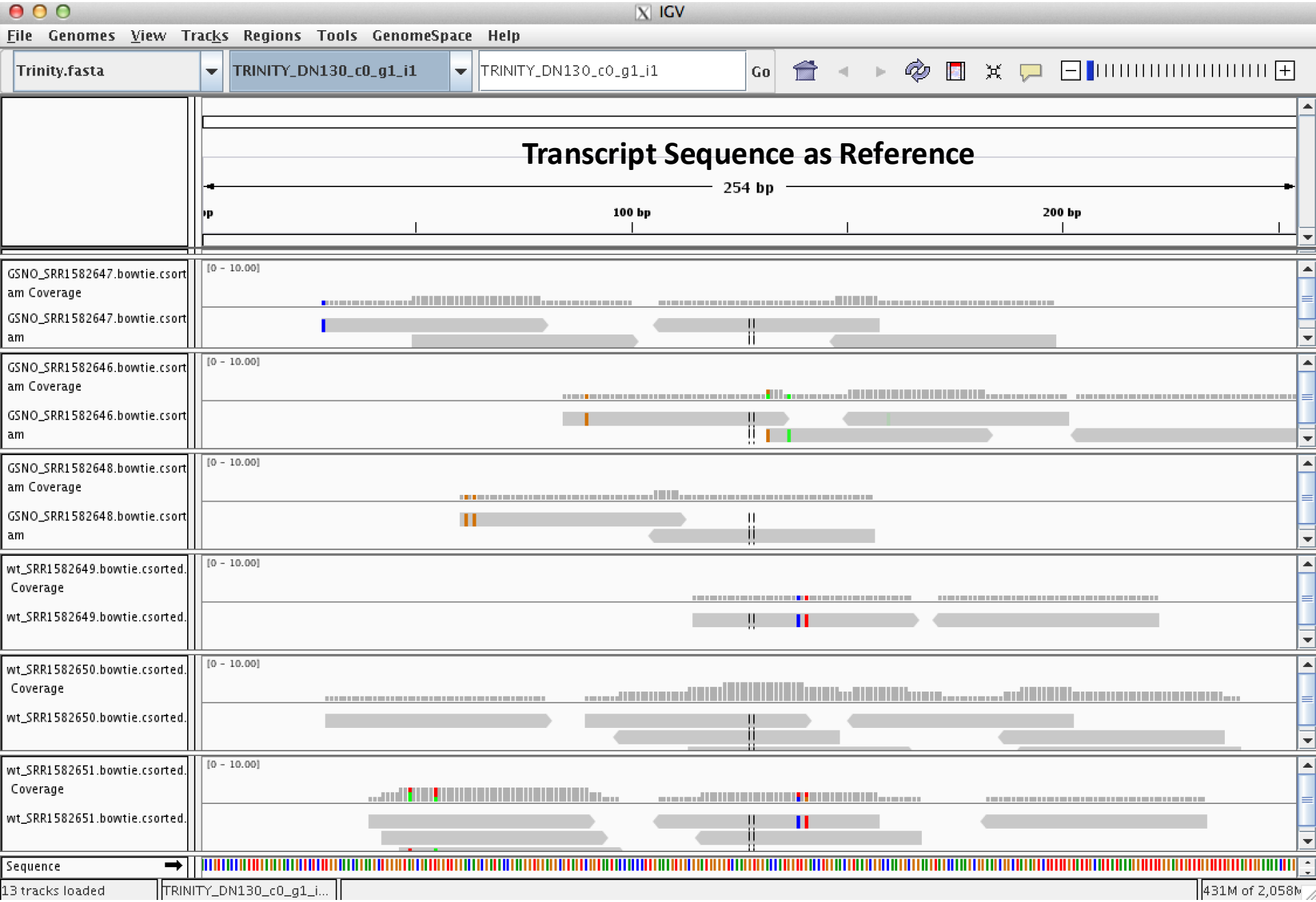
Citing IGV

To cite your use of IGV in your publication:

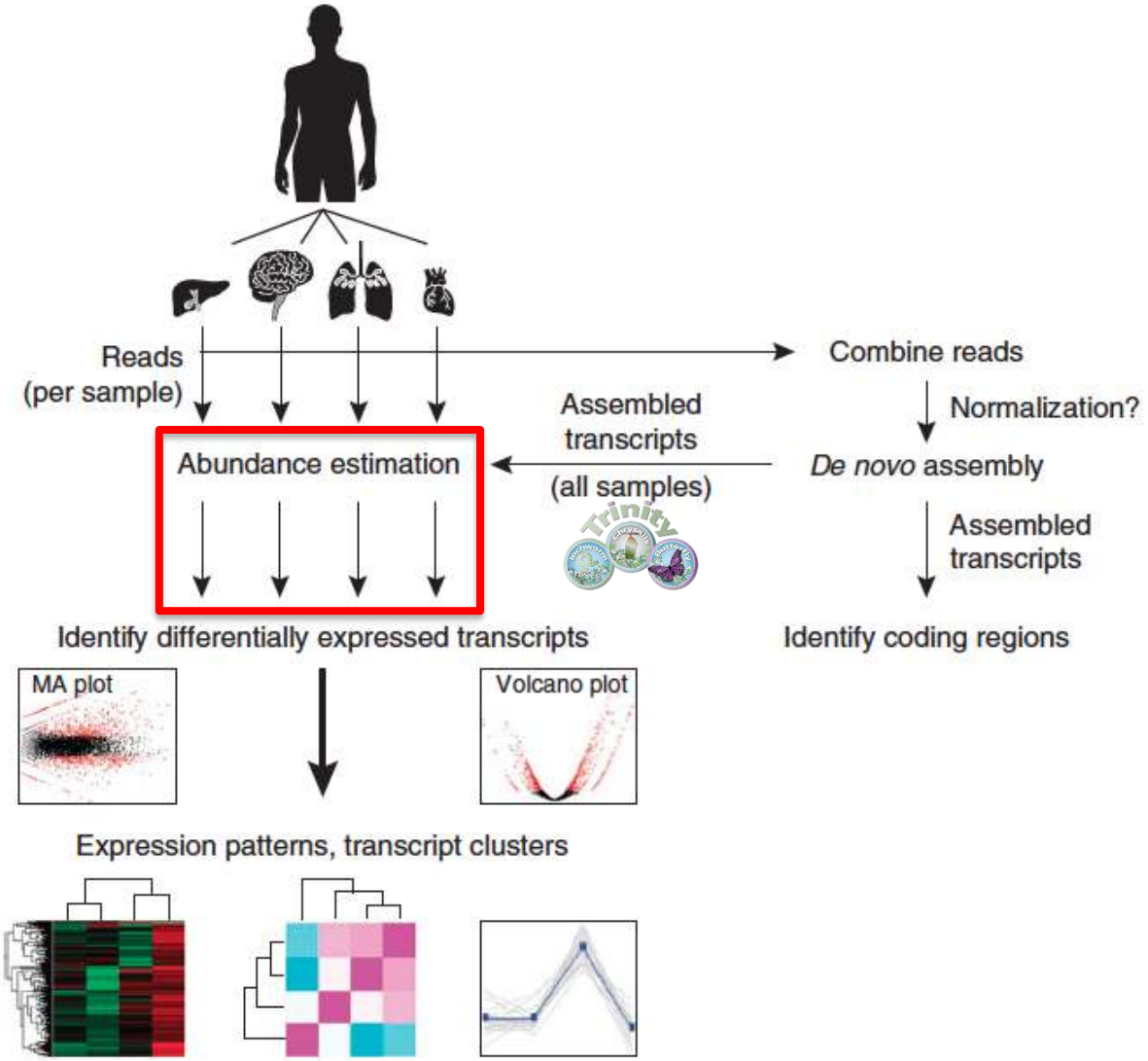
James T. Robinson, Helga Thorvaldsdóttir, Wendy Winckler, Mitchell Guttman, Eric S. Lander, Gad Getz, Jill P. Mesirov. **Integrative Genomics Viewer**. Nature Biotechnology 29, 24–26 (2011), or

Helga Thorvaldsdottir, James T. Robinson, Jill P. Mesirov. **Integrative Genomics Viewer (IGV): high-performance genomics data visualization and exploration.**

Can Examine Transcript Read Support Using IGV

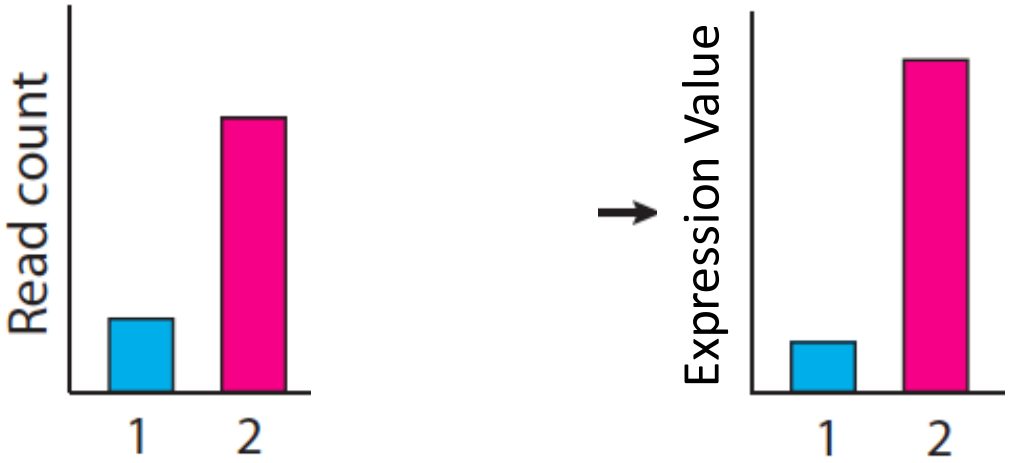


Part 4. Expression Quantification



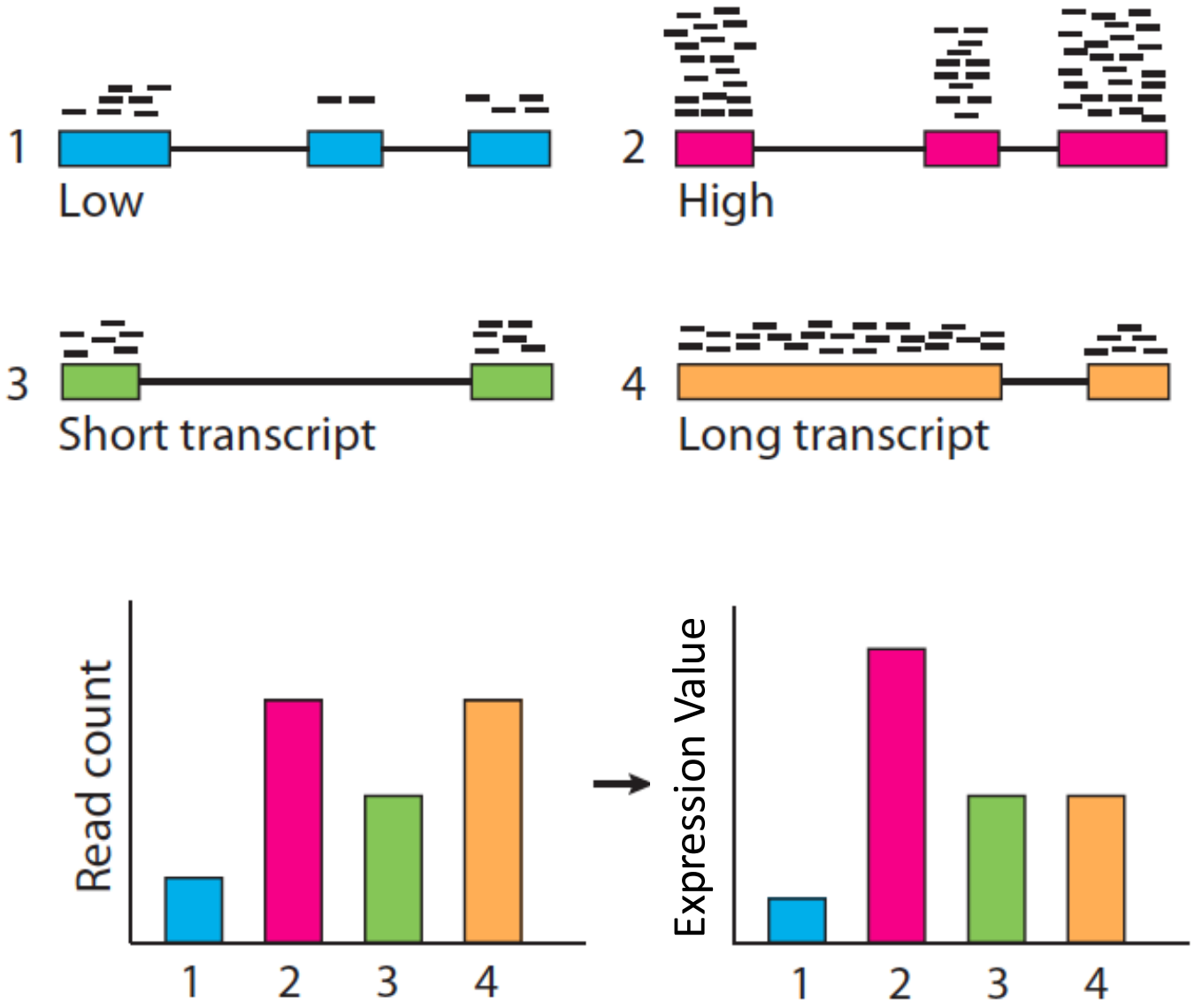
Bioconductor,
& Trinity

Calculating expression of genes and transcripts



Slide courtesy of Cole Trapnell

Calculating expression of genes and transcripts



Slide courtesy of Cole Trapnell

Normalized Expression Values

- Transcript-mapped read counts are normalized for both length of the transcript and total depth of sequencing.

- Reported as: Number of RNA-Seq **F**ragments
  Per **K**ilobase of transcript
  per total **M**illion fragments mapped

FPKM

RPKM (reads per kb per M) used with Single-end RNA-Seq reads
FPKM used with Paired-end RNA-Seq reads.

Transcripts per Million (TPM)

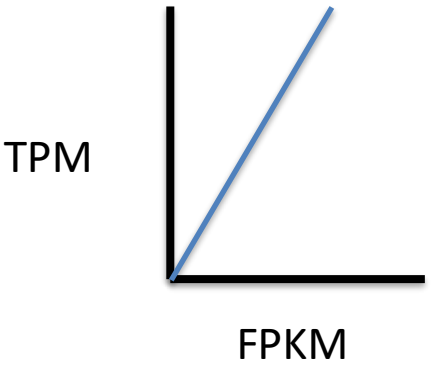
$$TPM_i = \frac{FPKM_i}{\sum_j FPKM} * 1e6$$

Preferred metric for measuring expression

- Better reflects transcript concentration in the sample.
- Nicely sums to 1 million

Linear relationship between TPM and FPKM values.

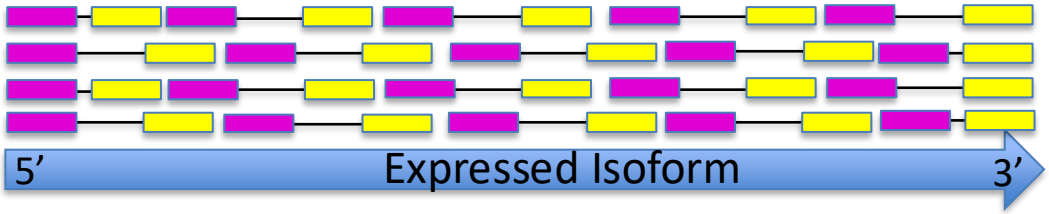
Both are valid metrics, but best to be consistent.



Transcript length normalization not required for 3’ QuantSeq

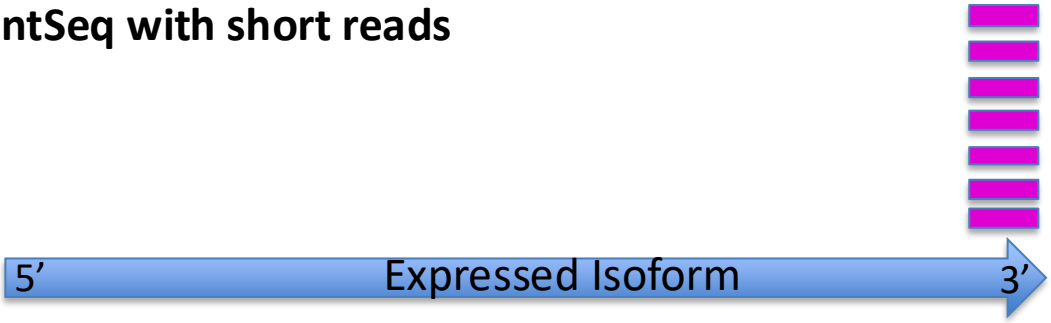
Length Normalization:

Standard full-length RNA-seq with short reads



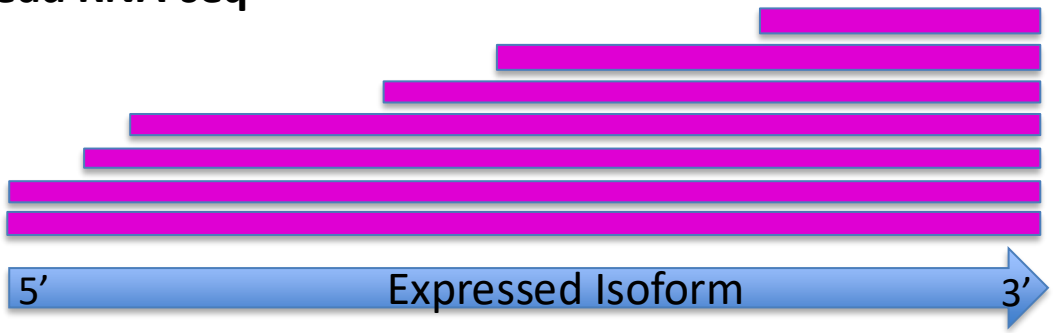
✓ **Yes** (TPM / FPKM)

3’ QuantSeq with short reads



✗ **No** (CPM / Counts)

Long read RNA-seq



✗ **No** (CPM / Counts)

CPM=“Counts per Million”

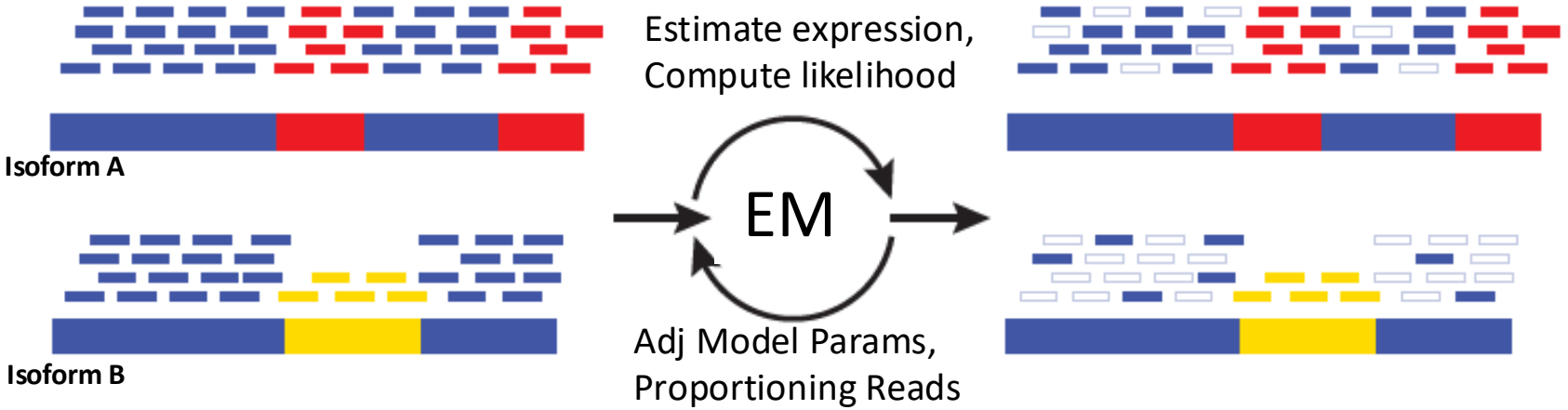
Multiply-mapped Reads Confound Abundance Estimation

Isoform A

Isoform B

Blue = multiply-mapped reads
Red, Yellow = uniquely-mapped reads

Multiply-mapped Reads Confound Abundance Estimation

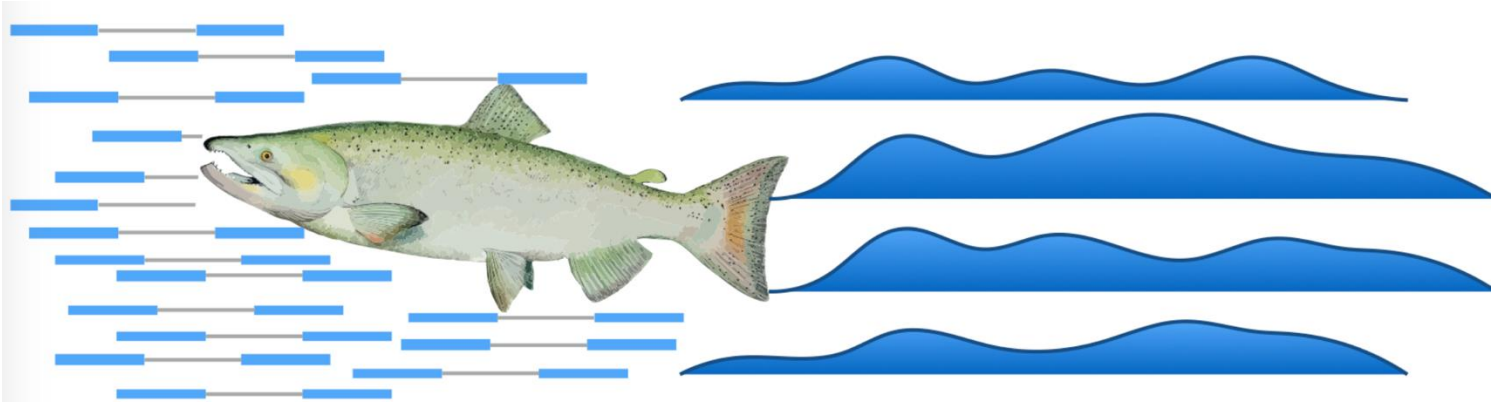


Blue = multiply-mapped reads
Red, Yellow = uniquely-mapped reads

Use Expectation Maximization (EM) to find the most likely assignment of reads to transcripts.

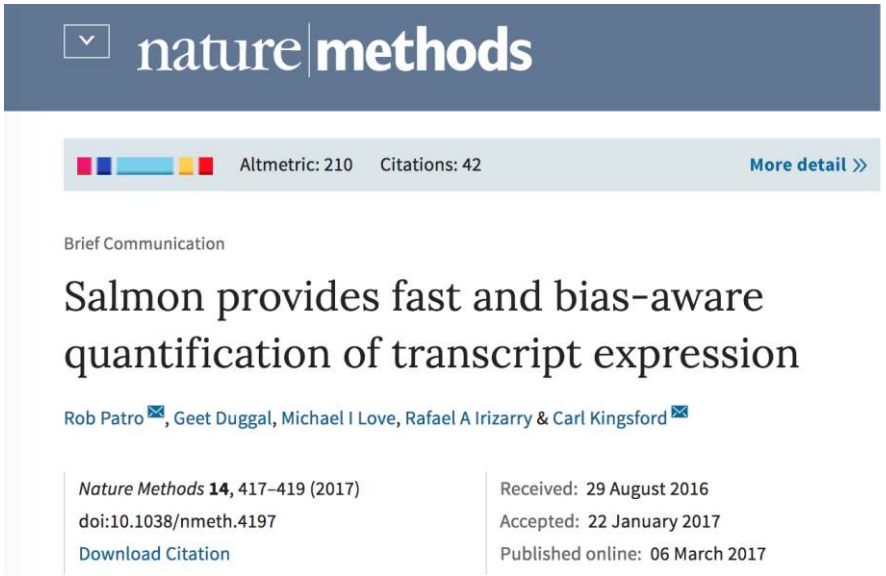
Performed by:

- RSEM (genome-free)
- Kallisto, Salmon (alignment-free)



Salmon —*Don't count . . . quantify!*

Uses a suffix array instead of the de Bruijn graph



nature methods

Altmetric: 210 Citations: 42 More detail »

Brief Communication

Salmon provides fast and bias-aware quantification of transcript expression

Rob Patro, Geet Duggal, Michael I Love, Rafael A Irizarry & Carl Kingsford

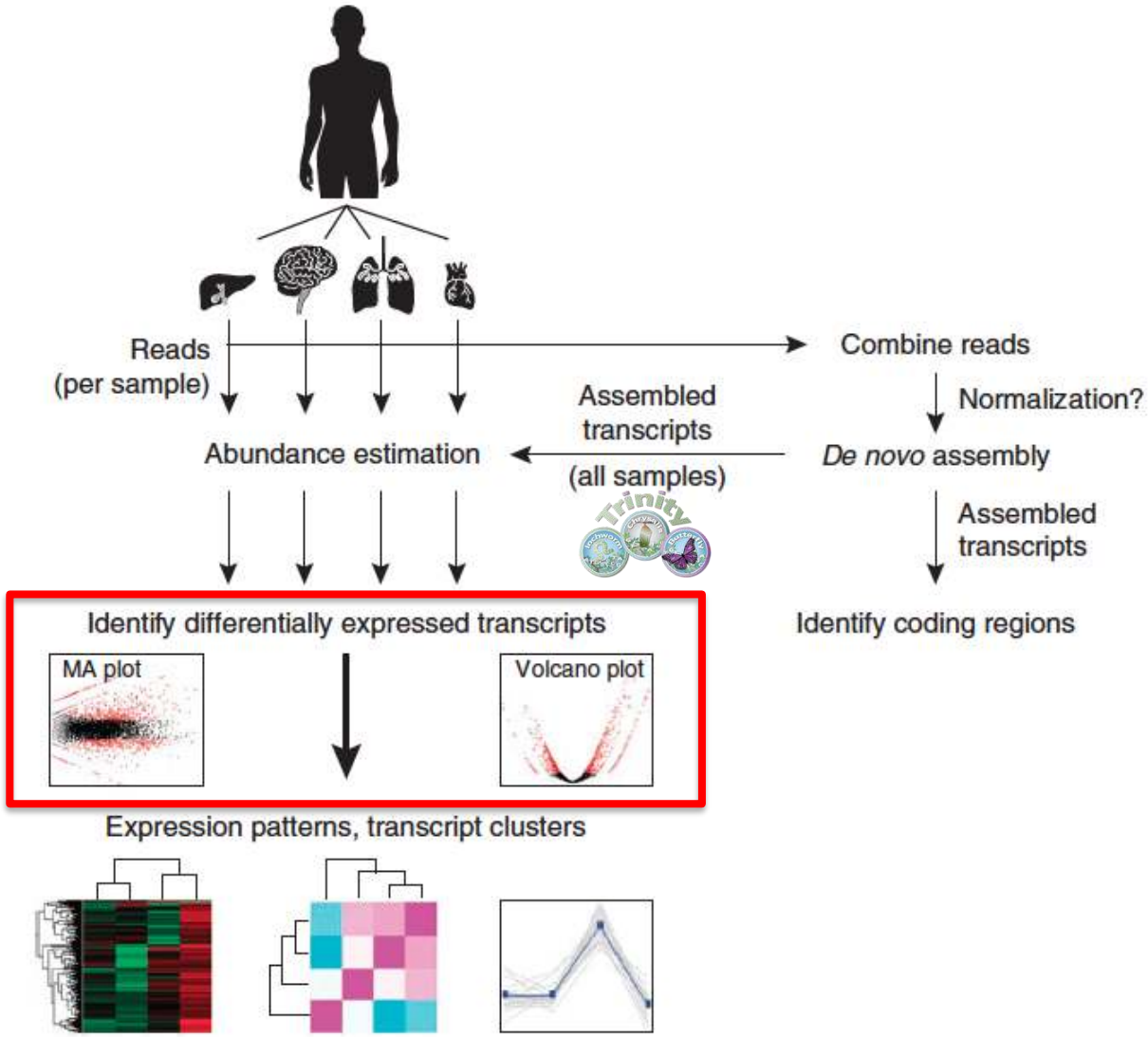
Nature Methods **14**, 417–419 (2017)
doi:10.1038/nmeth.4197
Download Citation

Received: 29 August 2016
Accepted: 22 January 2017
Published online: 06 March 2017

https://combine-lab.github.io/salmon/

Part 5. Differential Expression

More specifics in Rachel Steward's afternoon workshop



Bioconductor, & Trinity

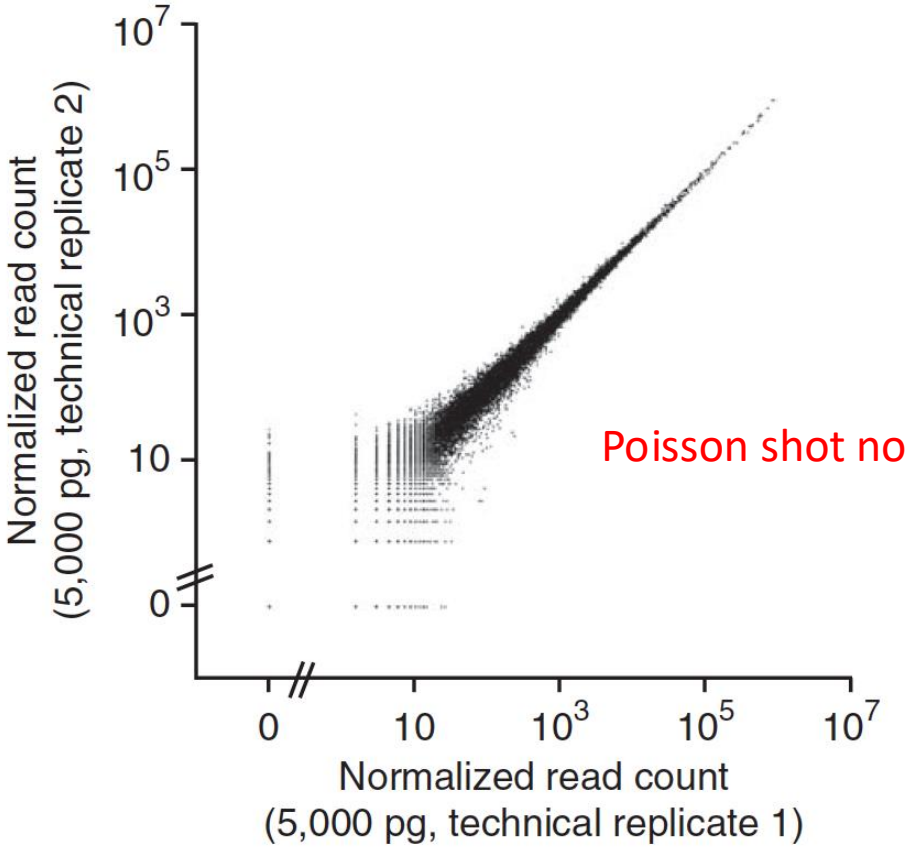
Differential Expression Analysis Involves

- Counting reads mapped to features
- Statistical significance testing

Beware of small counts leading to notable fold changes

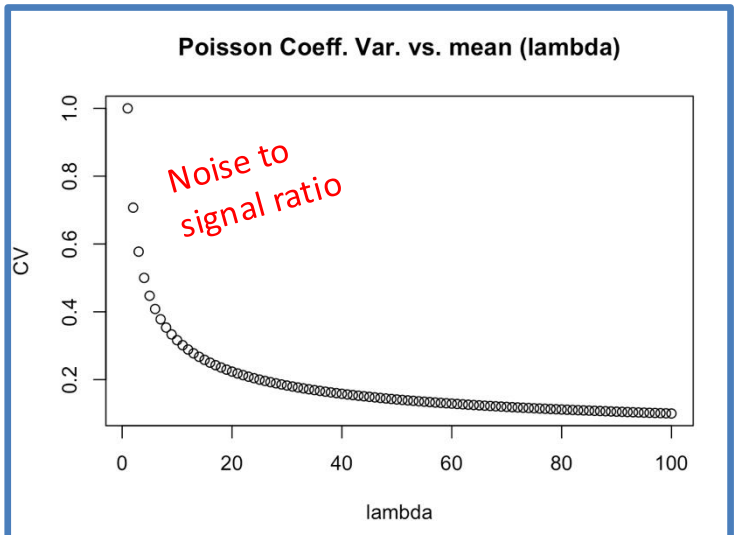
	Sample_A	**Sample_B**	**Fold_Change**	**Significant?**
Gene A	1	2	2-fold	No
Gene B	100	200	2-fold	Yes

Variation Observed Between Technical Replicates



Variation observed is well described by models of random sampling (Poisson Distribution)

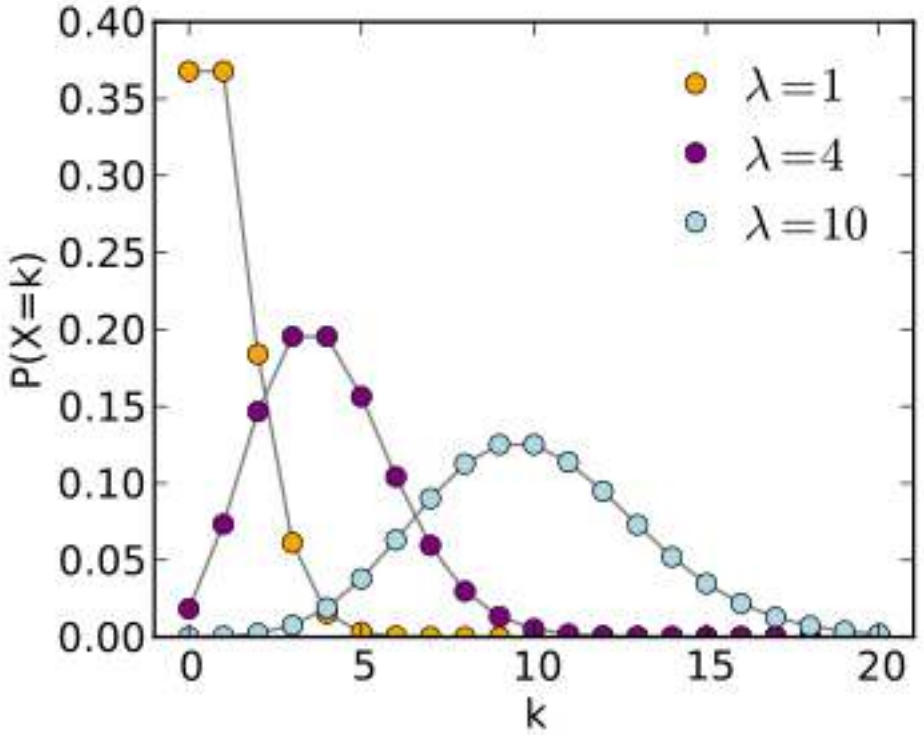
Poisson shot noise is high for small counts.



* plot from Brennecke, et al. Nature Methods, 2013

Observed RNA-Seq Counts Result from Random Sampling of the Population of Reads

Technical variation in RNA-Seq counts per feature is well modeled by the Poisson distribution



Mean # fragments

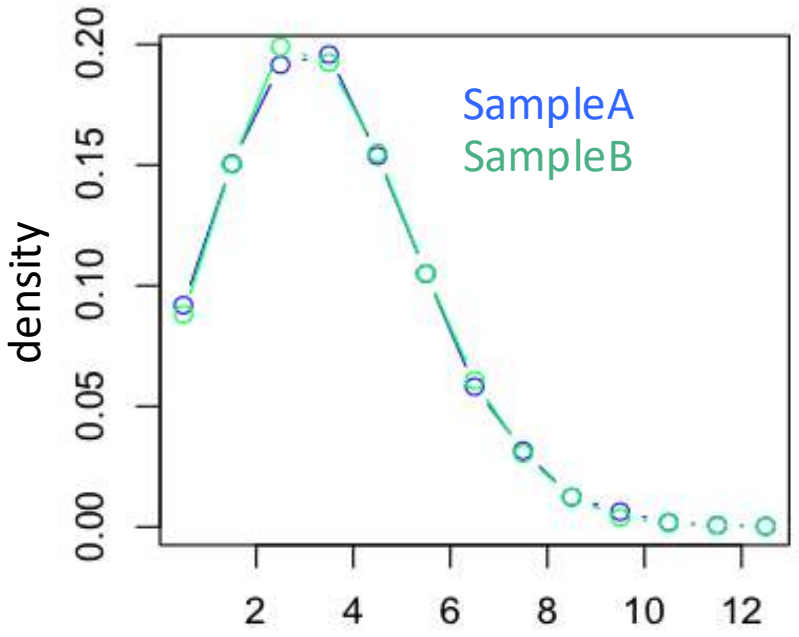
(observed read counts)

See: http://en.wikipedia.org/wiki/Poisson_distribution

Example: One gene*not* differentially expressed

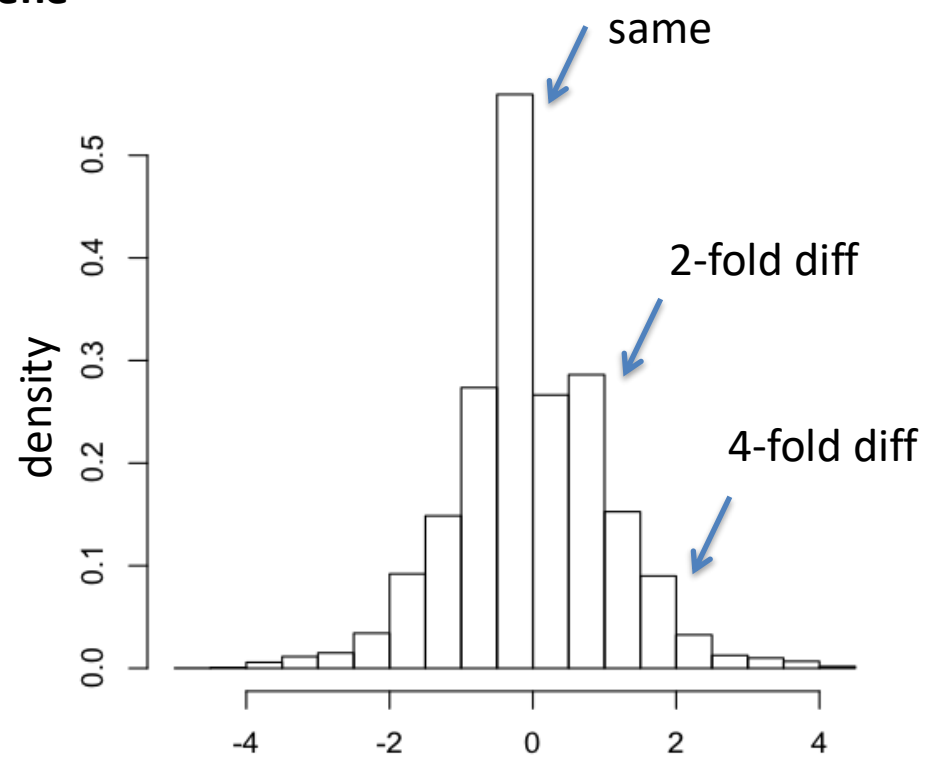
Example: SampleA(gene) = SampleB(gene) = 4 reads

Distribution of observed counts for single gene (under Poisson model)



(k) number of reads observed for a single gene

Dist. of $\log_2$(fold change) values



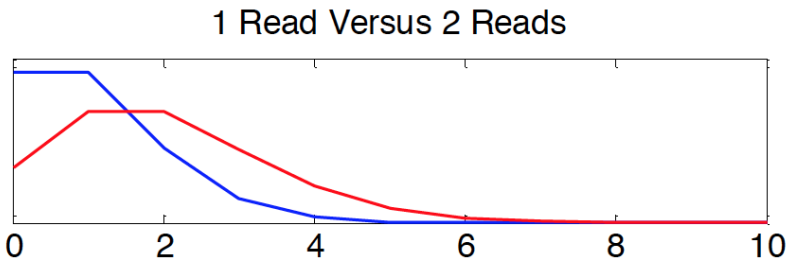
x = $\log_2$(SampleA/SampleB)

Sequencing Depth Matters

Poisson distributions for counts based on **2-fold** expression differences

Shallow sequencing

P(x=k)



No confidence in 2-fold difference. Likely observed by chance.

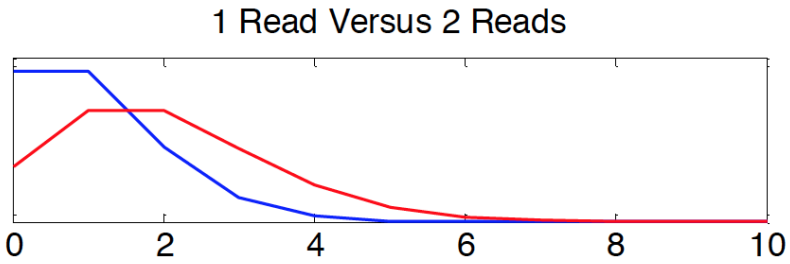
From: http://gkno2.tumblr.com/post/24629975632/thinking-about-rna-seq-experimental-design-for
and from supplementary text of Busby et al., Bioinformatics, 2013

Sequencing Depth Matters

Poisson distributions for counts based on **2-fold** expression differences

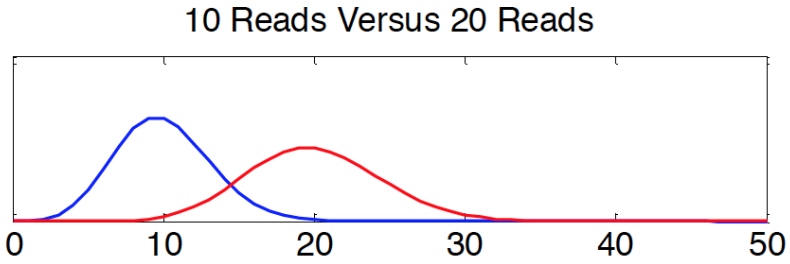
Shallow sequencing

P(x=k)



Deeper sequencing

P(x=k)

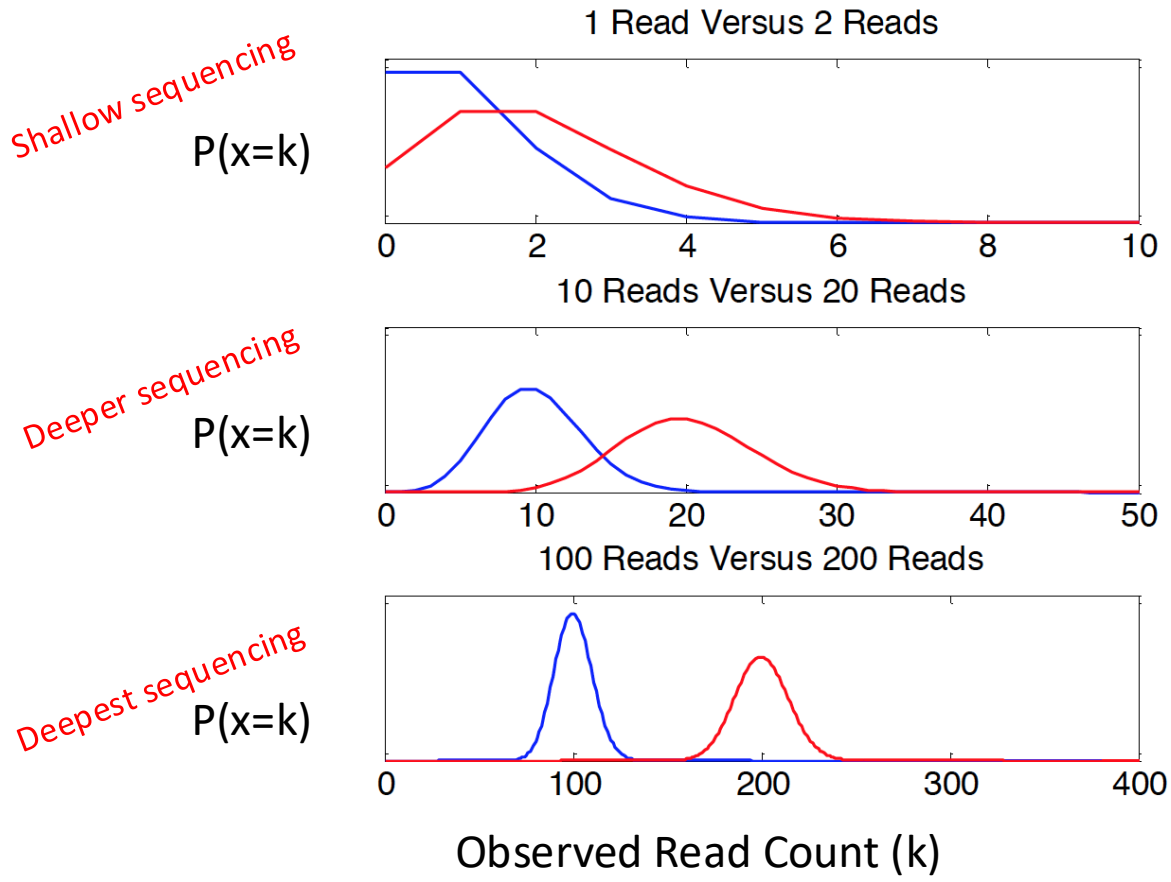


No confidence in 2-fold difference. Likely observed by chance.

From: http://gkno2.tumblr.com/post/24629975632/thinking-about-rna-seq-experimental-design-for
and from supplementary text of Busby et al., Bioinformatics, 2013

Sequencing Depth Matters

Poisson distributions for counts based on **2-fold** expression differences



No confidence in 2-fold difference. Likely observed by chance.

High confidence in 2-fold difference. Unlikely observed by chance.

From: http://gkno2.tumblr.com/post/24629975632/thinking-about-rna-seq-experimental-design-for
and from supplementary text of Busby et al., Bioinformatics, 2013

Greater Depth = More Statistical Power

Example: Single gene, reads sampled at different sequencing depths

Reads per sample	Sample A Number of reads	Sample B Number of reads	P-value (Fishers Exact Test)
100,000	1	2	1
1,000,000	10	20	0.099
10,000,000	100	200	**8.0e-09**

Technical vs. Biological Replicates

RNA-Seq Technical replicates aren't essential

(Technical variation is well-modeled by the Poisson distribution)

"We find that the Illumina sequencing data are highly replicable, with relatively little technical variation, and thus, for many purposes, it may suffice **to sequence each mRNA sample only once**" *Marioni et al., Genome Research, 2008*

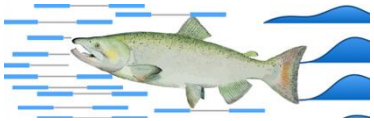
However, biological replicates *ARE* essential

total_variance = technical_variance + biological_variance

(Total variance well-modeled by negative binomial distribution)

"… **at least six biological replicates should be used**, rising to at least 12 when it is important to identify SDE genes for all fold changes." *Schurch et al., RNA, 2016*

DE analysis requires a counts matrix



Sample Type **A**, 3 Bio replicates

Sample Type **B**, 3 Bio replicates

Transcript_ID						
TR24|c0_g1_i1	90.00	67.00	85.00	36.00	35.00	34.00
TR2779|c0_g1_i1	186.00	137.00	217.00	147.00	186.00	197.00
TR127|c1_g1_i1	9.00	23.00	16.00	2.00	0.00	1.00
TR2107|c1_g1_i1	59.00	65.00	47.00	6.00	6.00	7.00
TR2011|c5_g1_i1	11.00	4.00	4.00	8.00	5.00	7.00
TR4163|c0_g1_i1	368.00	422.00	425.00	172.00	216.00	210.00
TR5055|c0_g2_i1	36.00	17.00	27.00	4.00	7.00	3.00
TR1449|c0_g1_i1	196.00	230.00	207.00	66.00	113.00	91.00
TR1982|c2_g1_i1	7.00	7.00	6.00	4.00	3.00	8.00
TR1859|c3_g1_i1	0.00	0.00	1.00	0.00	0.00	0.00
TR1492|c0_g1_i2	1895.00	1906.00	1921.00	1104.00	1263.00	1319.00
TR1122|c0_g1_i1	2.00	3.00	0.00	3.00	0.00	0.00
TR2278|c0_g1_i1	497.00	610.00	598.00	333.00	406.00	413.00
TR4084|c0_g1_i1	95.00	148.00	86.00	77.00	111.00	127.00
TR4761|c0_g1_i1	2089.00	1746.00	1875.00	155.00	174.00	165.00
TR3638|c0_g1_i1	647.00	676.00	712.00	117.00	184.00	174.00
TR2090|c0_g1_i1	0.00	0.00	0.00	22.00	0.00	0.02
TR3854|c0_g1_i1	1878.00	1734.00	1864.00	1775.00	2173.00	2151.00
TR131|c0_g1_i1	32.00	28.00	31.00	1001.00	1233.00	1208.00
TR5075|c0_g1_i1	13.00	22.00	21.00	6.00	8.00	10.00
TR2182|c3_g2_i6	1.44	2.70	3.84	3.35	0.00	0.00
TR3788|c0_g1_i1	17.00	30.00	22.00	91.00	132.00	125.00
TR4859|c0_g1_i1	6.00	12.00	8.00	4.00	1.00	3.00
TR2487|c0_g1_i1	386.00	383.00	424.00	689.00	866.00	806.00
TR2122|c0_g2_i2	145.00	135.00	136.00	155.00	157.00	201.00
TR4277|c0_g1_i1	4466.00	4701.00	4284.00	118.00	134.00	164.00
TR4669|c0_g2_i1	0.00	0.00	0.00	209.00	0.00	217.50
TR3091|c0_g1_i1	22.00	17.00	19.00	250.00	308.00	284.00

Typical output from DE analysis

Transcript_id	logFC	logCPM	PValue	FDR
TRINITY_DN876_c0_g1_i1	-7.15049572793027	10.6197708379285	0	0
TRINITY_DN6470_c0_g1_i1	-7.26777912190146	7.03987604865422	1.687485656951e-287	6.46813252309319e-284
TRINITY_DN5186_c0_g1_i1	-7.85623682454322	9.18570464327063	1.17049180235068e-278	2.99099671894011e-275
TRINITY_DN768_c0_g1_i1	7.72884741150304	9.7514619195169	4.32504881419265e-272	8.28895605240022e-269
TRINITY_DN70_c0_g1_i1	-12.7646078189688	7.86482982471445	3.92853491279431e-253	6.02322972829624e-250
TRINITY_DN1587_c0_g1_i1	-5.89392061881667	9.07366563894607	6.32919557933429e-243	8.08660221852944e-240
TRINITY_DN3236_c0_g1_i1	-7.27029815068473	8.02209568234202	3.64955175271959e-235	3.99678053376405e-232
TRINITY_DN4631_c0_g1_i1	-7.45310693639574	6.91664918183241	4.30540921272851e-229	4.1256583780971e-226
TRINITY_DN5082_c0_g5_i1	-5.33154406167545	10.6977538760467	2.74243356676259e-225	2.33594396920022e-222
TRINITY_DN1789_c0_g3_i1	10.2032564835076	7.32607652700285	1.44273728647186e-213	1.10600240380933e-210
TRINITY_DN4204_c0_g1_i1	4.81030233739325	9.88844409410644	9.27180216086162e-205	6.46160321501501e-202
TRINITY_DN799_c0_g1_i1	-4.22044475626154	6.9937398638711	1.24746518421083e-197	7.96922341846683e-195
TRINITY_DN196_c0_g2_i1	4.60597918494257	9.86878463857276	1.9819997623131e-192	1.16877001368402e-189
TRINITY_DN5041_c0_g1_i1	-4.27126549355785	9.70894399883	1.8930437900069e-185	1.03657669244235e-182
TRINITY_DN1619_c0_g1_i1	-4.47156415953777	9.22535948721718	1.76766063029526e-181	9.03392426122899e-179
TRINITY_DN899_c0_g1_i1	-4.90914328409143	7.93768691394594	1.11054513767547e-180	5.32089939088761e-178
TRINITY_DN324_c0_g2_i1	4.87160837667488	6.84850312231775	2.20092562166991e-179	9.92487989160089e-177
TRINITY_DN3241_c0_g1_i1	-4.77760618069256	7.94111259715689	1.60585457735621e-173	6.83915621667372e-171
TRINITY_DN4379_c0_g1_i1	3.85133572453294	7.23712813663389	3.48140532848425e-164	1.4046554341137e-161
TRINITY_DN1919_c0_g1_i1	4.05998814332136	6.95937301668582	1.8588621194715e-161	7.12501850393425e-159
TRINITY_DN2504_c0_g1_i1	-6.92417817059644	6.20370039359785	2.42022459856956e-160	8.83497227268296e-158
…				

Up vs. Down regulated

Avg. expression level

Significance

Tools for DE analysis with RNA-Seq

edgeR
ShrinkSeq
DESeq
baySeq
Vsf
Limma/Voom
mmdiff
cuffdiff

ROTS
TSPM
DESeq2
EBSeq
NBPSeq
SAMseq
NoiSeq
Sleuth

(italicized not in R/Bioconductor but stand-alone)

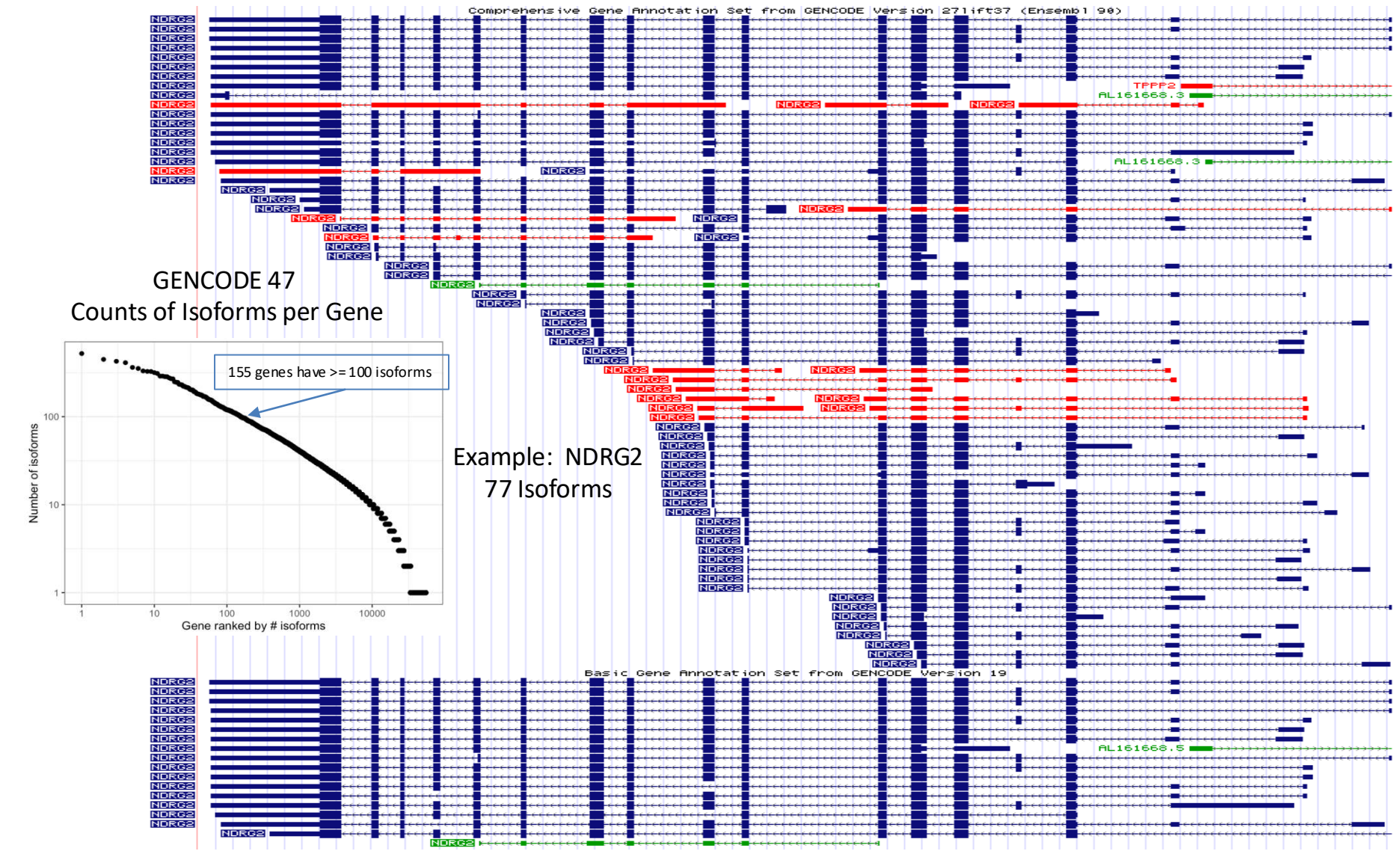
See: http://www.biomedcentral.com/1471-2105/14/91
A comparison of methods for differential expression analysis of RNA-seq data
Soneson & Delorenzi, 2013

Part 6. Latest advancements in long read isoform sequencing

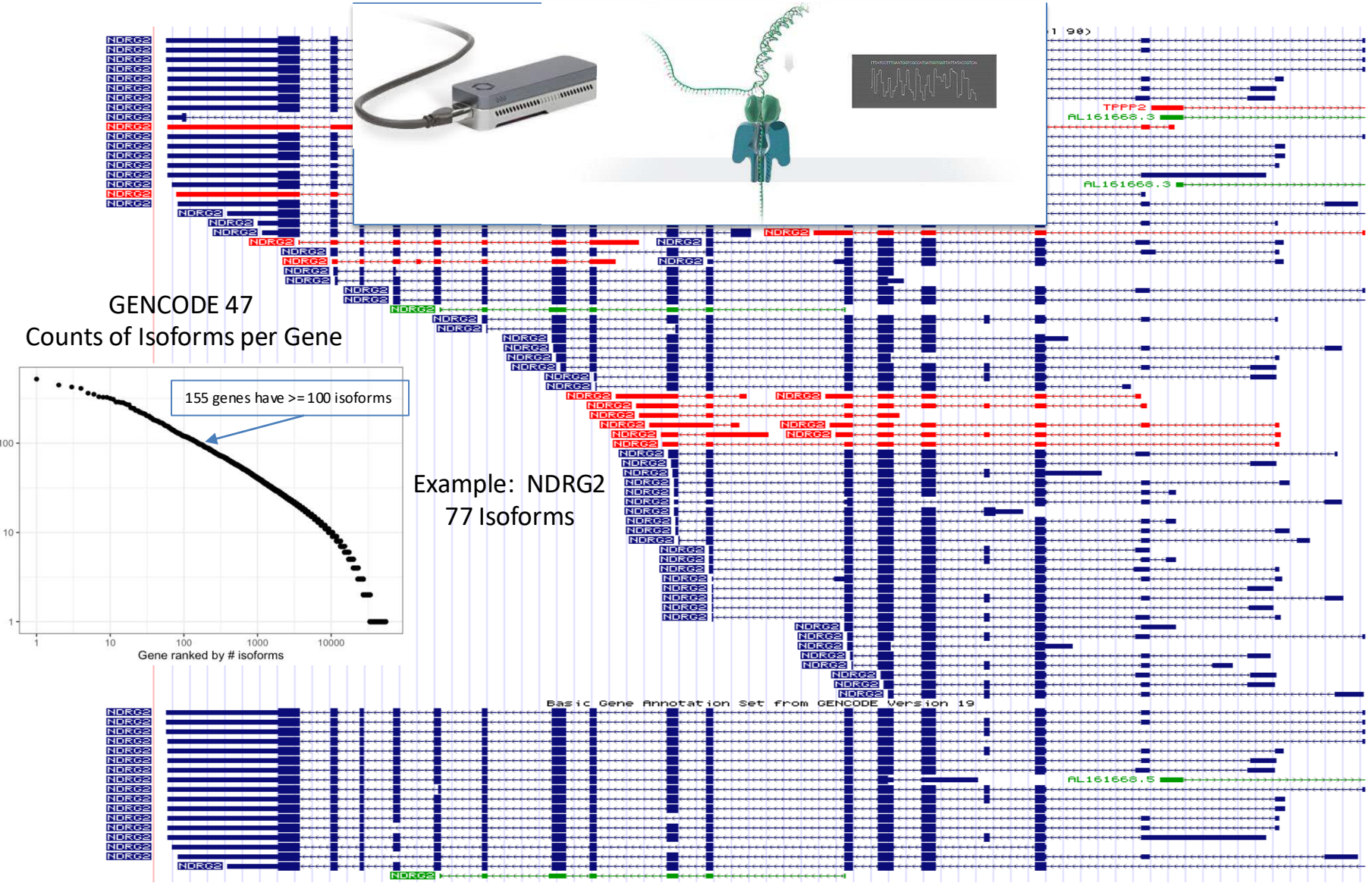
Some transcripts can be challenging to reconstruct from short reads

- Complex alternative splicing (many isoforms)
- Very long RNAs (ex. Titin – up to 36 kb)
- Transcripts containing repetitive sequences

Long Isoform Reads are Essential for Resolving Transcriptome Complexity



Long Isoform Reads are Essential for Resolving Transcriptome Complexity



Long Read Isoform Sequencing via PacBio MAS-Iso-Seq (Kinnex)

Editorial | Published: 12 January 2023

Method of the Year 2022: long-read sequencing

The variables on RNA molecules: concert or cacophony? Answers in long-read sequencing

Long read

	PacBio SMRT sequencing	ONT MinION	PacBio Sequel	ONT GridION	ONT PromethION	PacBio Sequel II	PacBio Revio
	2011	2015	2015	2017	2018	2021	2023
							
Throughput :	75k	1–10 million	500k	10–30 million	30–150 million	4M	8M
Error rate :	10%	5–10%	10%	5–10%	5–10%	<1%	<1%

Inflection point for LR transcriptomics

Aziz Al'Khafaji

MAS-seq

(commercially **Kinnex**)

40-120 million cDNA reads

Info on error rates for long reads – impressive!!

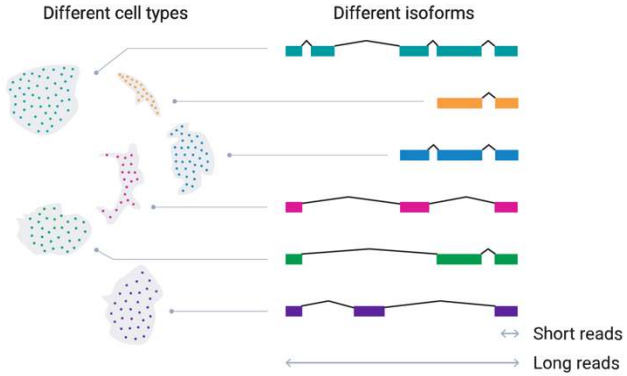
https://nanoporetech.com/accuracy

https://www.pacb.com/technology/hifi-sequencing/

99% .... 99.9% .....

Q20 Q30

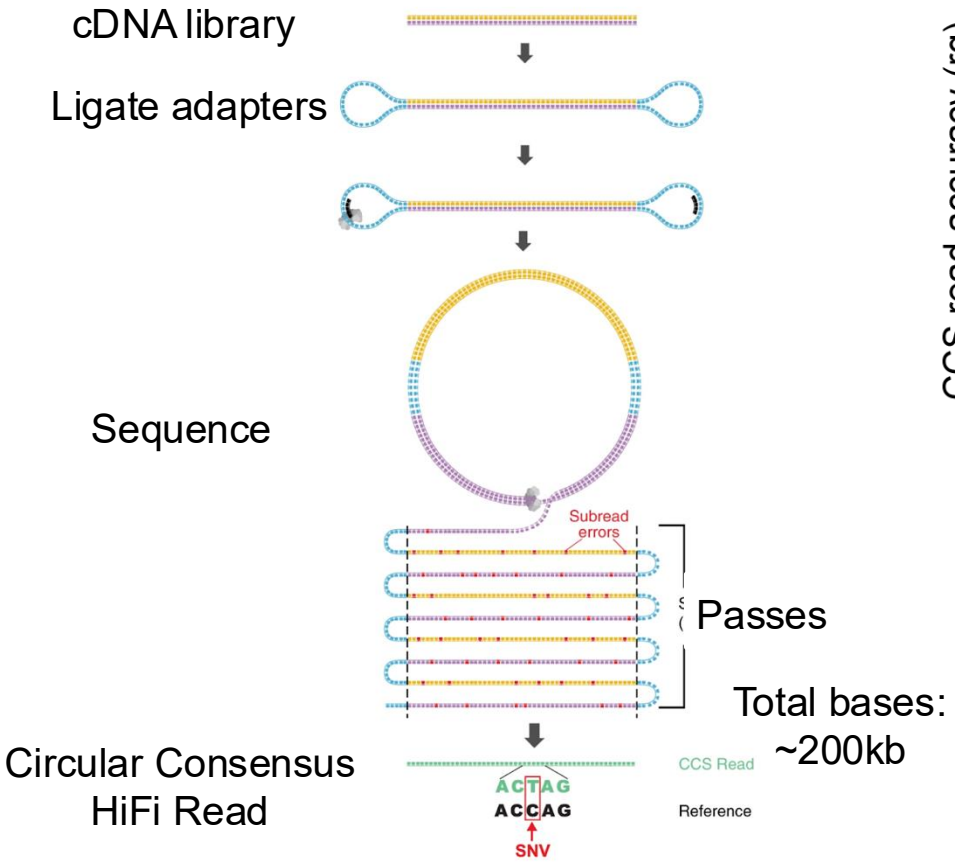
Long reads for Single Cell Transcriptomes*!!*



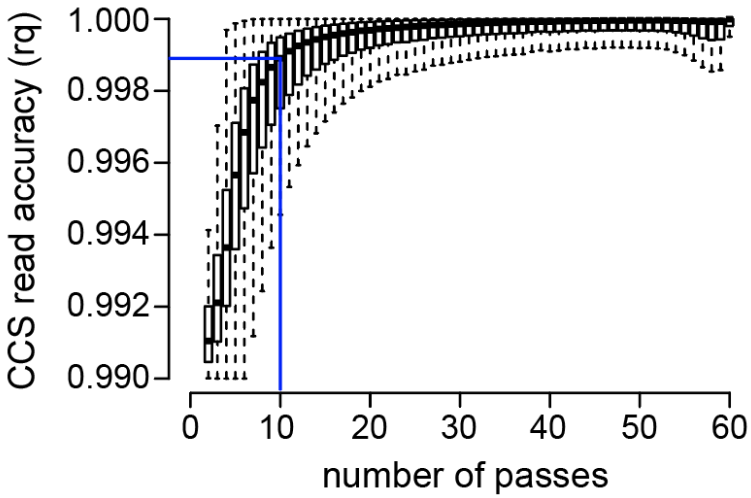
BROAD CLINICAL LABS

Standard isoform sequencing is inefficient on the PacBio platform

PacBio HiFi Sequencing



CCS read accuracy ~ # passes



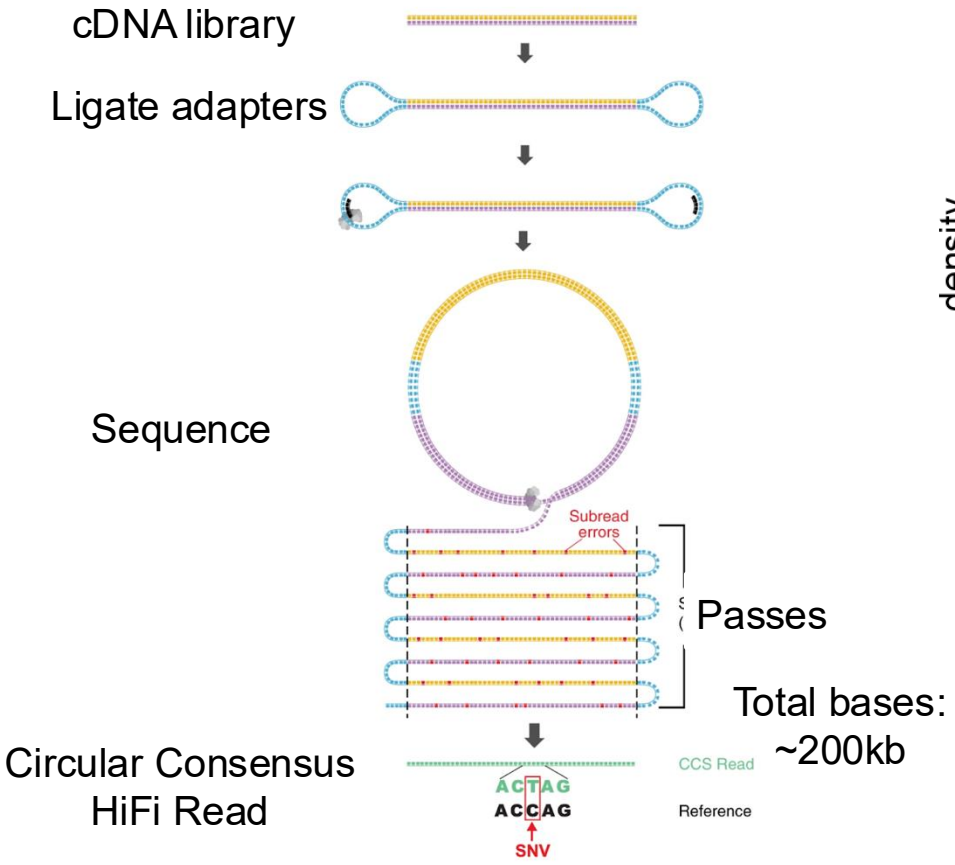
Base calling accuracy increases with the number of consensus reads.
~Q30 (99.9%) @ 10 passes.

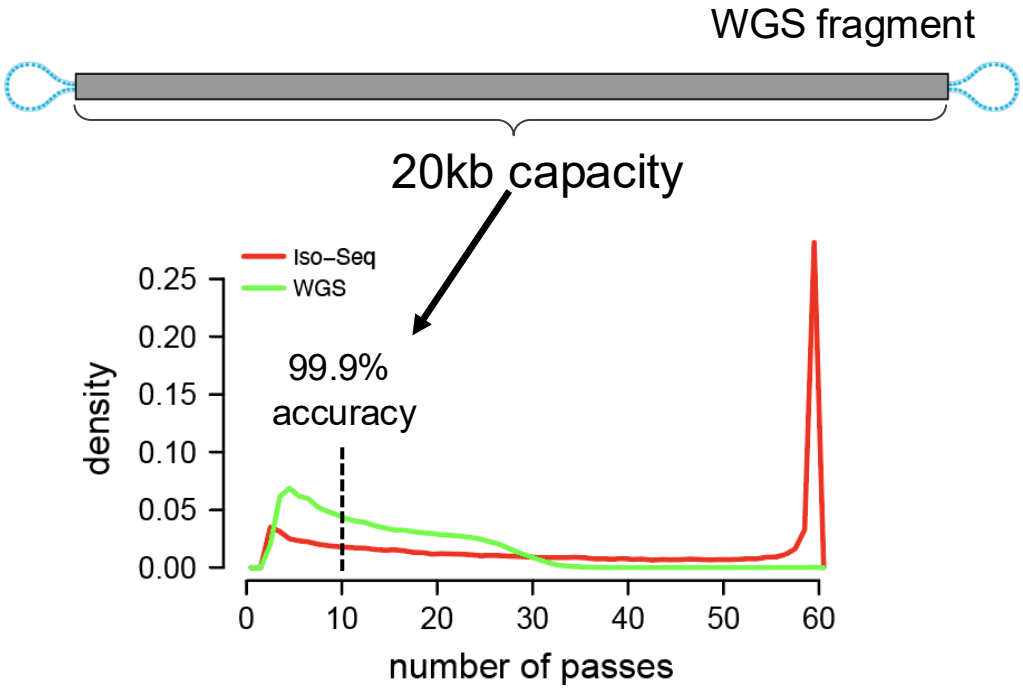
200kb total = 20kb / pass

From Aziz Al'Khafaji, Broad Inst.

Standard isoform sequencing is inefficient on the PacBio platform

PacBio HiFi Sequencing



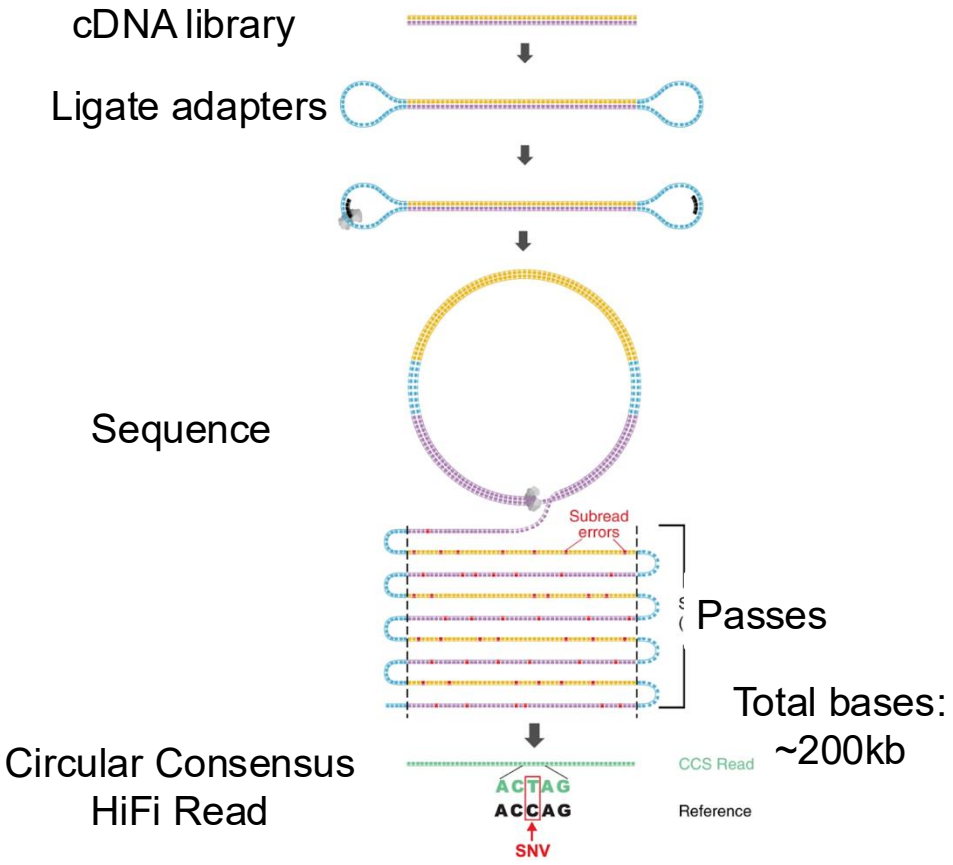


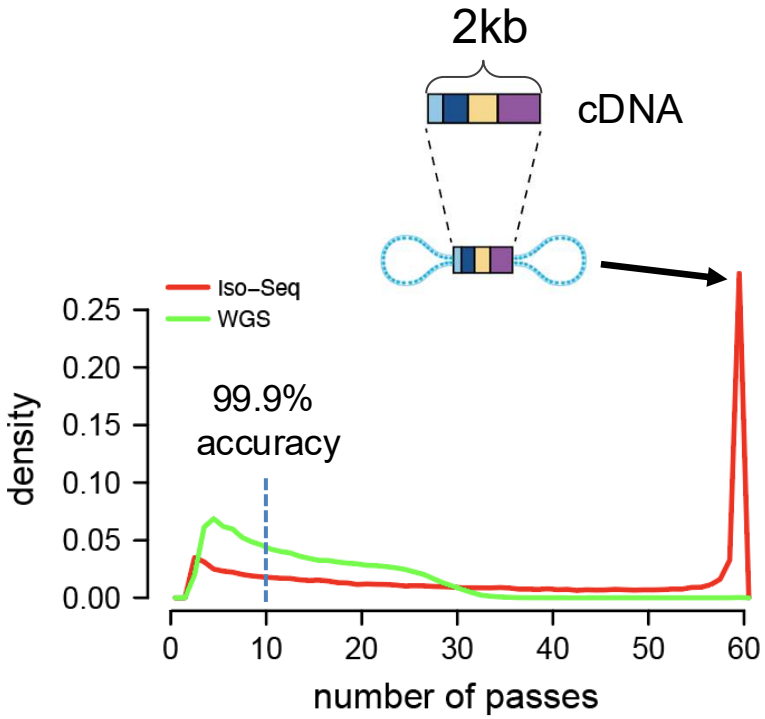
HiFi for WGS involves 20kb segments

From Aziz Al'Khafaji, Broad Inst.

Standard isoform sequencing is inefficient on the PacBio platform

PacBio HiFi Sequencing



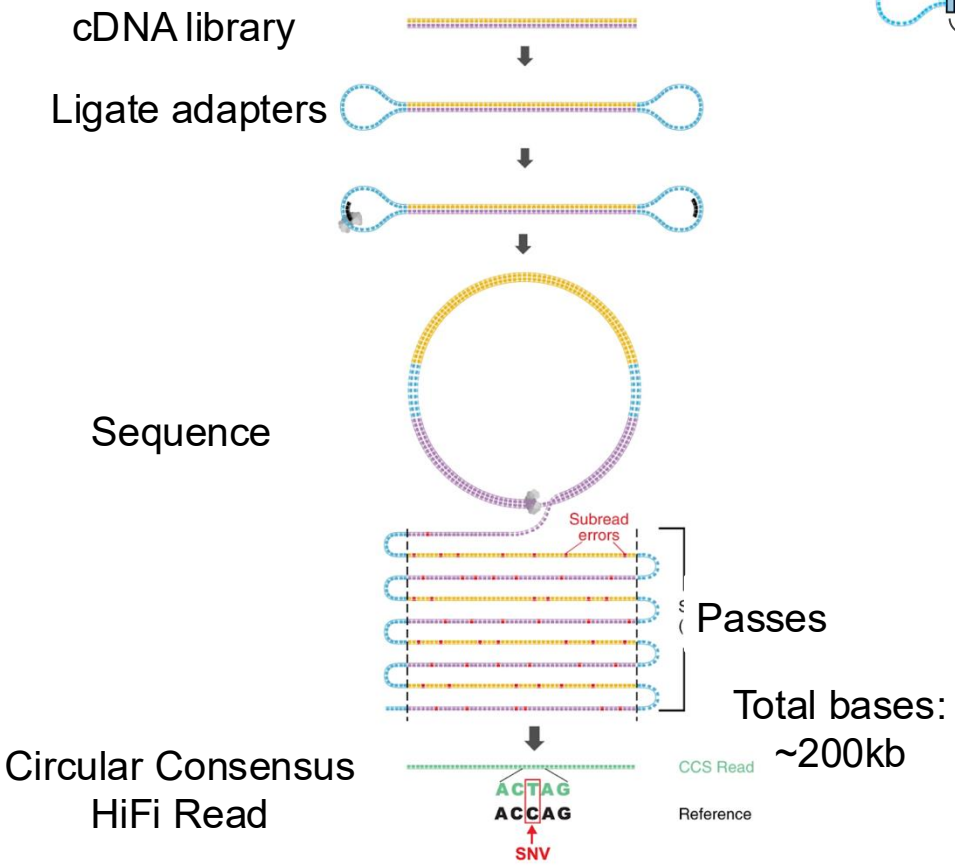


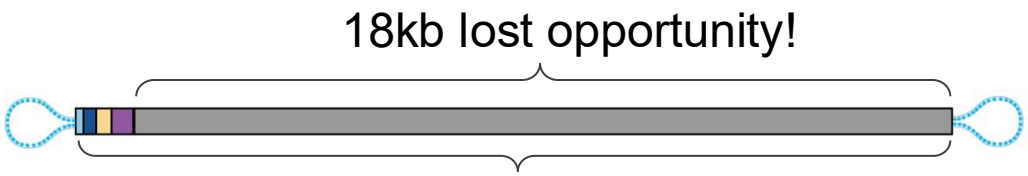
Most transcripts are <5kb and get >60 passes. Wasted sequencing potential!

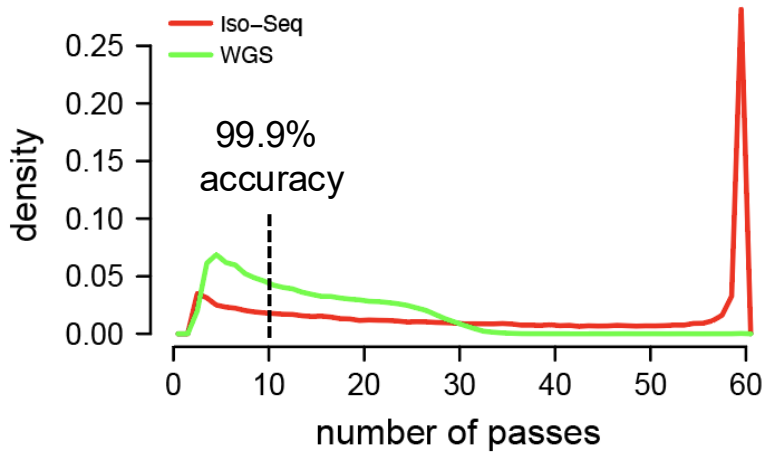
From Aziz Al'Khafaji, Broad Inst.

Standard isoform sequencing is inefficient on the PacBio platform

PacBio HiFi Sequencing





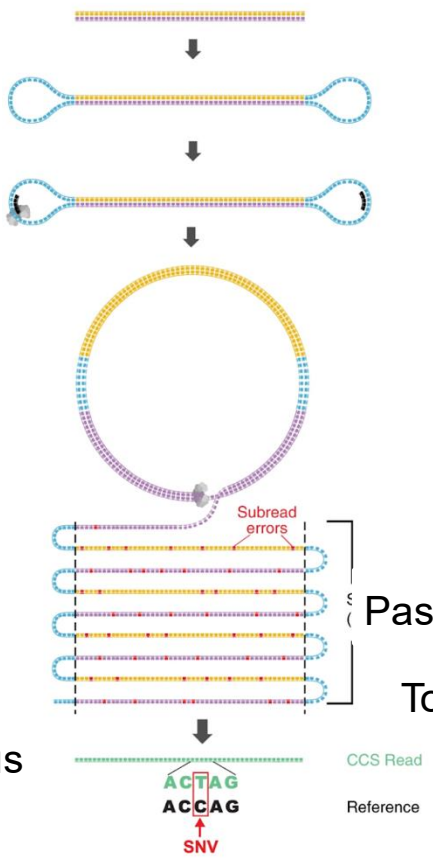


Of the 20kb segment, RNAs only use ~2kb

From Aziz Al'Khafaji, Broad Inst.

Standard isoform sequencing is inefficient on the PacBio platform

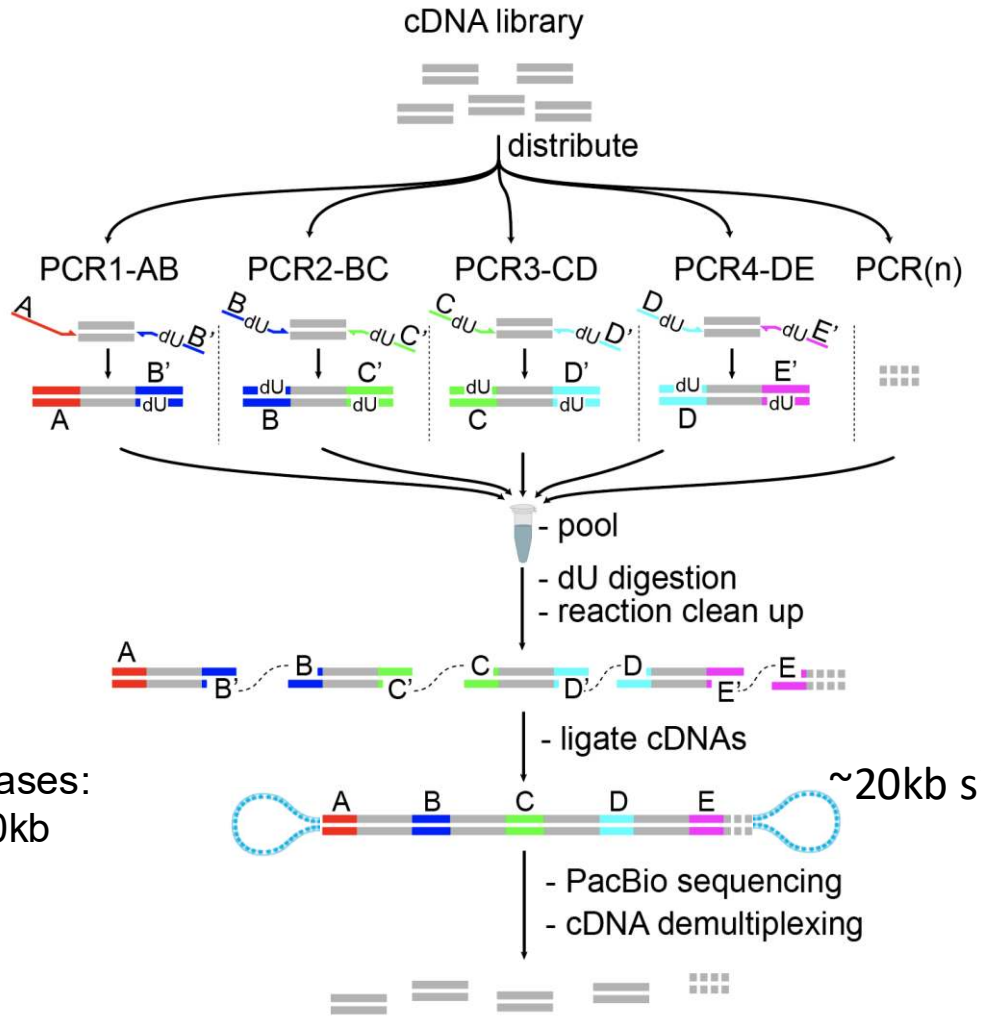
PacBio HiFi Sequencing



Total bases: ~200kb

From Aziz Al'Khafaji, Broad Inst.

Multiplexed Array Sequencing (MAS-Seq)



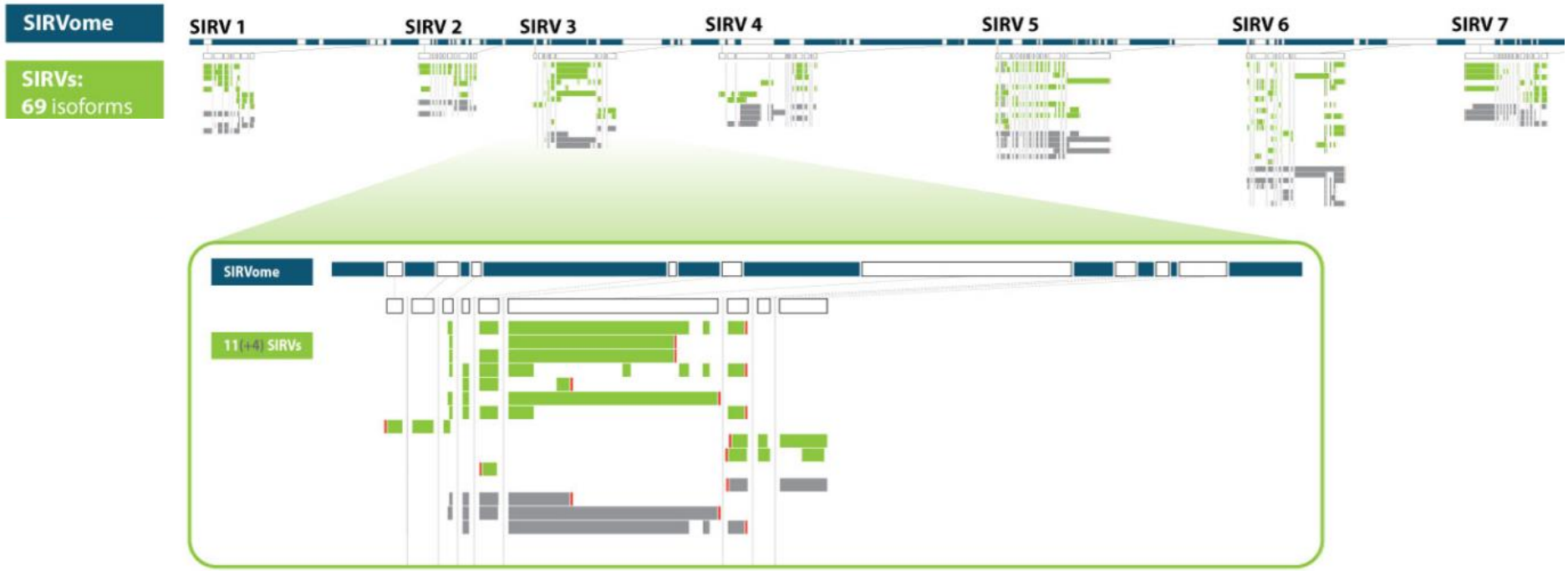
~20kb size

>15-fold increase in throughput

Al'Khafaji et al., NBT 2024

Technical validation using RNA isoform standards

SIRVs (Spike-in RNA Variant Control Mixes) are synthetic gene isoforms

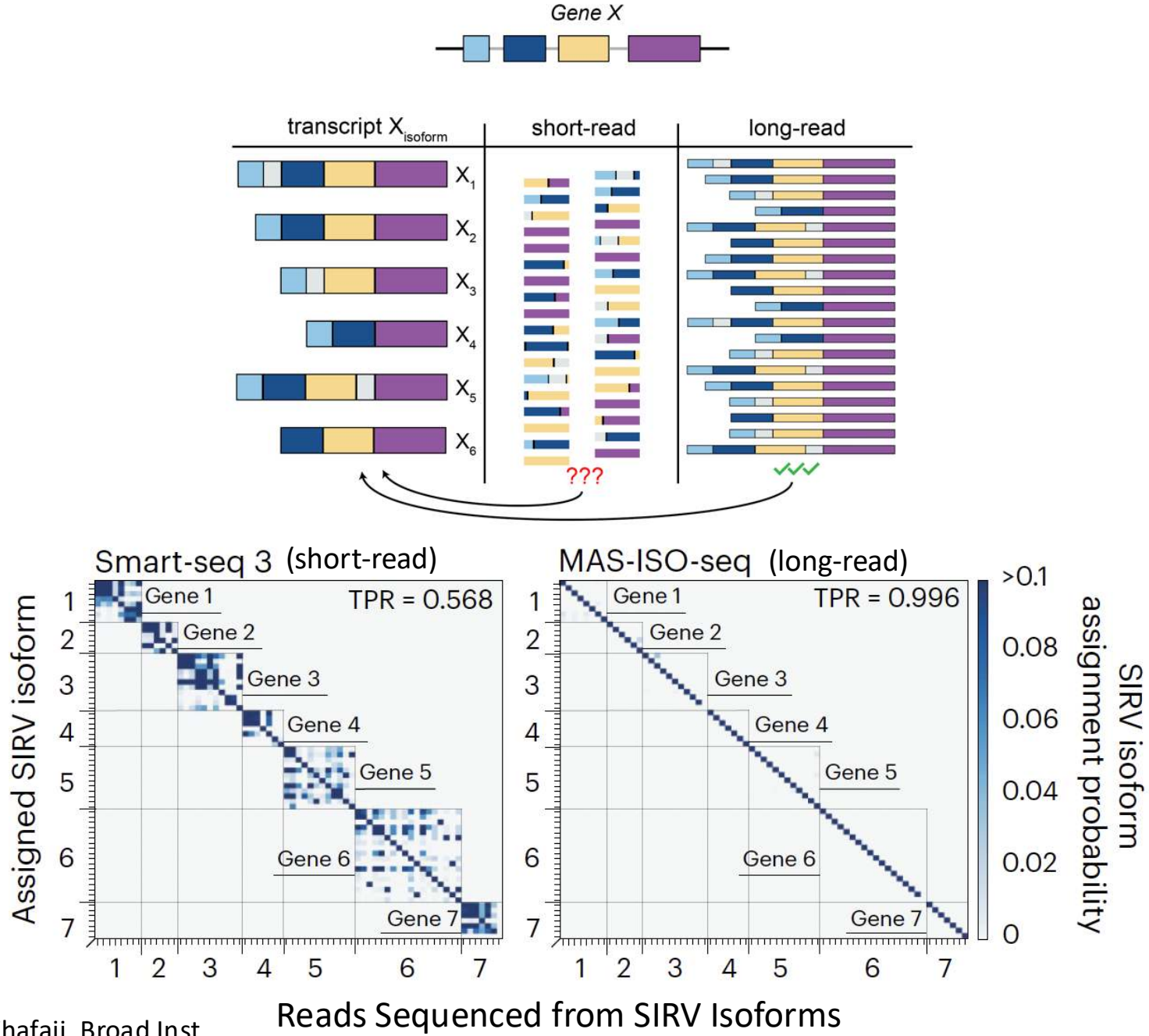


SIRVS serve as truth dataset to evaluate MAS-seq's ability to accurately identify RNA isoforms.

LEXOGEN

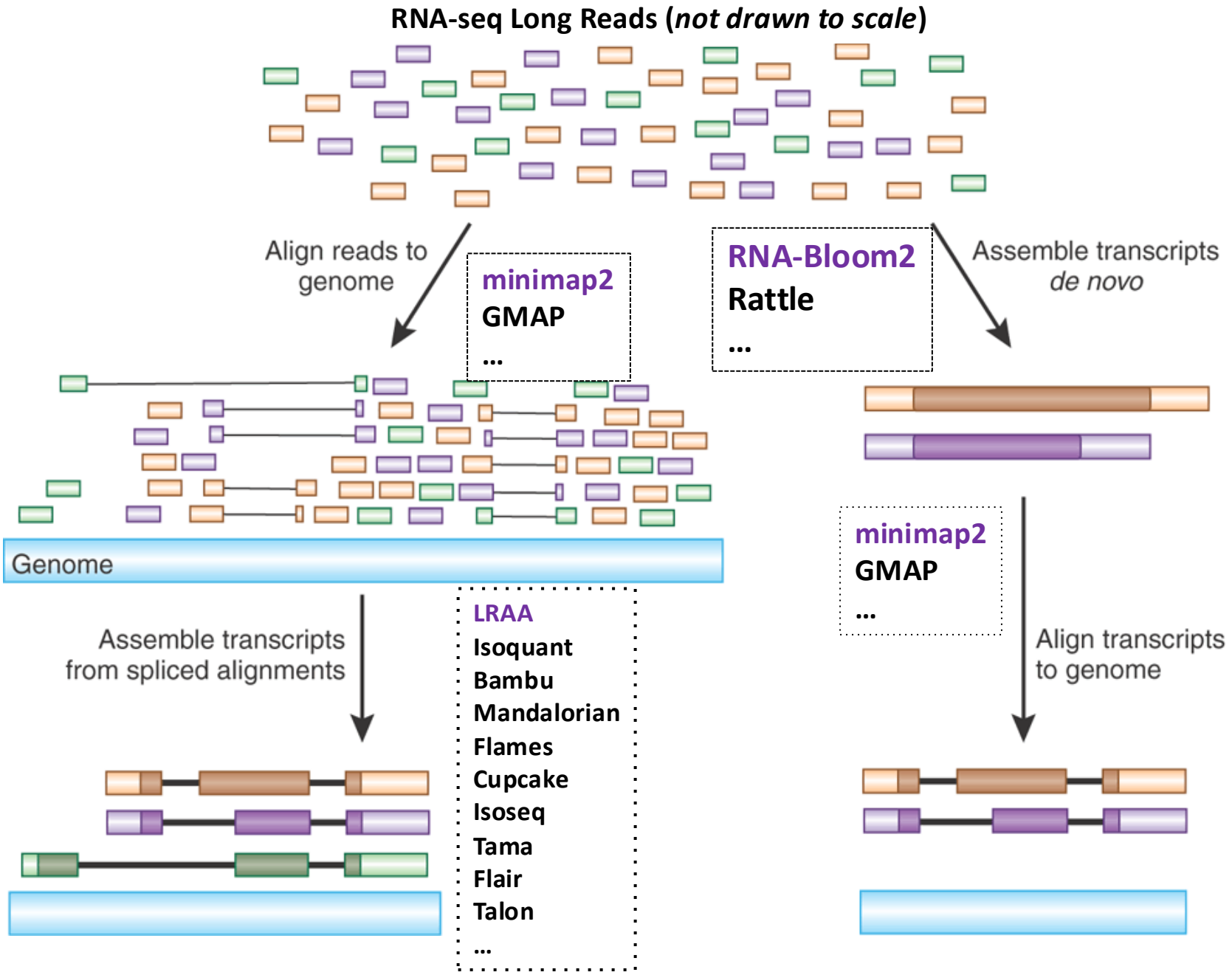
From Aziz Al'Khafaji, Broad Inst.

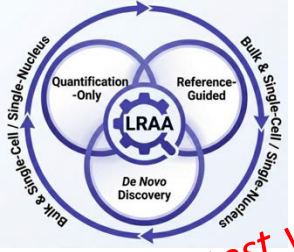
Long-read sequencing accurately identify RNA isoform standards



From Aziz Al'Khafaji, Broad Inst.

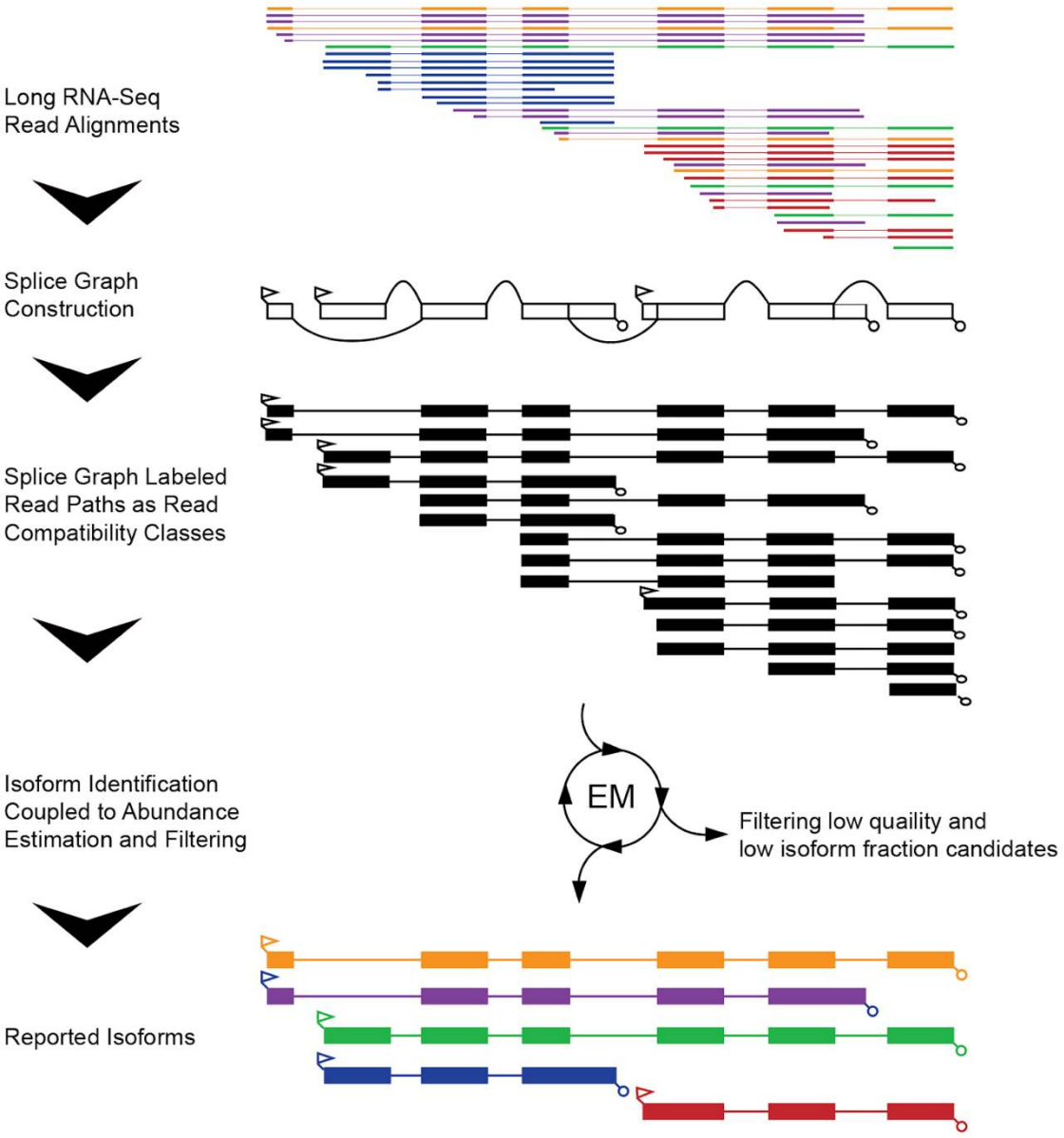
Transcript Reconstruction from (Long) RNA-Seq Reads





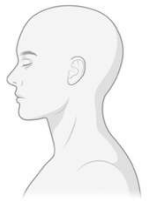
Our latest work

LRAA algorithm for isoform identification and quantification for bulk and single-cell long-read transcriptomics



https://github.com/MethodsDev/LongReadAlignmentAssembler/wiki

Pilot Study to Survey Long Reads and Isoform Structures Across Humans and Non-human Primates



Human

	Colon	Liver	Lung	Muscle
Male				
16 days		X		
7 months		X		X
1 year		X	X	X
14 months	X		X	
9 years	X			
Female				
2 days	X		X	
1 month				X

Rhesus Macaque

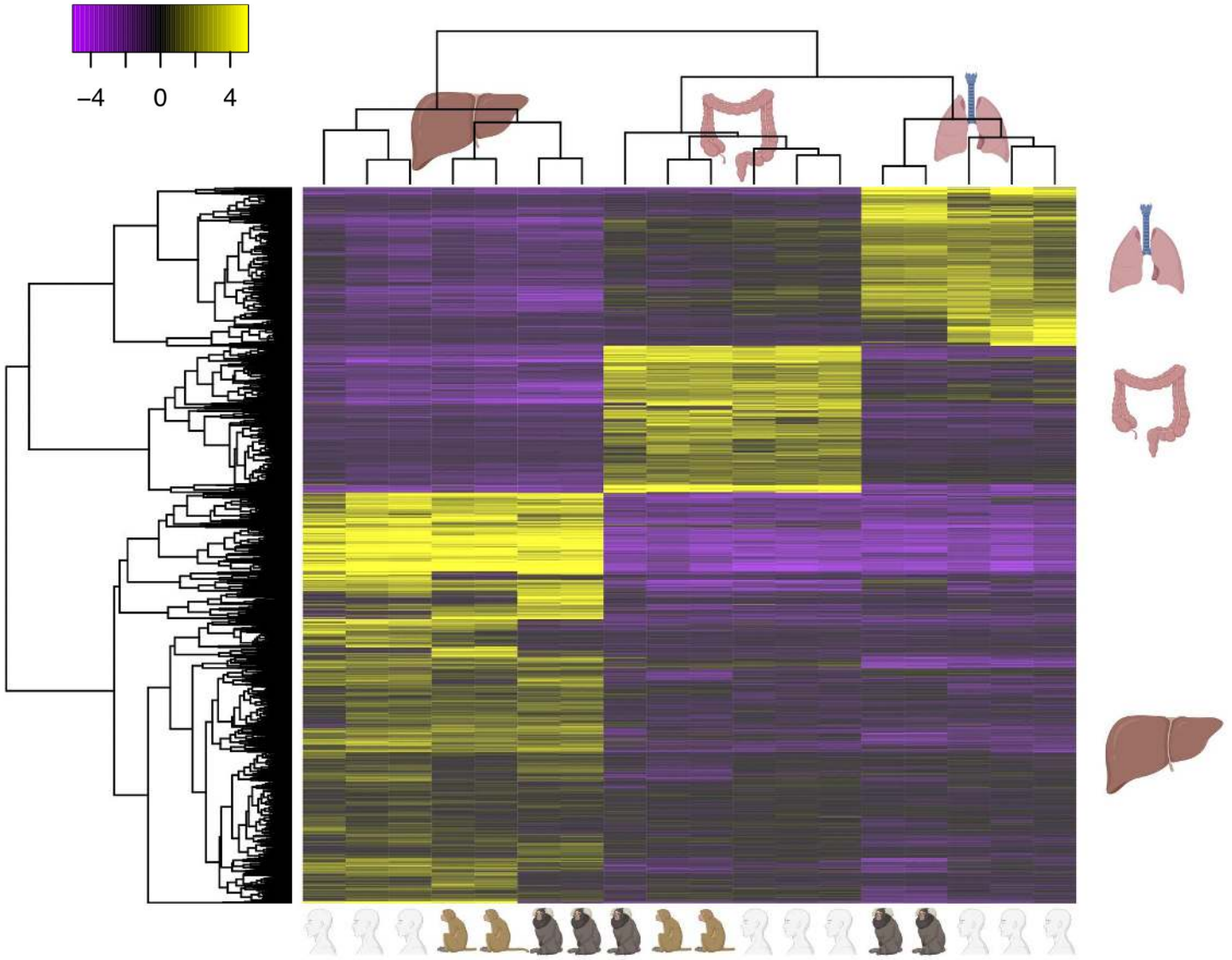
	Colon	Liver	Ovary	Testis
Male				
42 days	X	X		X
Female				
36 days	X	X	X	

Marmoset

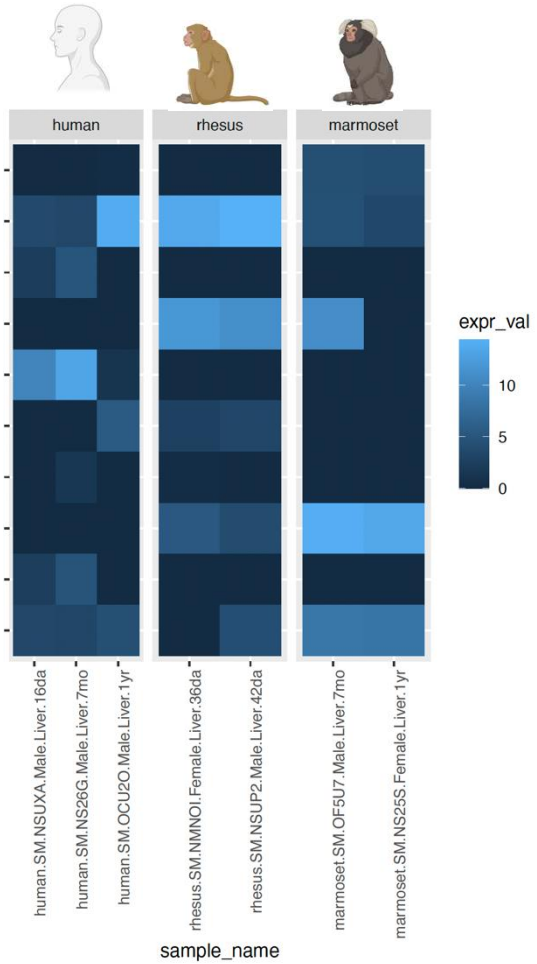
	Colon	Liver	Lung
Male			
7 months	X	X	X
Female			
1 year		X	X

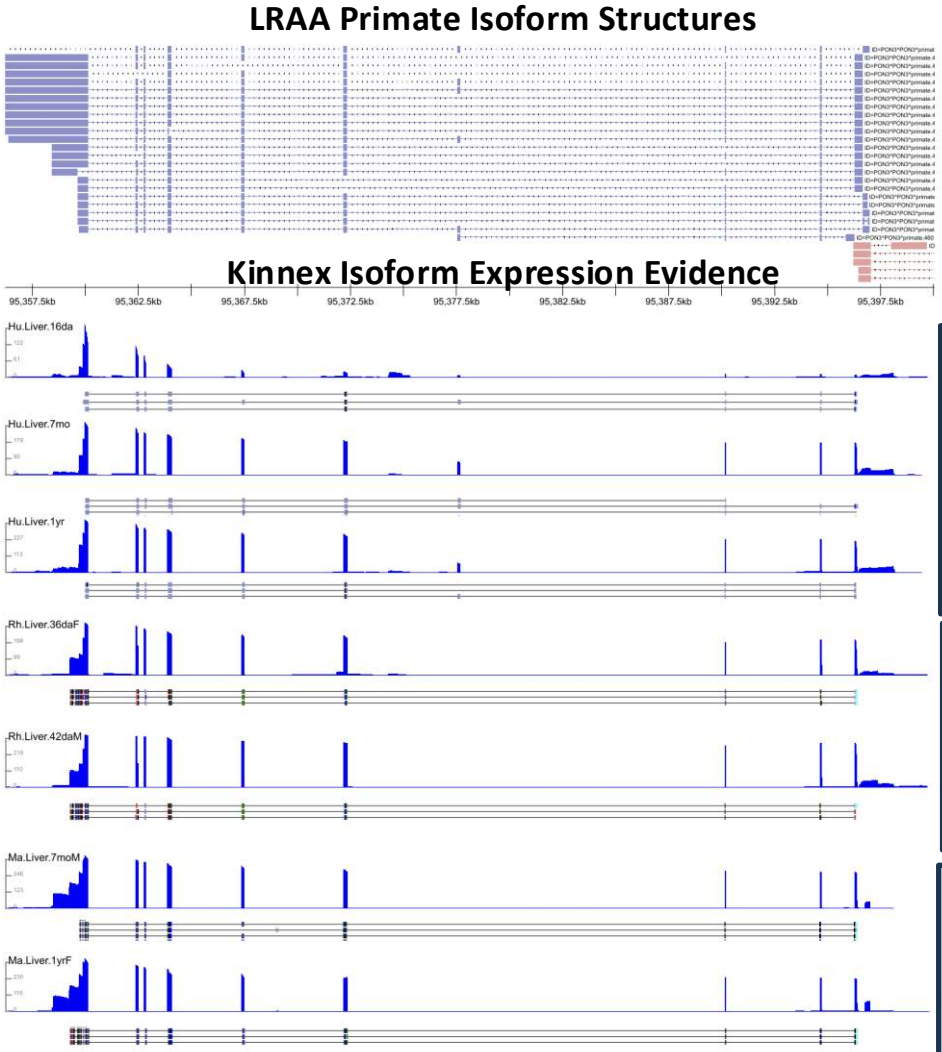
Bulk Kinnex of Primate Tissue in Collaboration with the GTEx Consortium

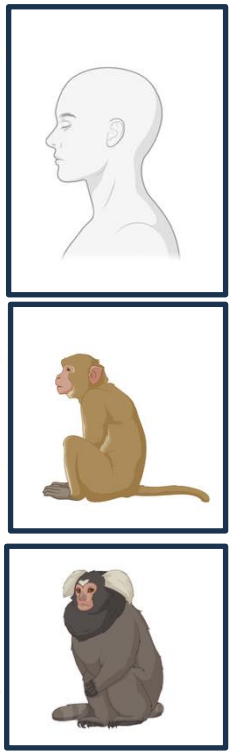
Thousands of tissue-specific genes expressed



Example: Paraoxonase3 Isoform Expression in Liver

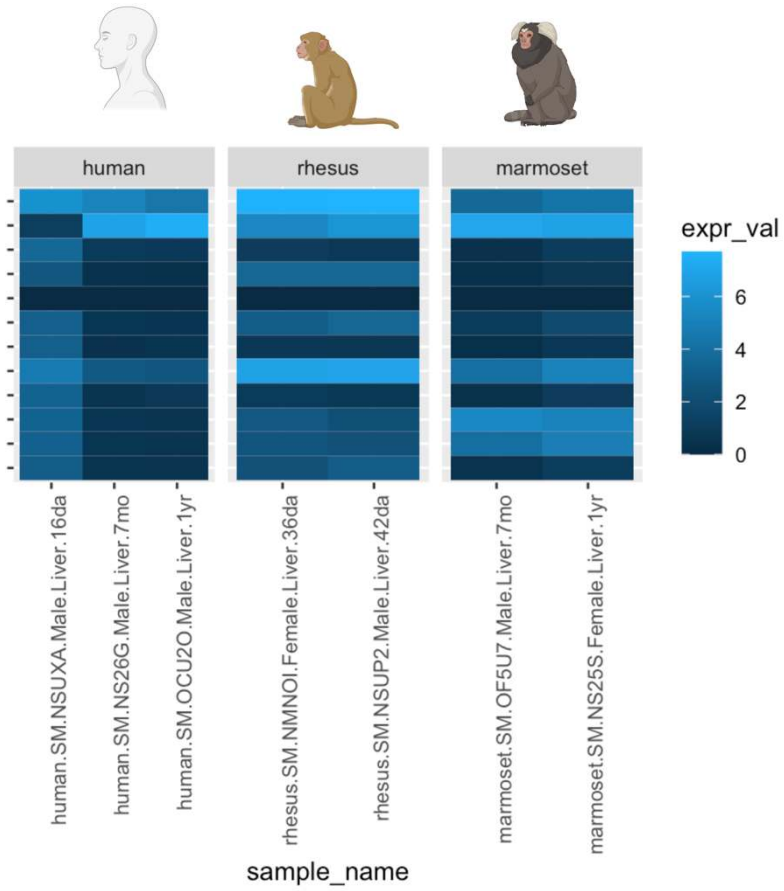


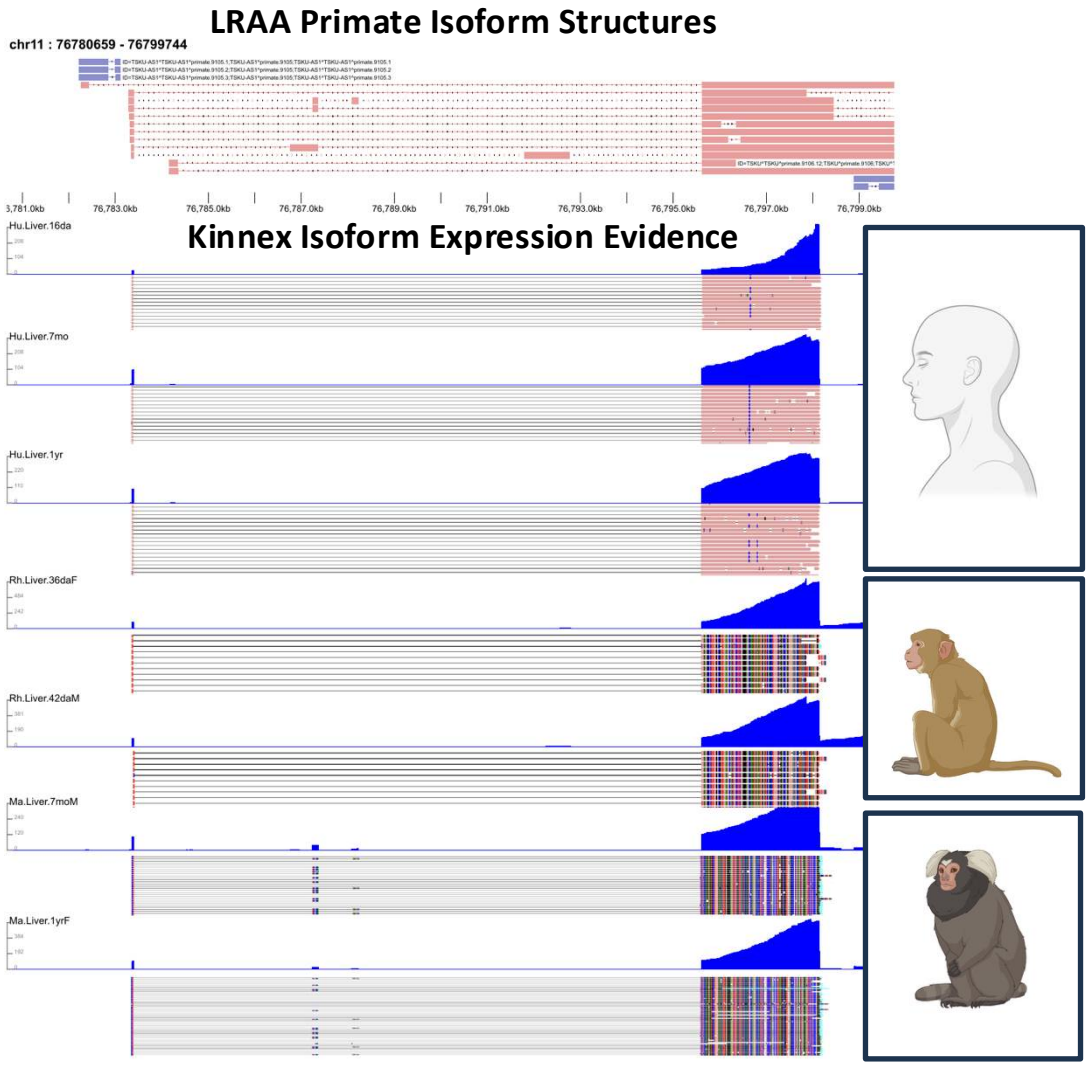




The PON3 gene, a member of the paraoxonase family, encodes a protein that associates with high-density lipoprotein (HDL) and is involved in the hydrolysis of lactones and the inhibition of low-density lipoprotein (LDL) oxidation.

Example: Tsukushi (TSKU) Isoform Expression in Liver





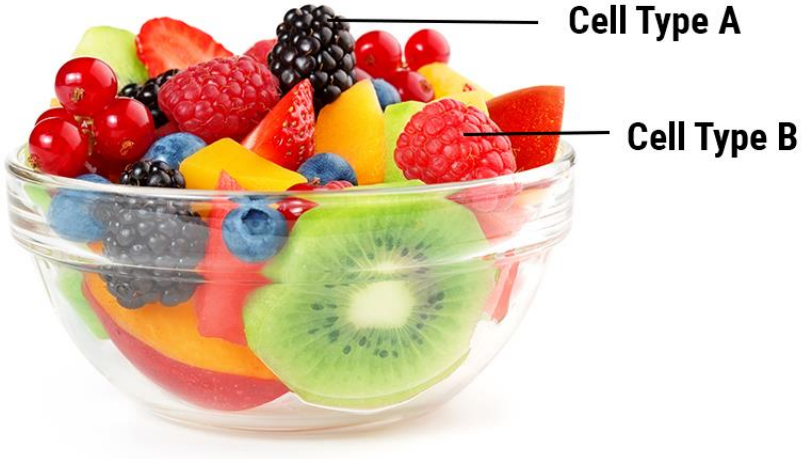
The TSKU gene, encoding the protein Tsukushi, plays a role in cholesterol homeostasis and is released in response to non-alcoholic fatty liver disease (NAFLD). It impacts systemic cholesterol homeostasis, reducing circulating HDL cholesterol, lowering cholesterol efflux capacity, and decreasing cholesterol-to-bile acid conversion in the liver.

Part 7. Overview of Single Cell Transcriptomics

The Quintessential “Fruit Smoothie Metaphor” for Bulk RNA-seq

Bulk RNA Seq

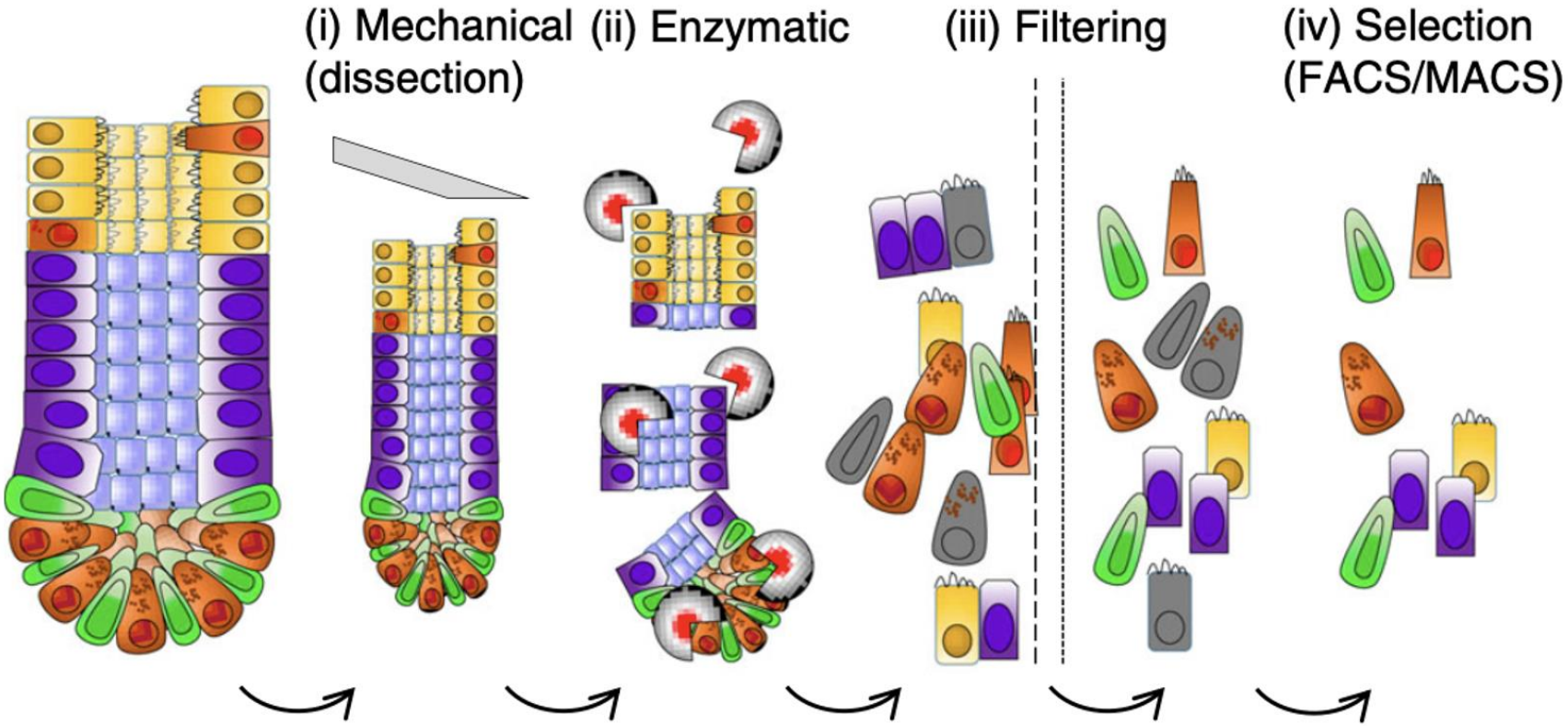
vs.



SCRNA Seq

From: https://perkinelmer-appliedgenomics.com/2022/02/15/single-cell-rna-seq-intro/

Step 1: Break down tissue to single cells (or nuclei)



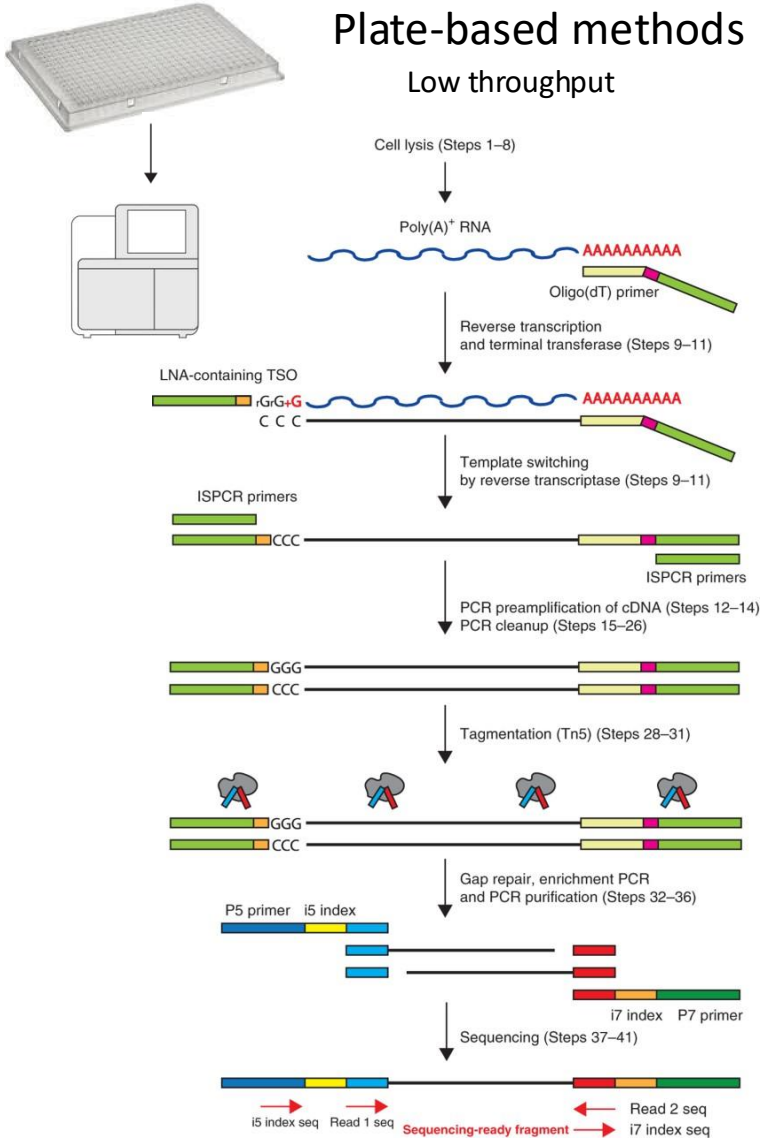
Can also extract and sequence nuclei instead of whole cells – popular in neurobiology

Lafzi et al., Nat Protocols, 2018

Examples of Different Popular Classes of Single Cell Sequencing

Plate-based methods

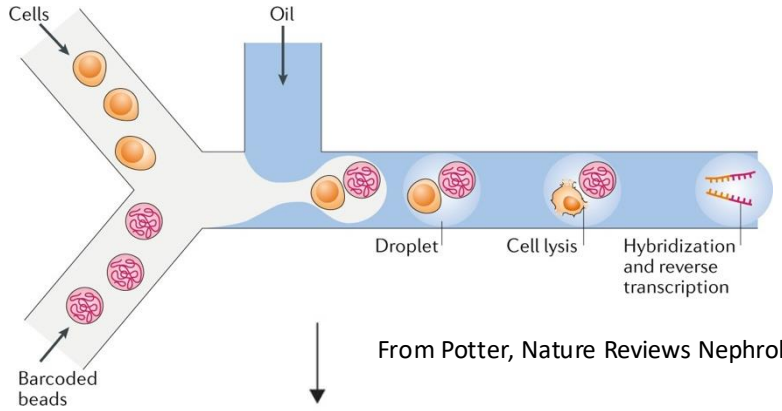
Low throughput



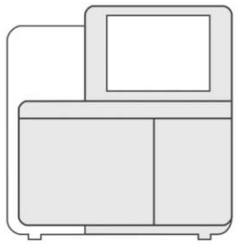
Smart-seq2 Method: Get reads covering the full length of the RNA molecule.

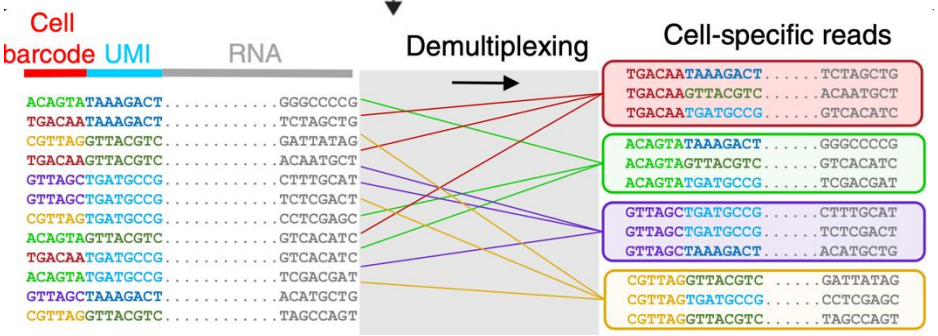
Picelli et al., Nature Protocols, 2014

Droplet-based methods



From Potter, Nature Reviews Nephrology, 2018

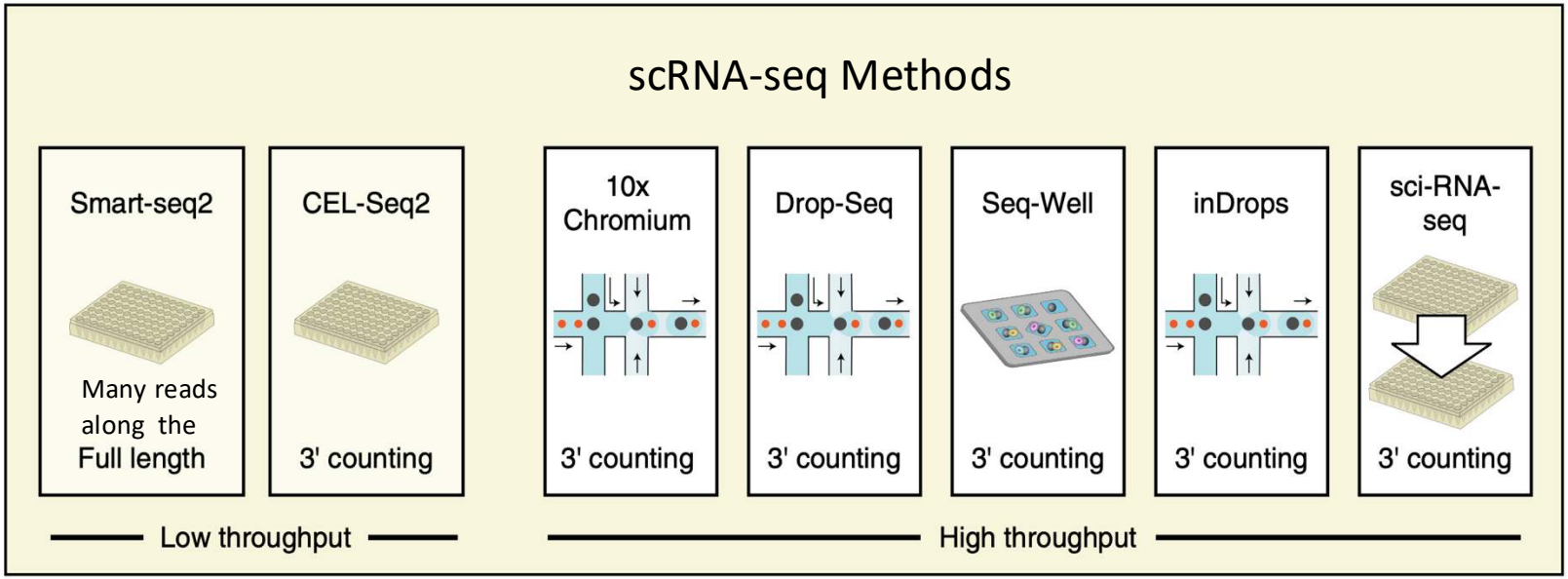




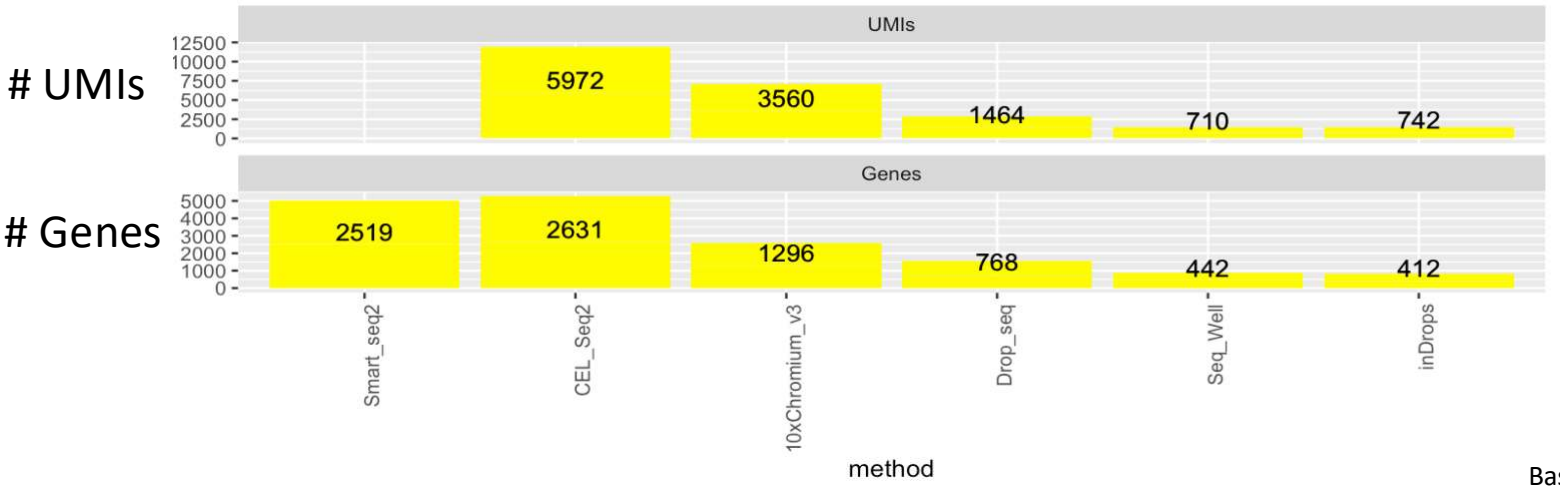
Lafzi et al., Nat Protocols, 2018

Unique Molecular Identifier (UMI)

Single Cell Transcriptome Sequencing Methods

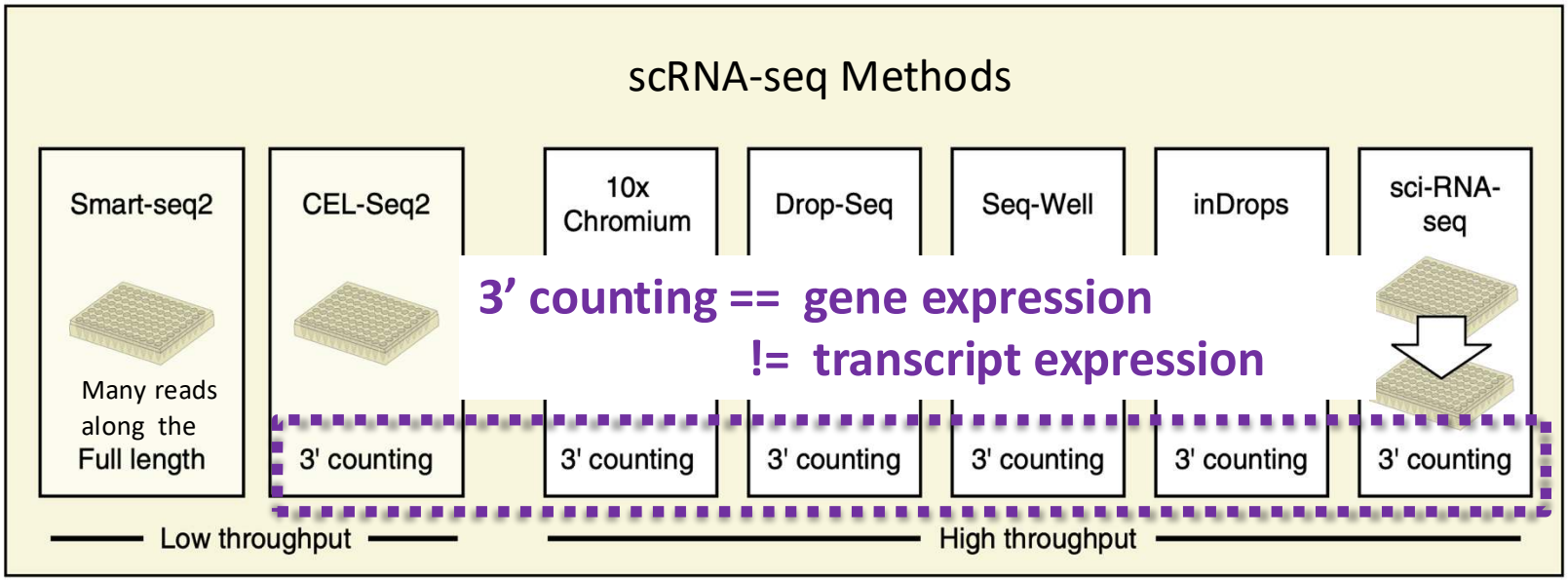


Averaged counts of UMIs and Genes per cell by method

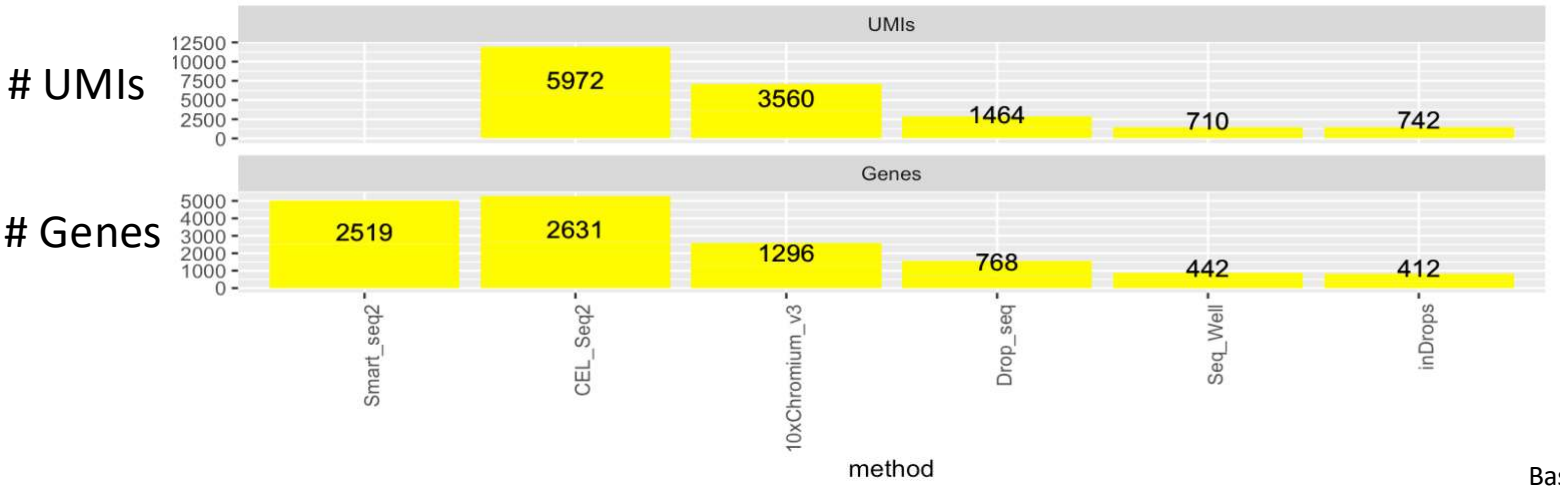


Based on Ding et al., NBT 2020

Single Cell Transcriptome Sequencing Methods

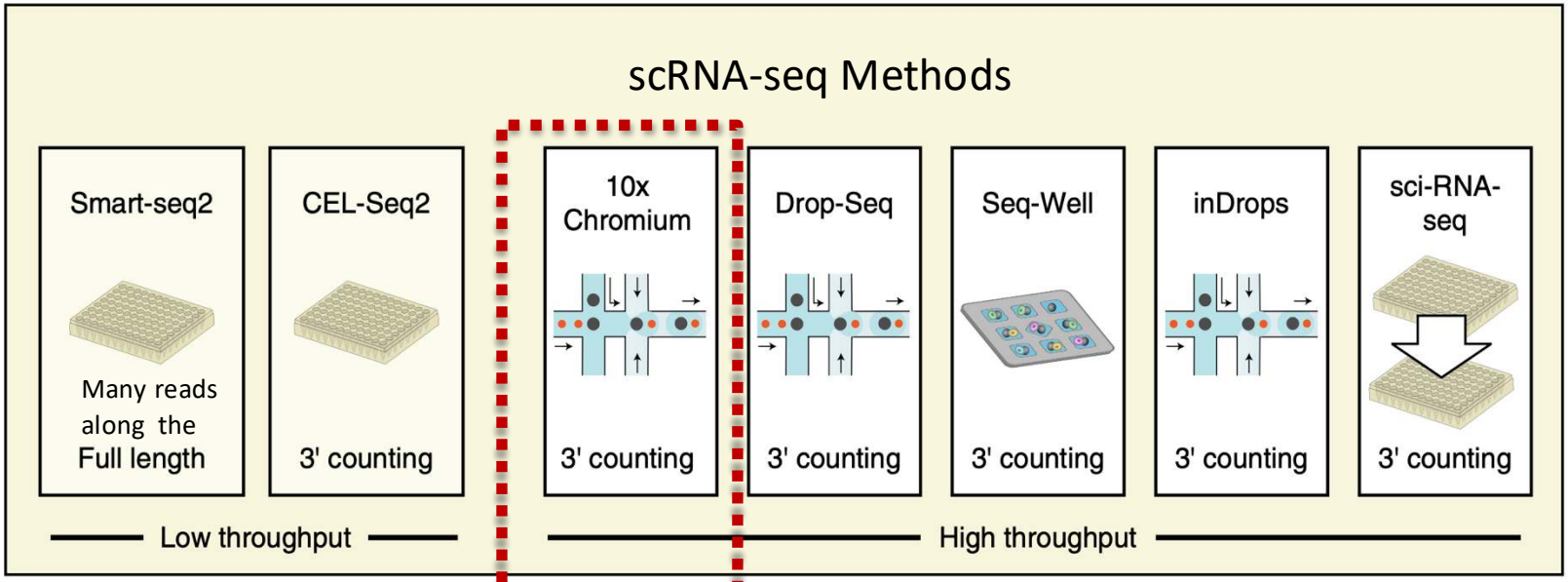


Averaged counts of UMIs and Genes per cell by method

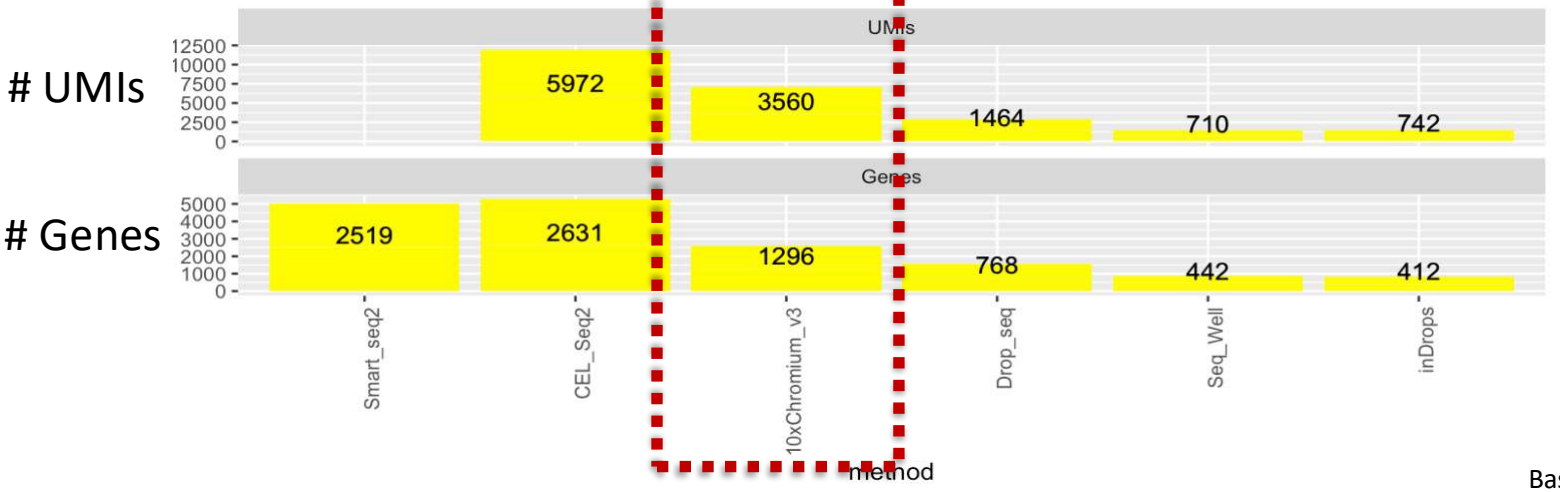


Based on Ding et al., NBT 2020

Single Cell Transcriptome Sequencing Methods

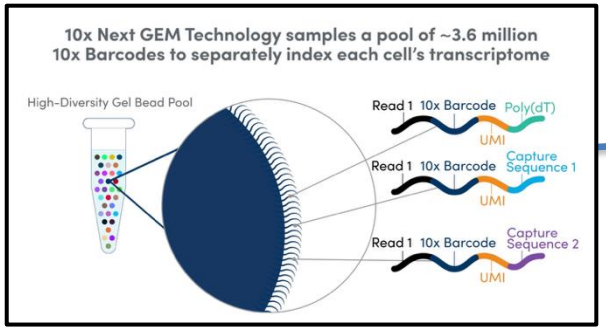


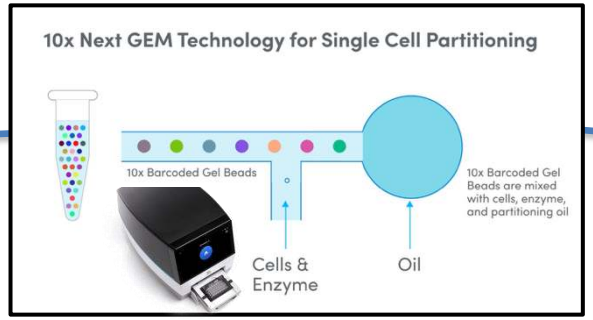
Averaged counts of UMIs and Genes per cell by method

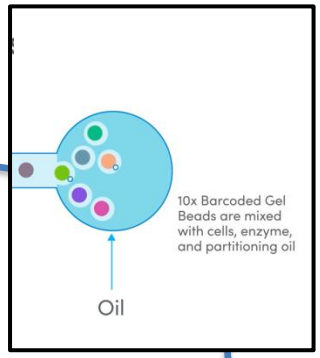


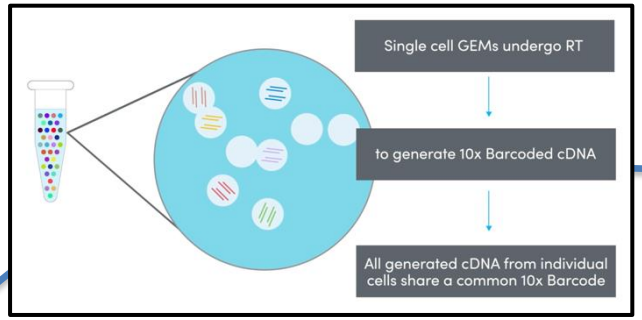
Based on Ding et al., NBT 2020

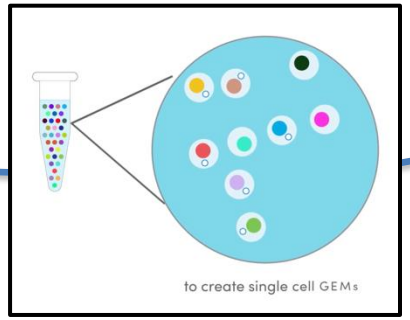
10x Genomics Chromium Single Cell Transcriptome Sequencing

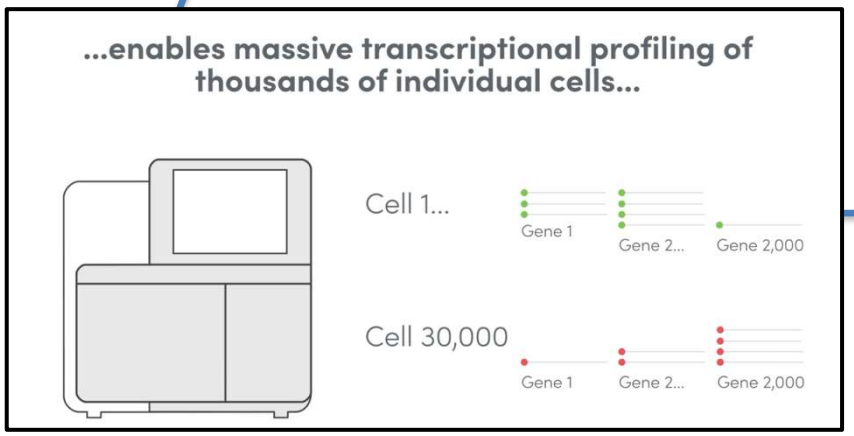


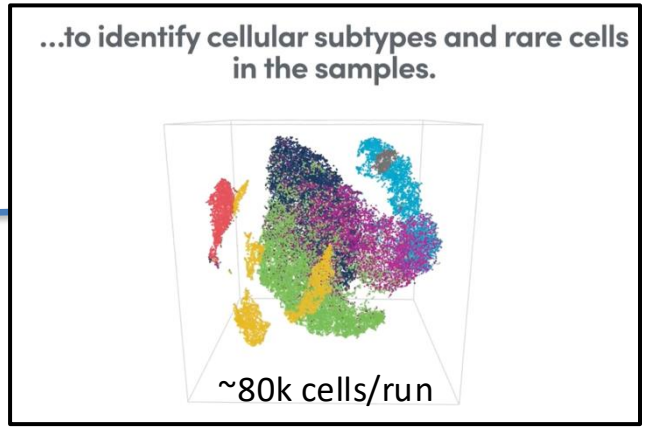






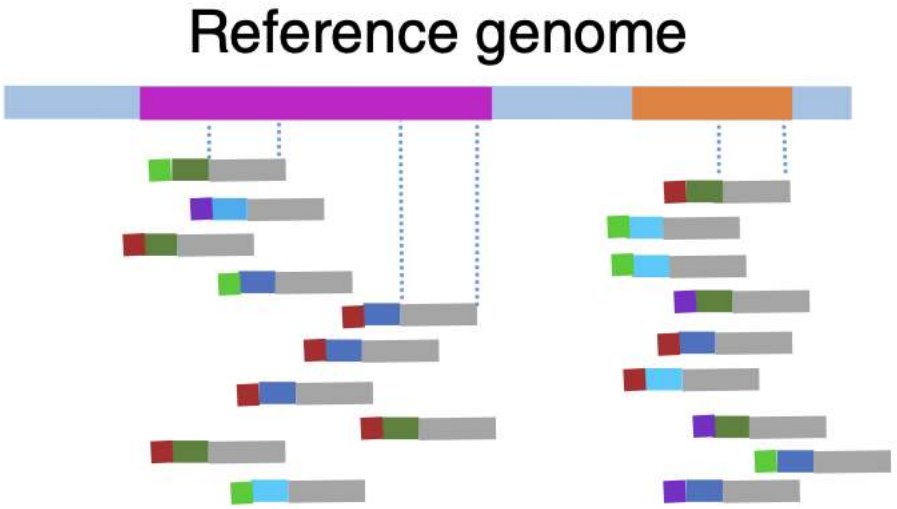






https://www.10xgenomics.com/platforms/chromium

Analysis Workflow for Single Cell Transcriptomics



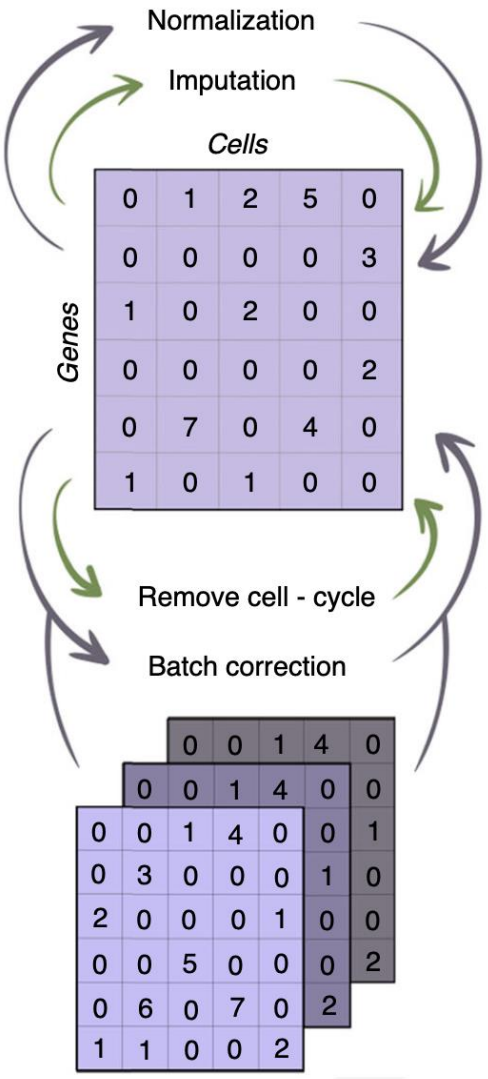
- Align reads to the reference genome

- Collapse PCR duplicates (by UMIs)

	Cell1	Cell2	...	CellN
Gene1	3	2	.	13
Gene2	2	3	.	1
Gene3	1	14	.	18
...	.	.	.	.
...	.	.	.	.
...	.	.	.	.
GeneM	25	0	.	0

- Build a {Gene X Cell} UMI counts matrix

Single Cell Transcriptomics Data Processing Workflow



Gene 'count' matrices for single cell data tend to be very large and very sparse

eg. 25k genes x 100k cells

(almost all zeros – no reads detected)

Various processing needed:

- Which cells are 'good' cells? vs dying/stressed cells, doublets, or empty droplets?

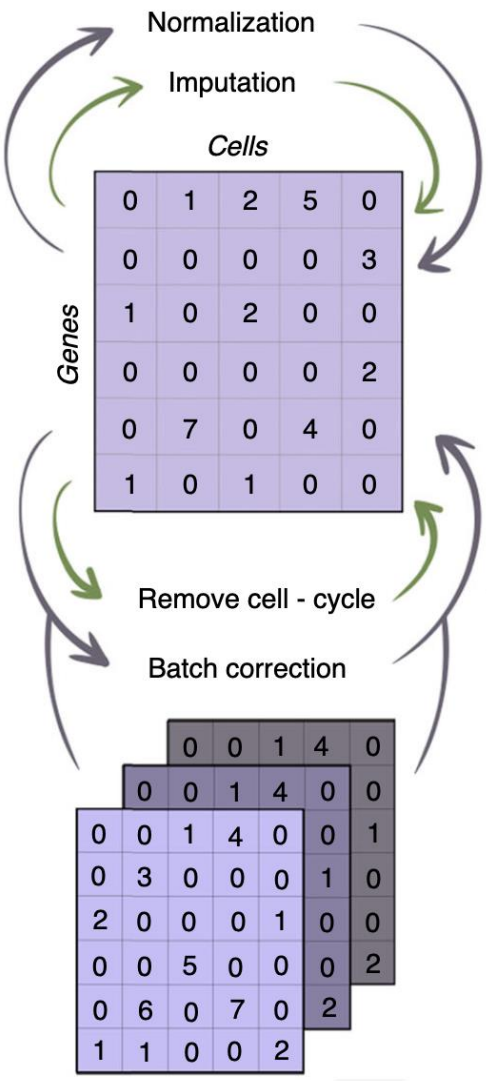
- possibly remove confounding cell cycle signatures from expression data.

- Multiple experiments/replicates - batch correction or harmonization?

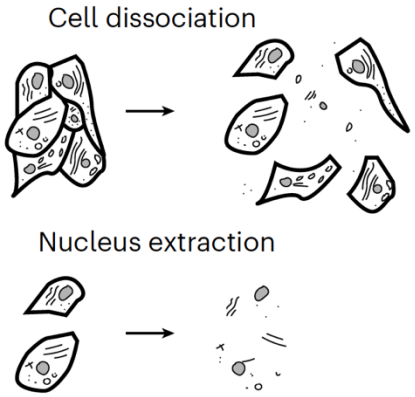
Andrews, 2021, review

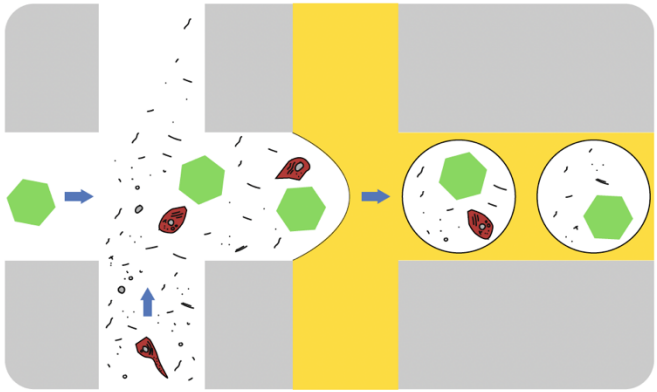
In Silico Removal of Ambient RNA

(by Cellbender)

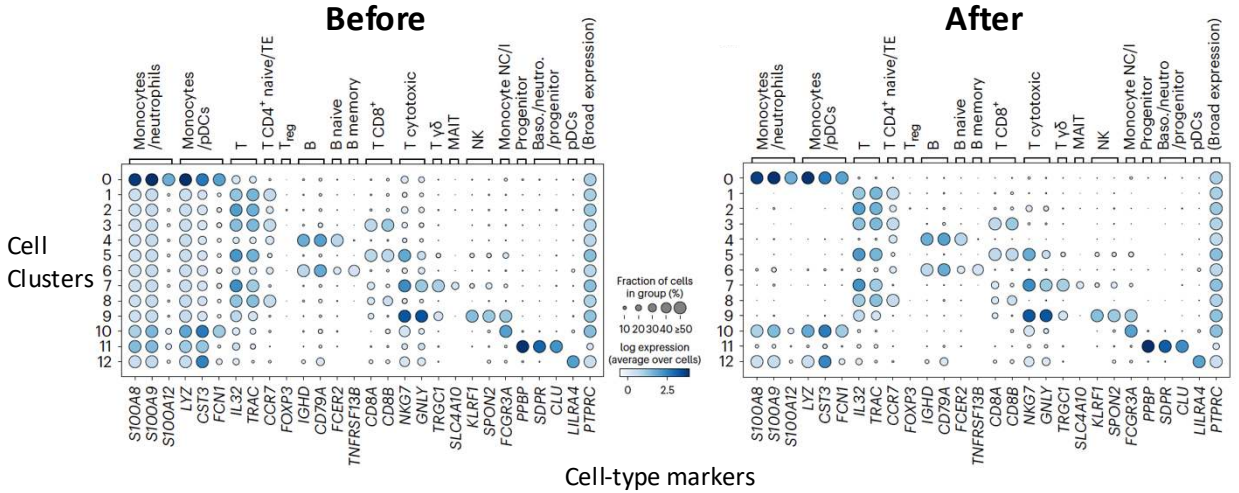


Phenomenology of ambient RNA





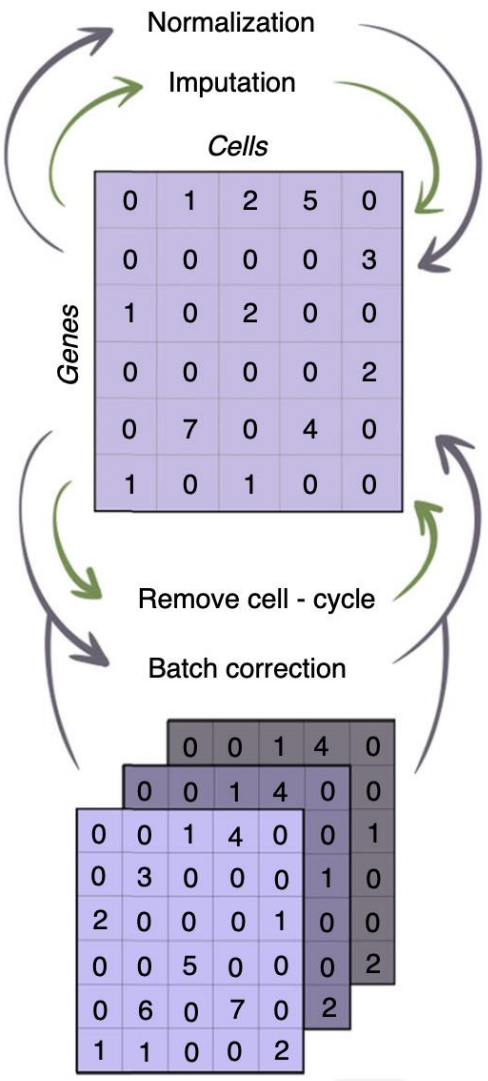
Cell Markers and Read Quantities by Cell Type



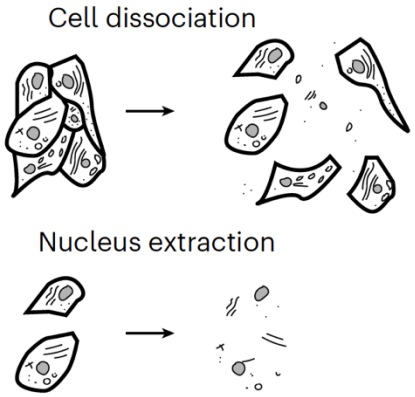
Andrews, 2021, review

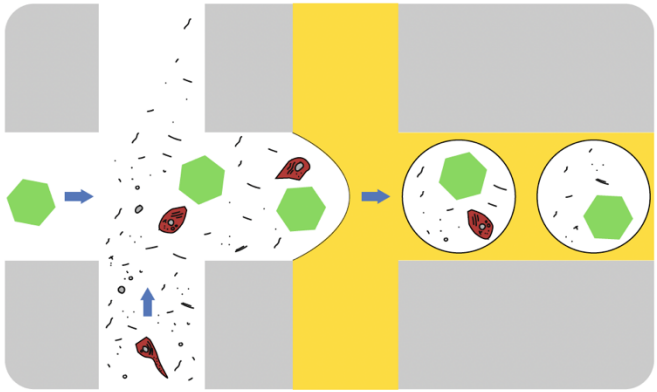
In Silico Removal of Ambient RNA

(by Cellbender)



Phenomenology of ambient RNA



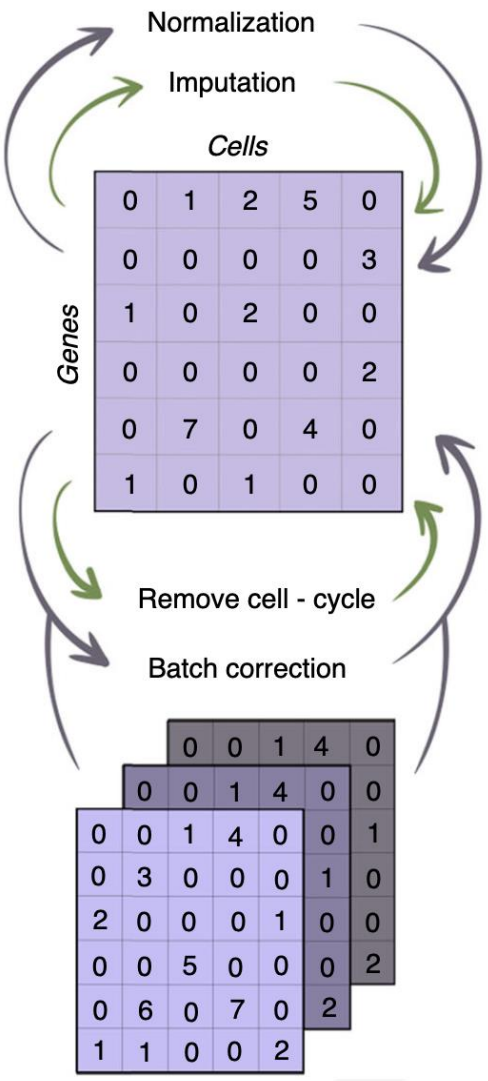


CellBender

https://github.com/broadinstitute/CellBender

Andrews, 2021, review

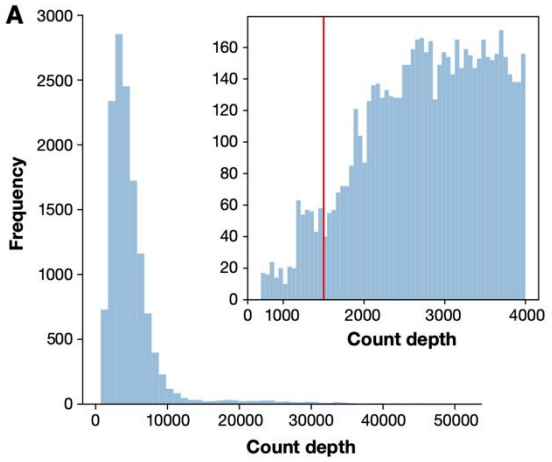
Metrics for Filtering Cells – Keep the Good Ones



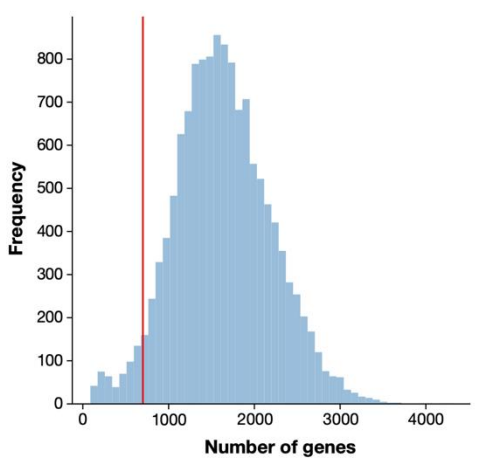
Andrews, 2021, review

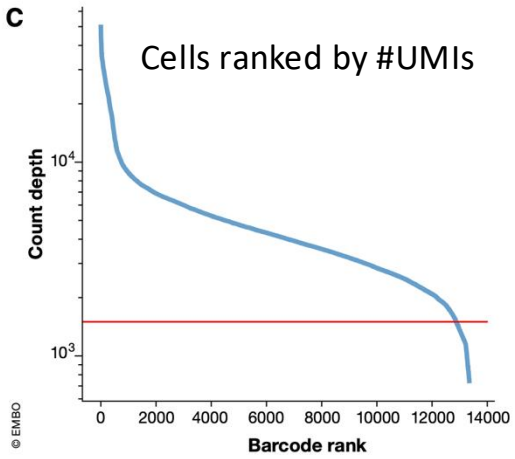
Filter cells based on #genes, #UMIs, and %Mito RNA

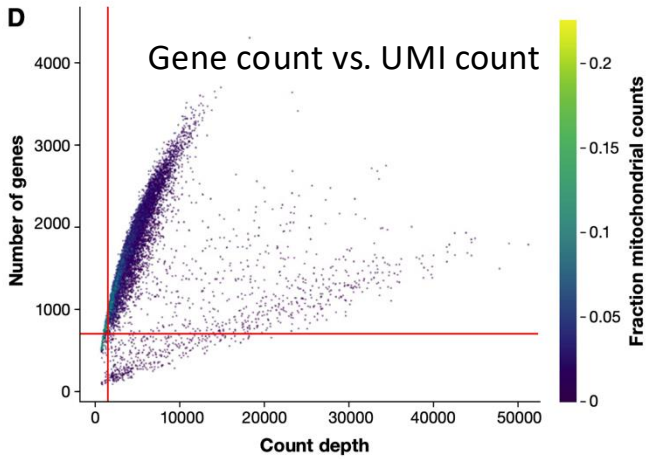
Histogram of #UMIs per cell



Histogram of #genes per cell

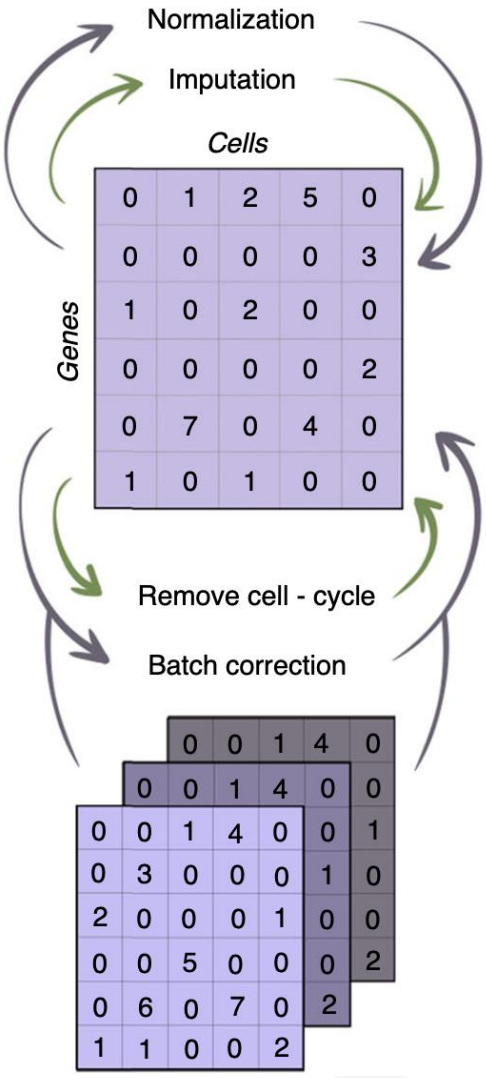






Exclude cells with high mitochondrial RNA content

Batch Correction for Single Cell Transcriptomes



Plot your cells and paint by batch to examine this.
Batch correction methods are available

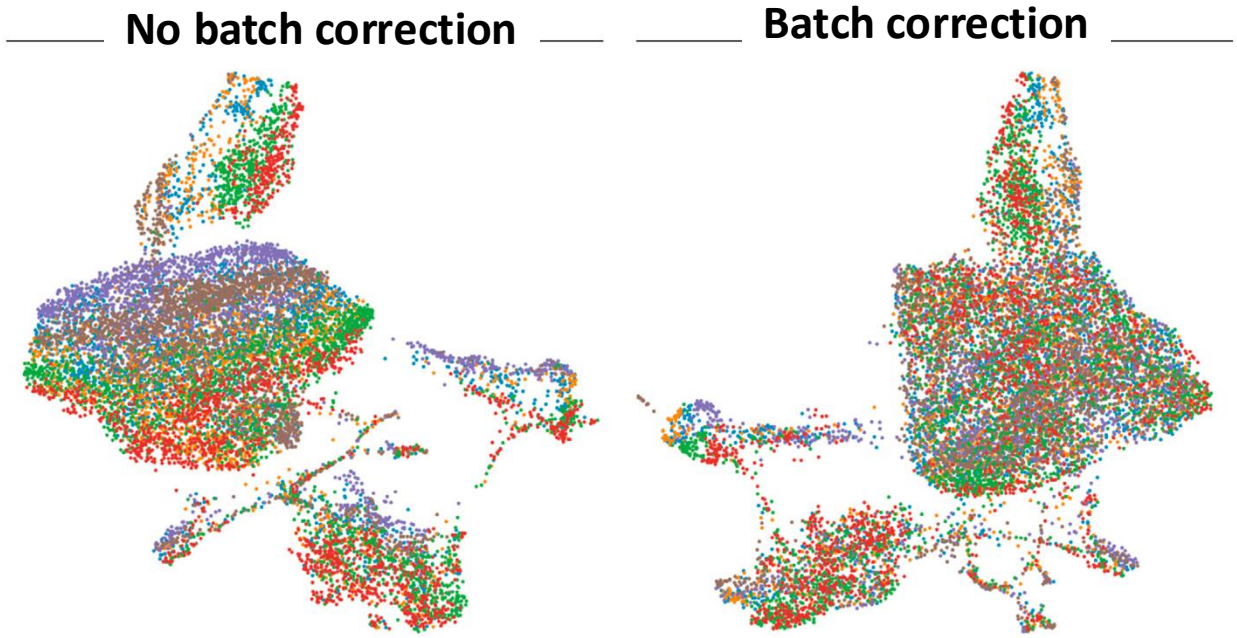
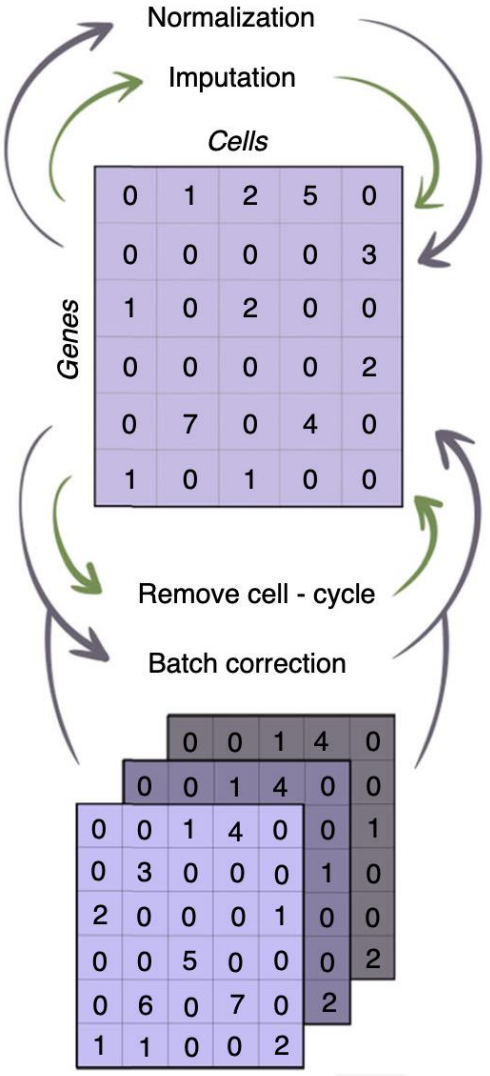


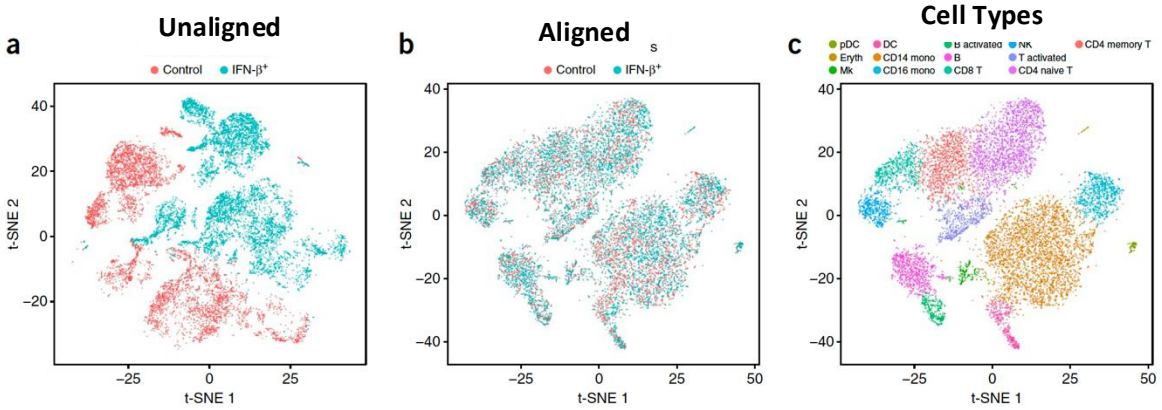
Figure 3. UMAP visualization before and after batch correction.
Cells are coloured by sample of origin. Separation of batches is clearly visible before batch correction and less visible afterwards. Batch correction was performed using ComBat on mouse intestinal epithelium data from Haber *et al* (2017).

Andrews, 2021, review

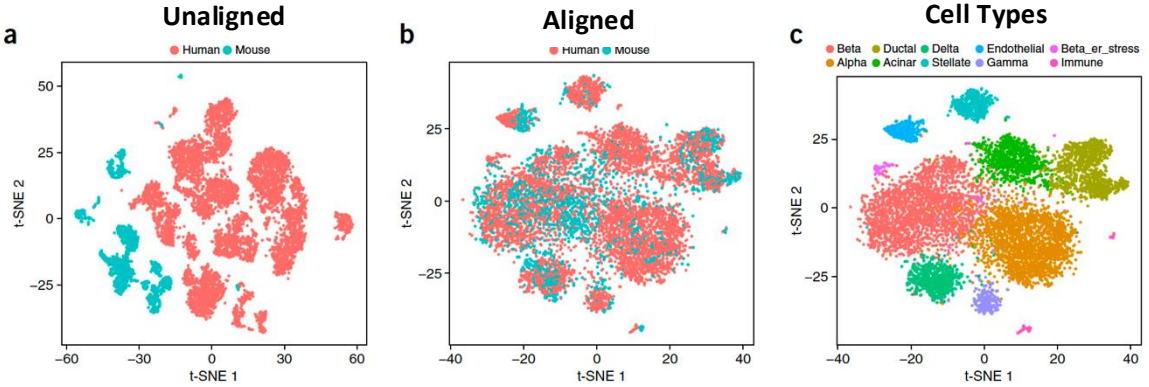
Integrating scRNA-seq data sets based on common sources of variation



Peripheral blood mononuclear cells (PBMCs) +/- stimulation



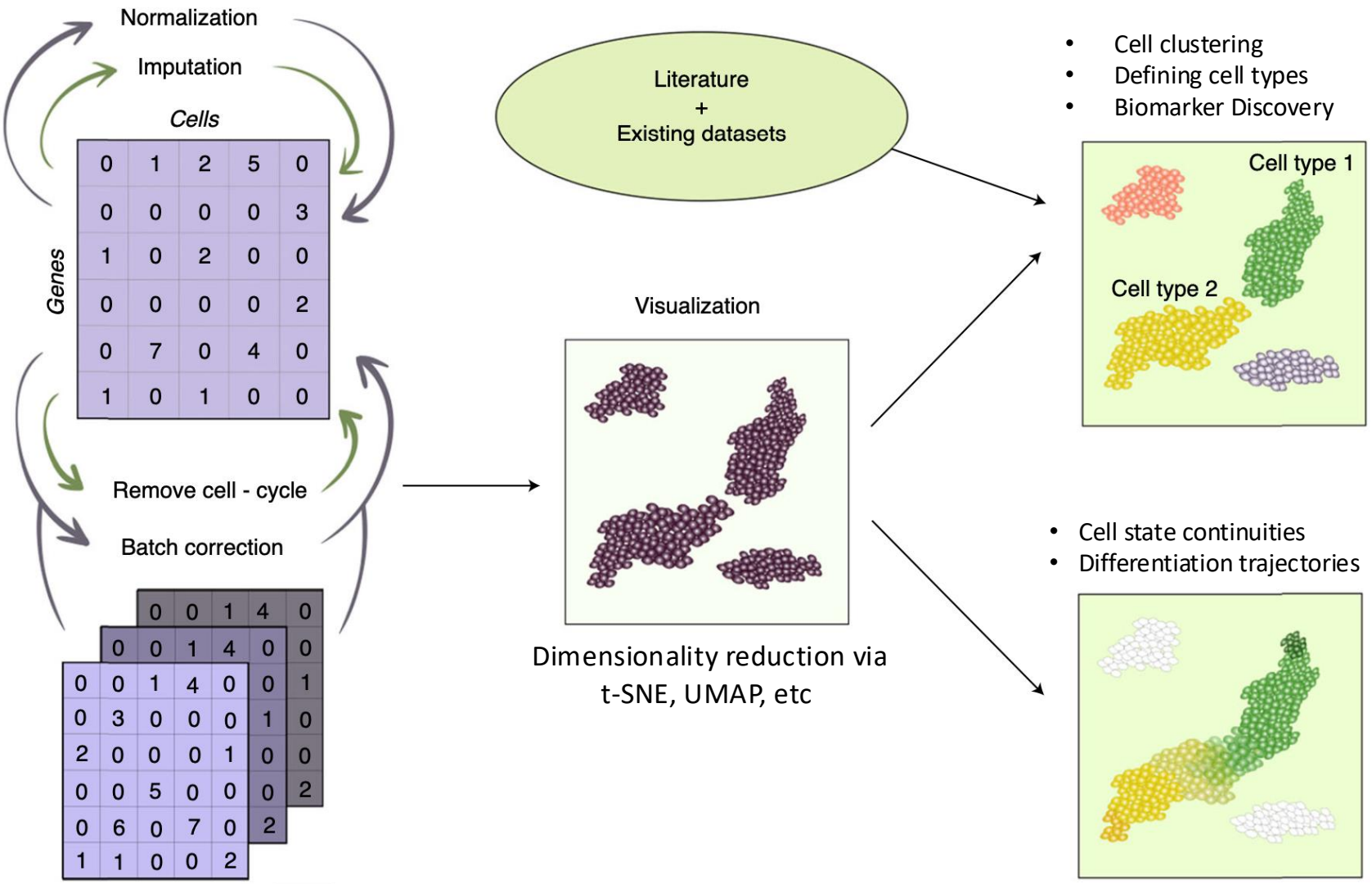
Mouse and human pancreas islet cells



Aligned using Seurat via canonical correlation analysis (CCA)
Butler et al., Nature Biotech, 2018

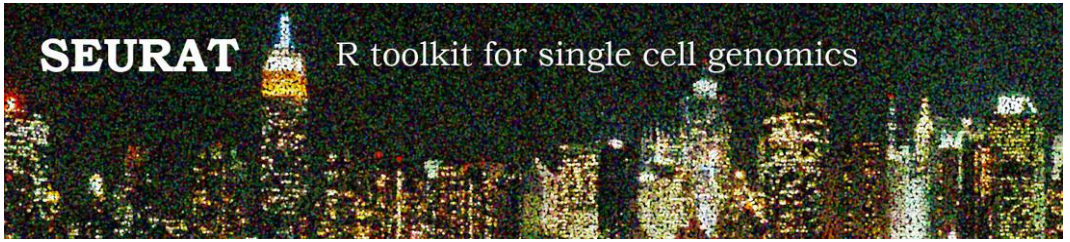
Andrews, 2021, review

Finally, Single Cell Data Exploration and Biological Discovery



Andrews, 2021, review

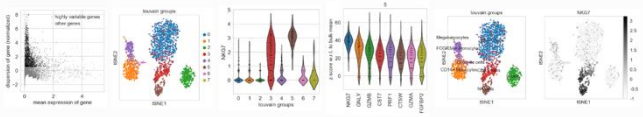
Popular Software Packages for Single Cell Transcriptome Studies



Tutorials

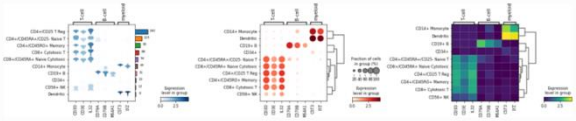
Clustering

For getting started, we recommend Scanpy's reimplementation → tutorial: pbmc3k of Seurat's [^cite_satija15] clustering tutorial for 3k PBMCs from 10x Genomics, containing preprocessing, clustering and the identification of cell types via known marker genes.



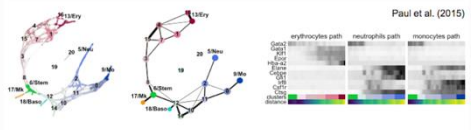
Visualization

This tutorial shows how to visually explore genes using scanpy. → tutorial: plotting/core

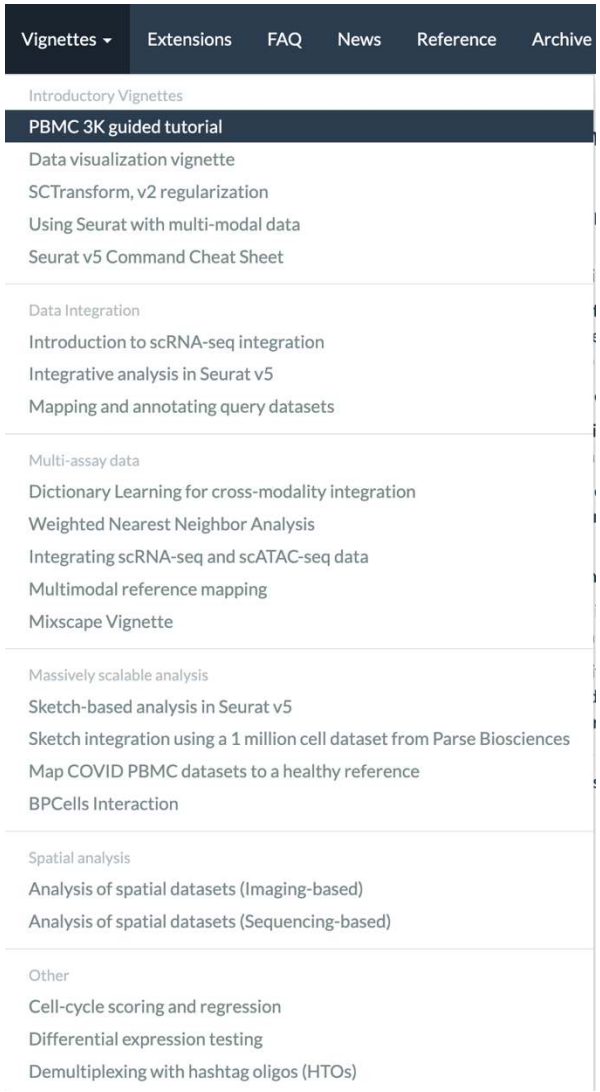


Trajectory inference

Get started with the following example for hematopoiesis for data of [^cite_paul15]:
→ tutorial: paga-paul15

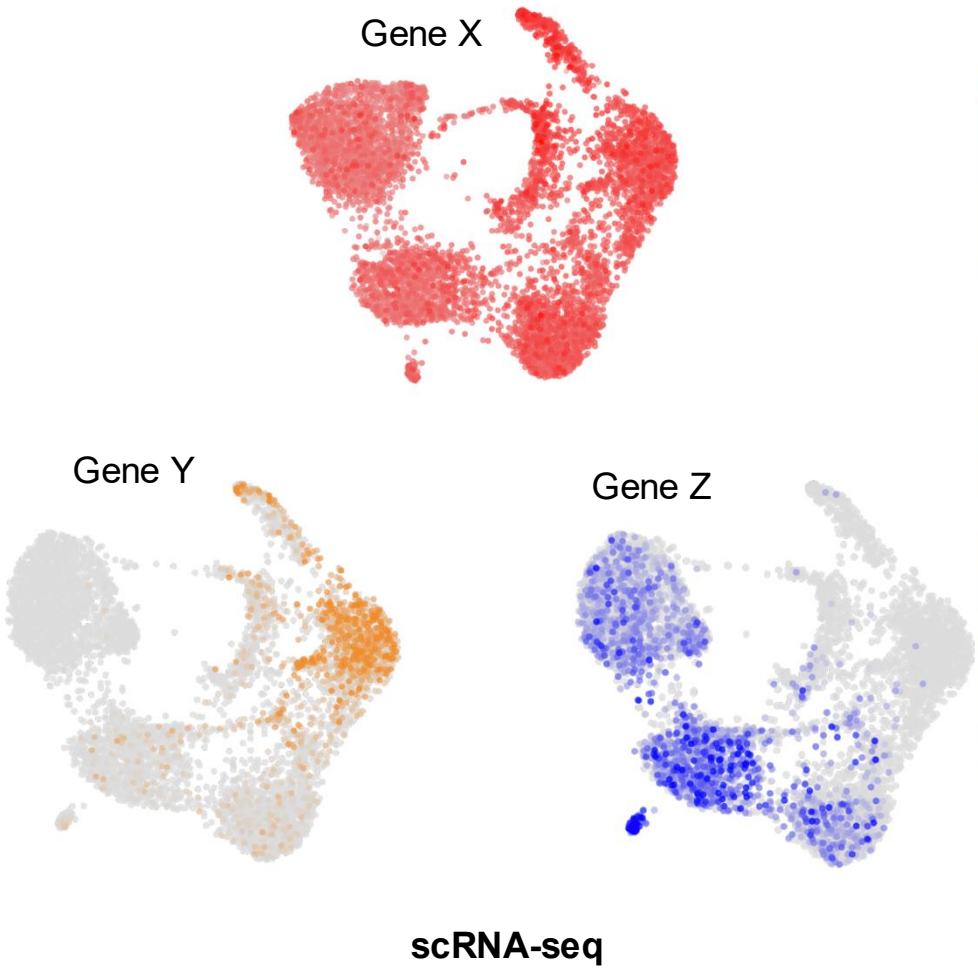


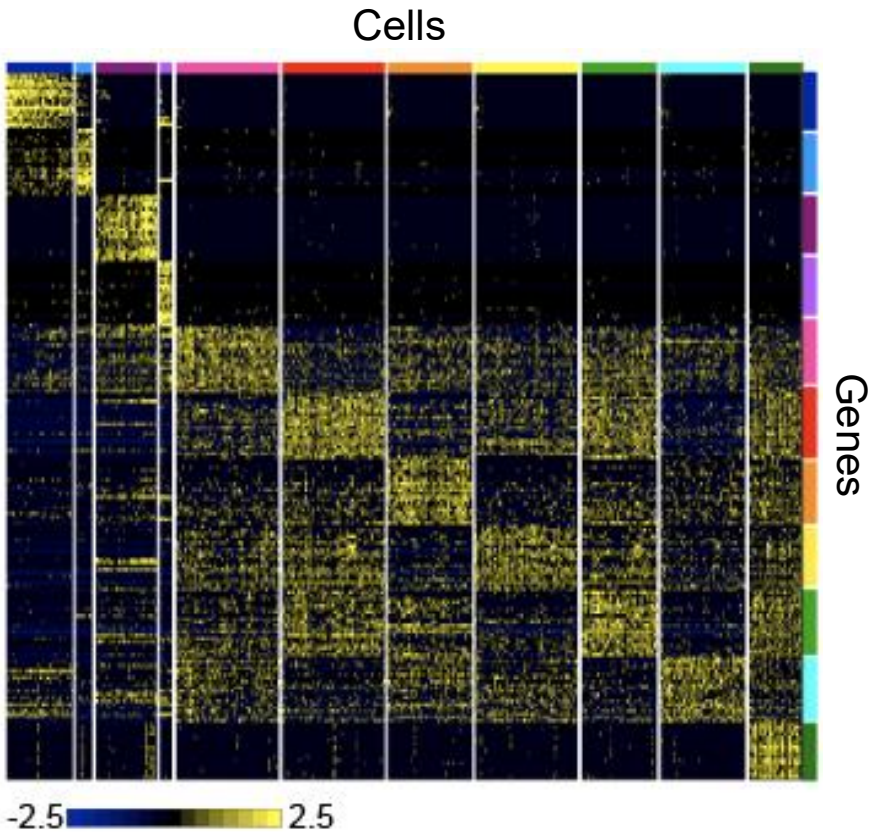
F. Alexander Wolf, Philipp Angerer & Fabian J. Theis,
Genome Biology, 2018;
Isaac Virshup: lead developer since 2019



From Rahul Satija's lab

Gene expression ≠ transcript expression





But – long isoform reads to the rescue!!

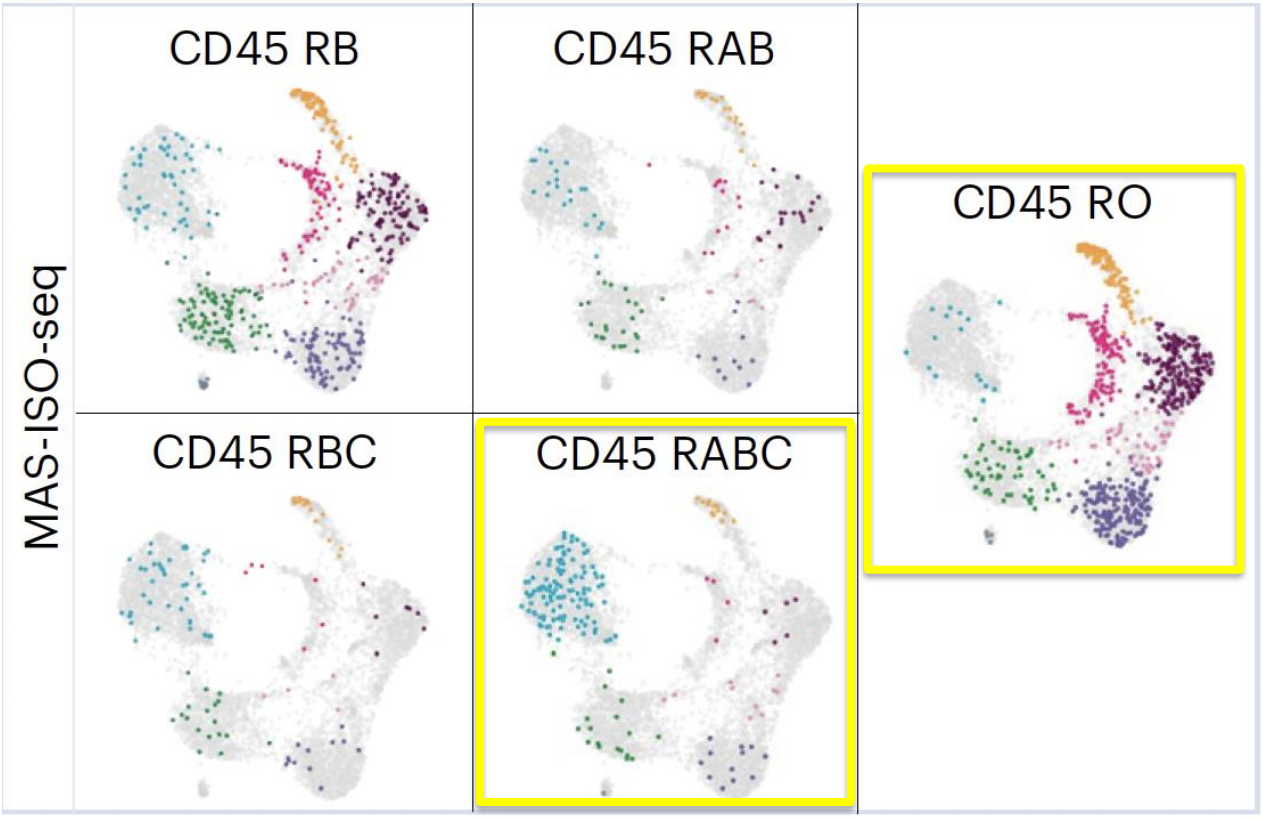
From Aziz Al'Khafaji, Broad Inst.

Long read scRNA-seq (Kinnex) of tumor infiltrating CD8 T cells

CD45 epitope expression

(by CITE-seq)

CD45 T-cell Marker Isoform expression resolved via long reads

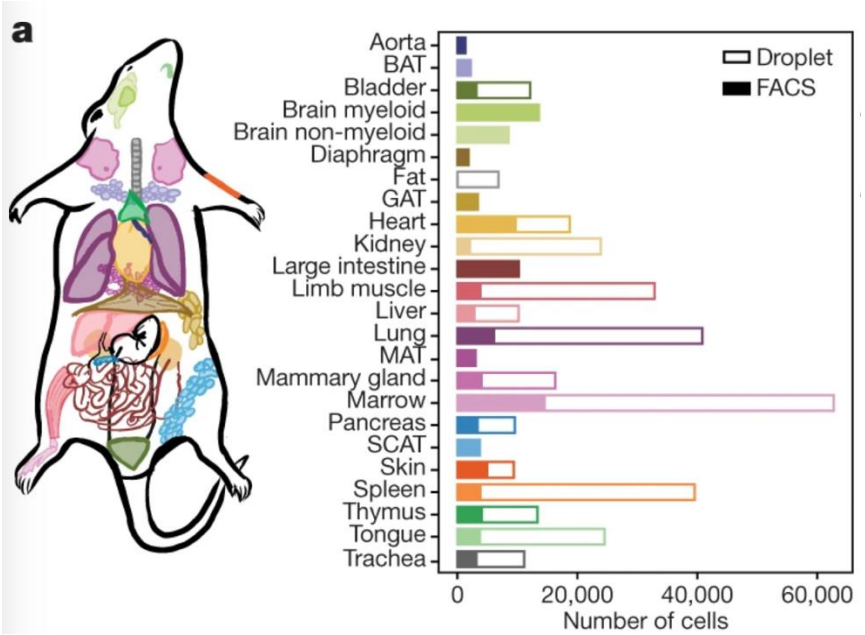


Perform MAS-Iso-seq on the 10x sc libraries to get long isoform reads at single cell resolution

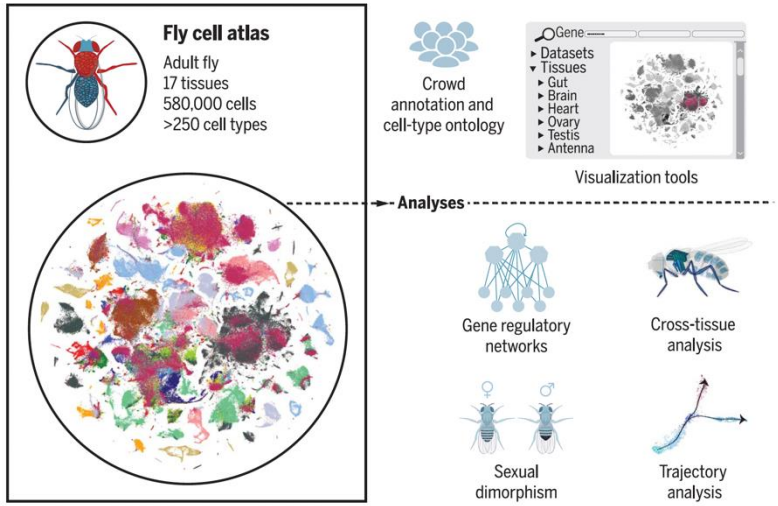
Al'Khafaji et al., Nature Biotechnology, 2023

Cataloguing Cell Types and Building Cell Atlases

Tabula Muris



Tabula Drosophila



Tabula *Drosophilae*. In this single-cell atlas of the adult fruit fly, 580,000 cells were sequenced and >250 cell types were annotated. They are from 15 individually dissected sexed tissues as well as the entire head and body. All data are freely available for visualization and download, with featured analyses shown at the bottom right.

HUMAN CELL ATLAS

Characterize the ~37 trillion cells in the human body

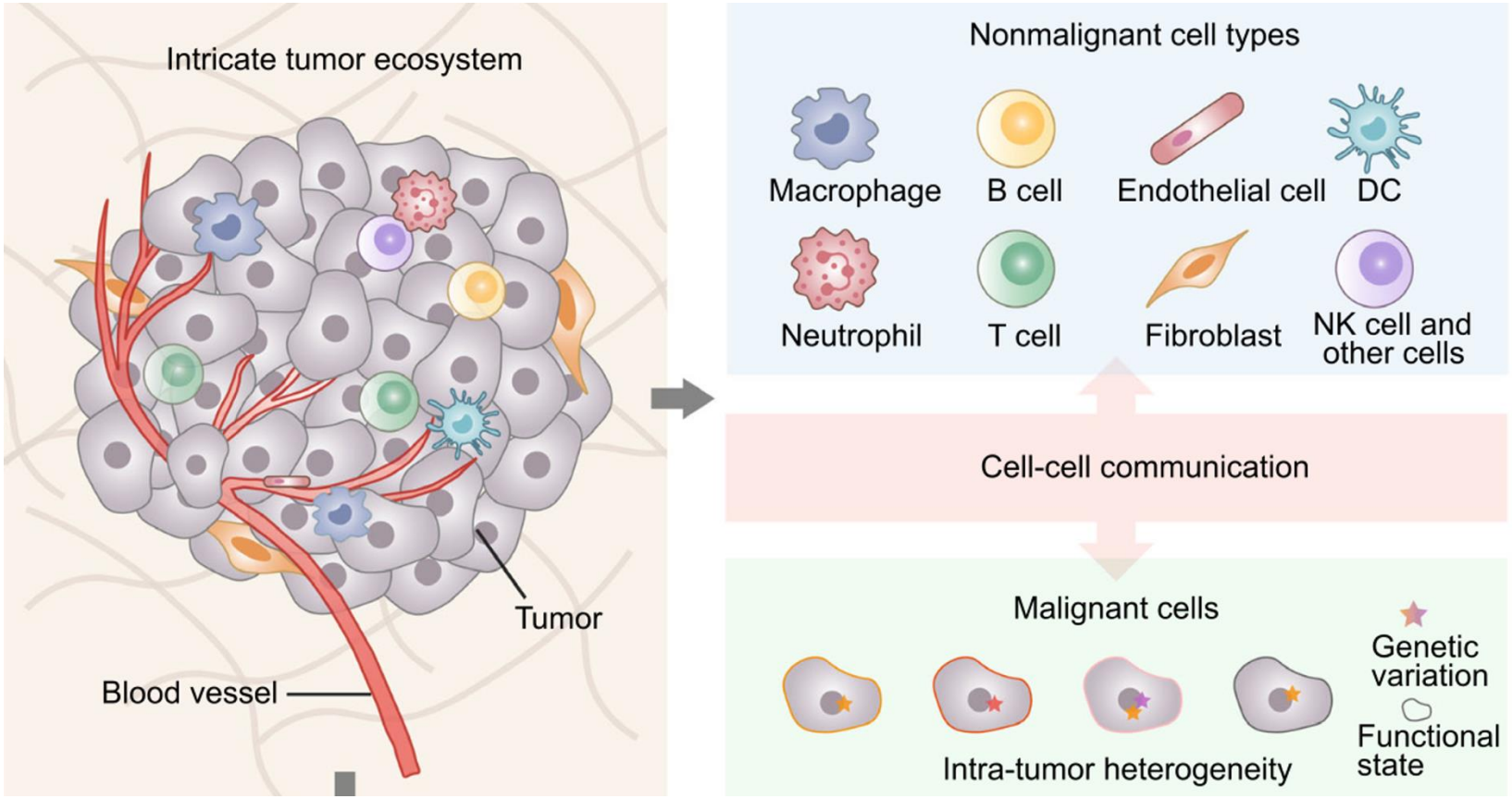
HCA is a global initiative of > 3k members

Initially targeting 18 biological networks of organs and tissues

Currently at >70M across 25k specimens. Cells (Jan, 2026)

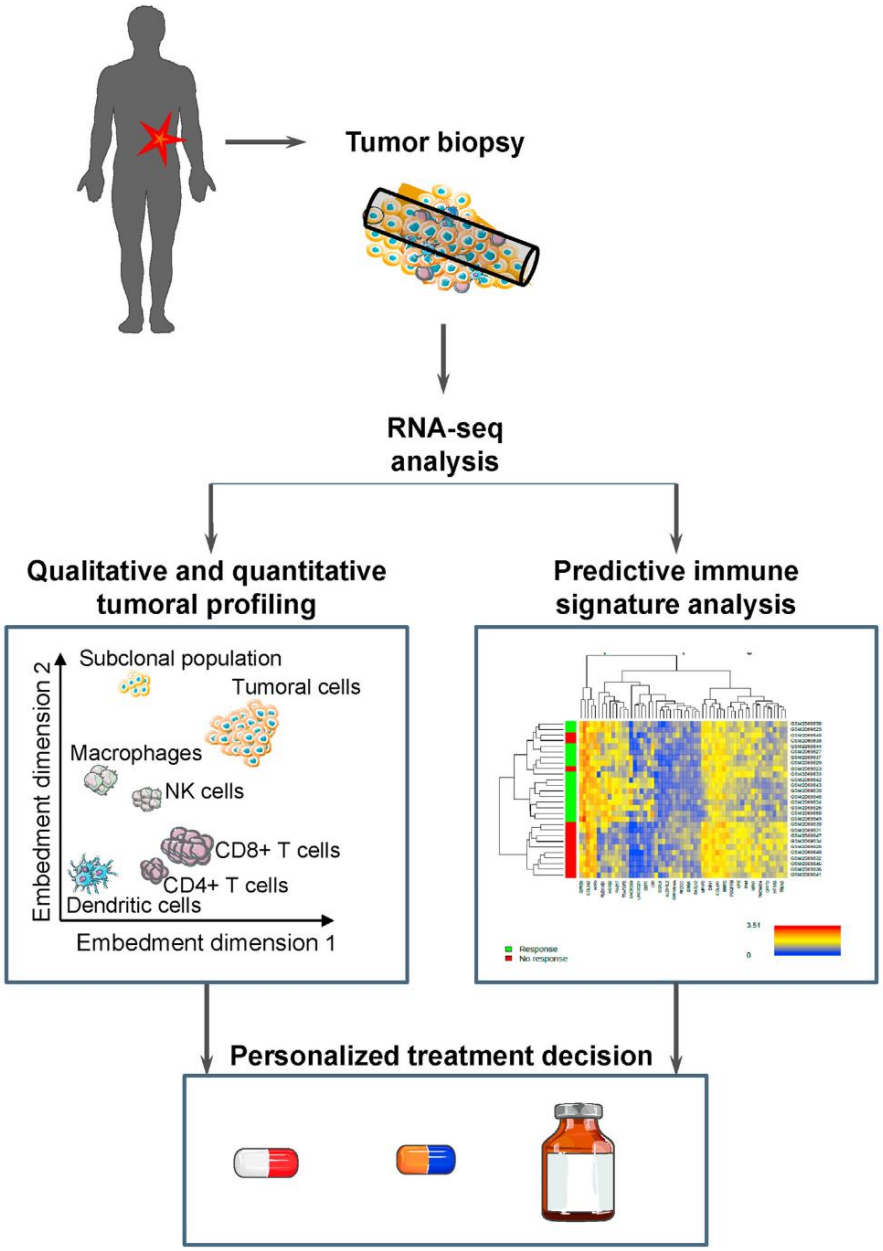
https://www.humancellatlas.org/

Single cell analysis is revolutionizing cancer research



From Li, Jin, & Bai, Protein & Cell, 2022

Clinical Application for Tumor Single Cell Transcriptomics

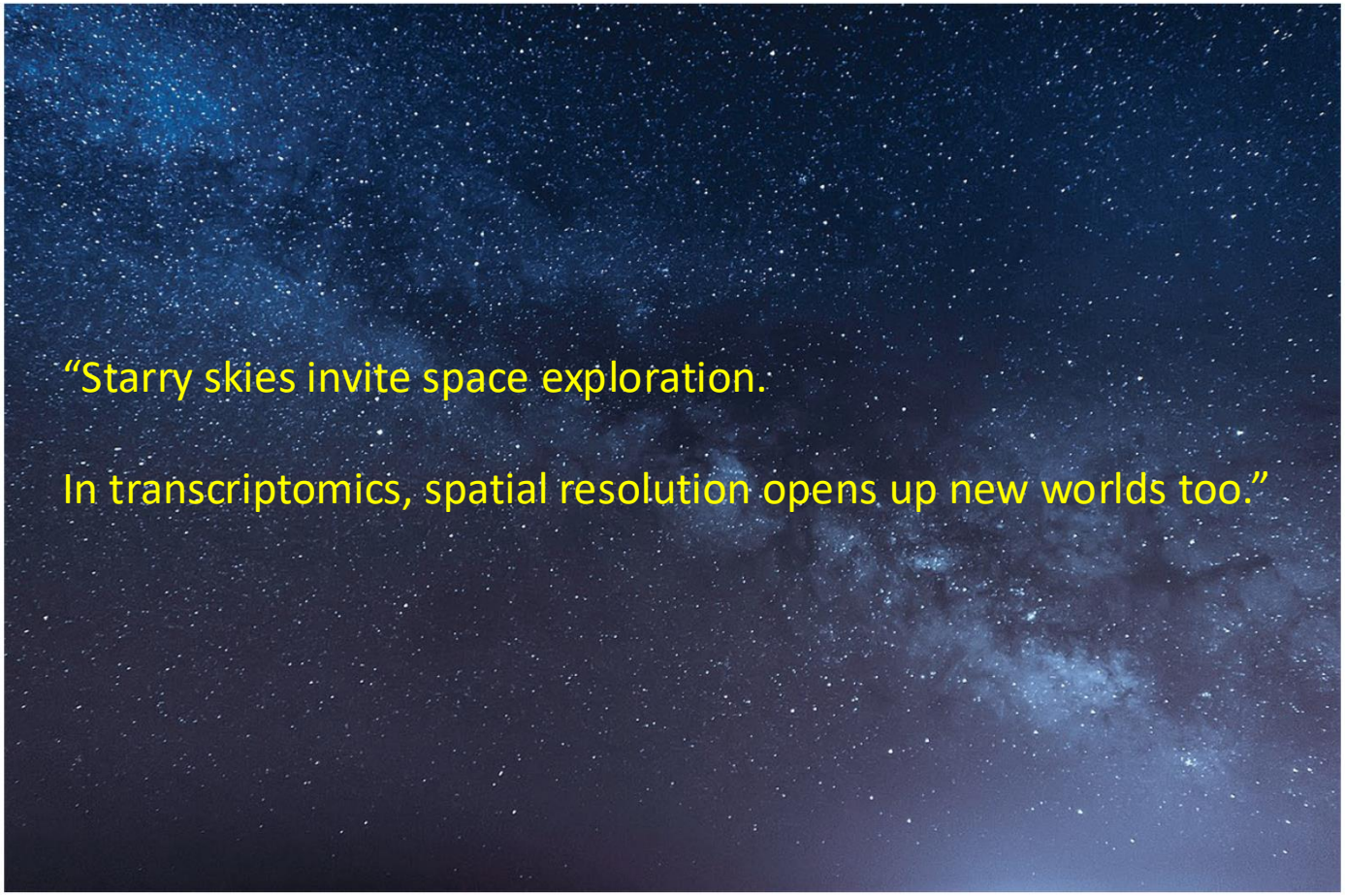


From Kuksin et al, EJC, 2021

Part 8. Overview of Spatial Transcriptomics

Method of the Year: spatially resolved transcriptomics

Nature Methods has crowned spatially resolved transcriptomics Method of the Year 2020.



Starry skies invite space exploration. In transcriptomics, spatial resolution opens up new worlds too.
Credit: bjdlzx/Getty Images

Vivien Marx

Method of the Year: spatially resolved transcriptomics

Nature Methods has crowned spatially resolved transcriptomics Method of the Year 2020.

Starry skies invite space exploration. In transcriptomics, spatial resolution opens up new worlds too.
Credit: bjdlzx/Getty Images

Vivien Marx

Single Cells vs. Spatial Transcriptomics

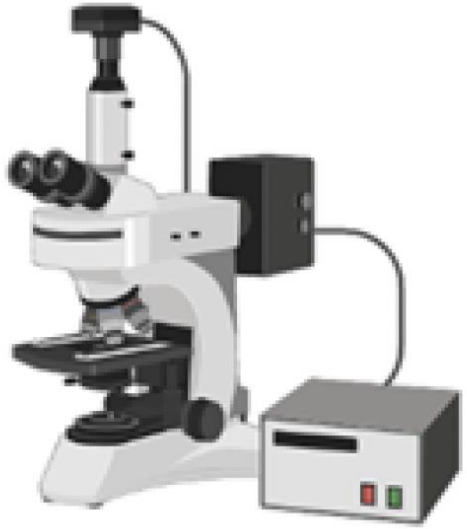
Vs.

Car parts ~ single cells

Car ~ tissue

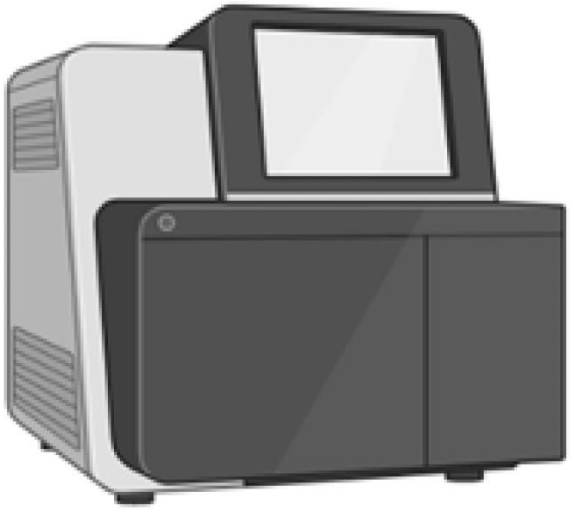
Classes of Spatial Transcriptomics

Imaging Readout



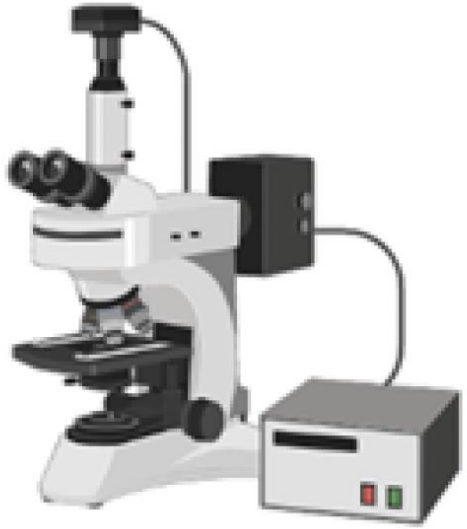
Based on In Situ Hybridization (ISH) and fluorescent tags

Sequencing Readout



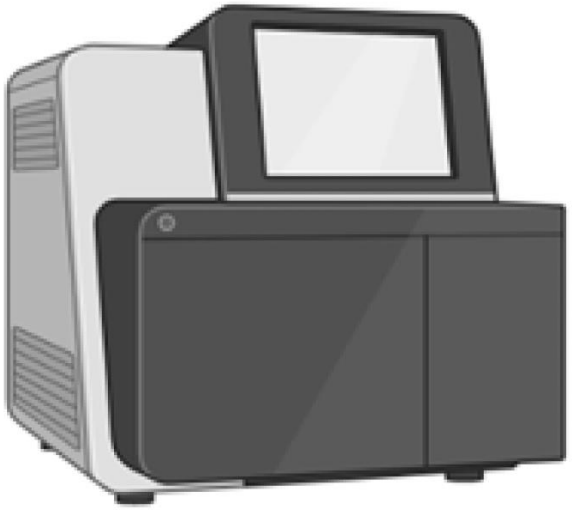
Classes of Spatial Transcriptomics

Imaging Readout



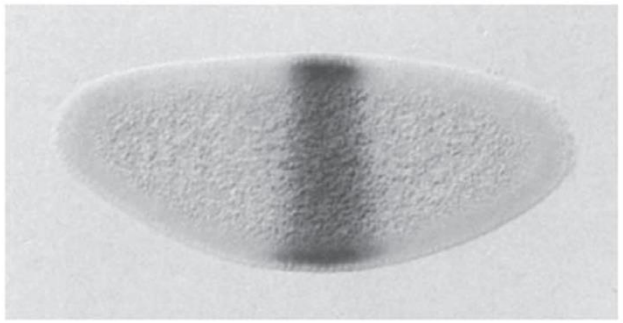
Based on In Situ Hybridization (ISH) and fluorescent tags

Sequencing Readout



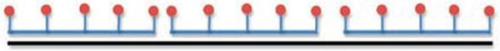
Single Molecule Fish (smFISH) Methods for Visualizing RNA Molecules at Sub-cellular Resolution

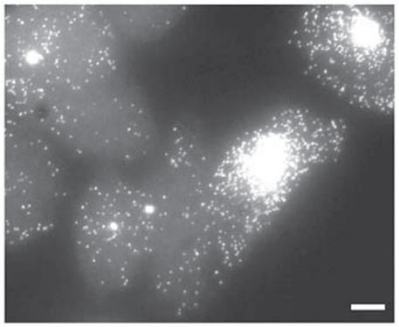
a Long probe, many labels



Target: hunchback RNA in Drosophila embryo

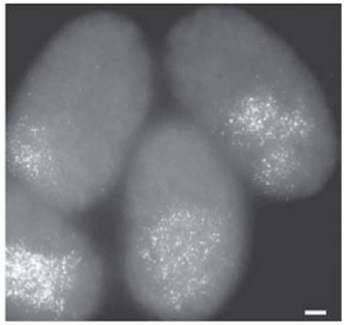
b Shorter probes, fewer labels





Target: single transcripts in mammalian cells

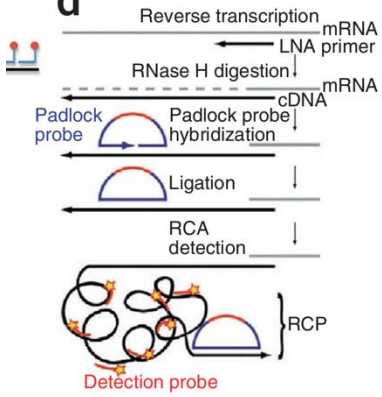
c Many probes, single label ea.

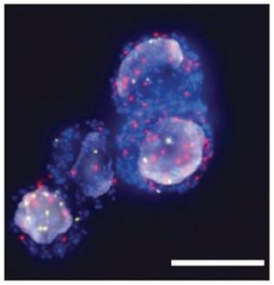


Target: end-1 gene in C.elegans embryos

Rolling circle amplification (RCA) of 'padlock probes'. Labels hyb to RCA product.

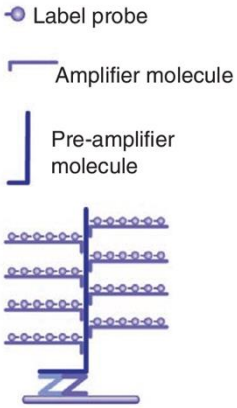
d



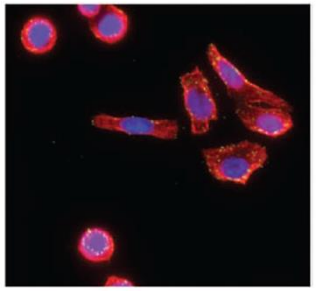


TARGET: ERBB2 (aka. HER2) in human fibroblasts

e



Branched oligo sets that amplify labeling

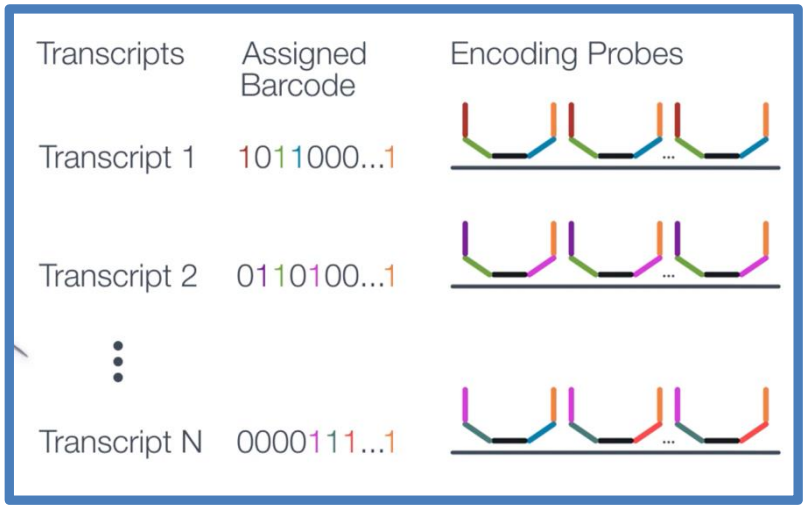


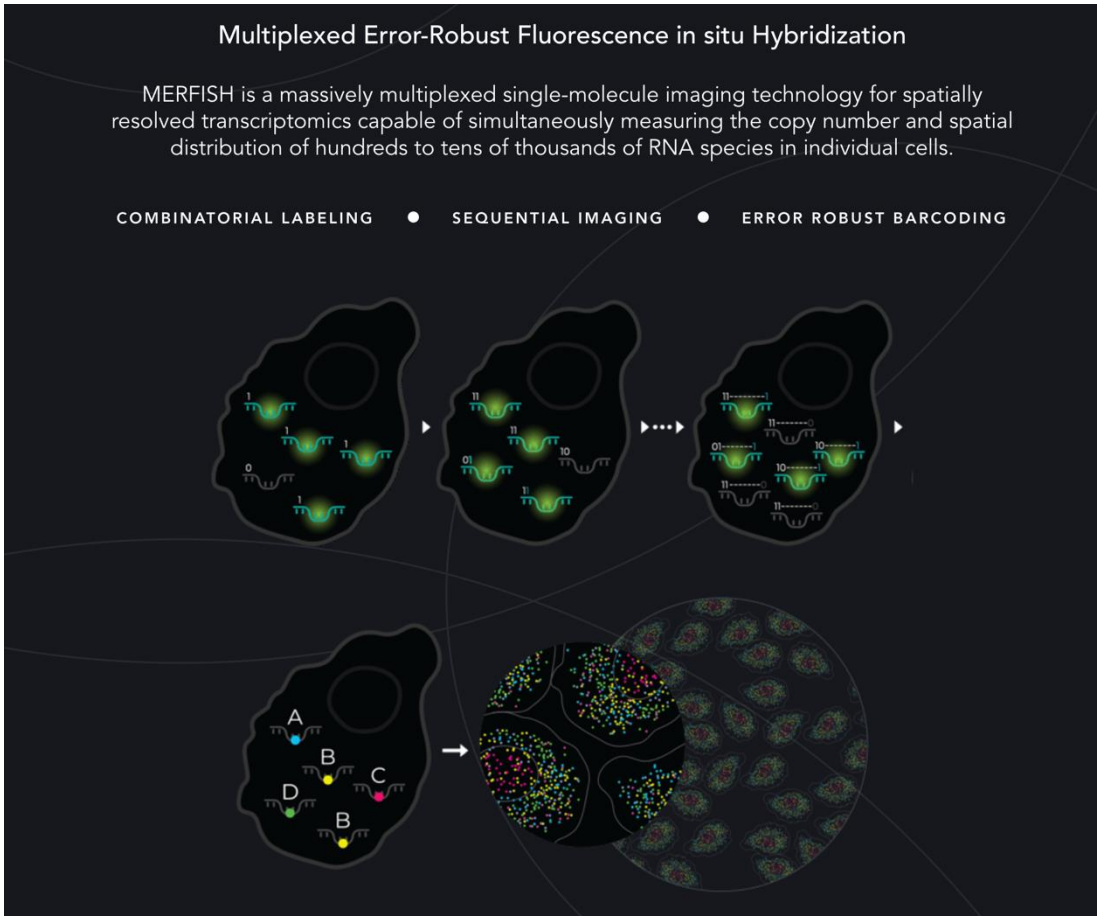
Target: ERBB2 (green) and 18SrRNA (red)

Itzkovitz & van Oudenaarden, Nature Methods Supplement, 2011

MERFISH – smFISH adapted for hundreds to thousands of transcripts

Each transcript target probe has a unique combination of beacon landing pads



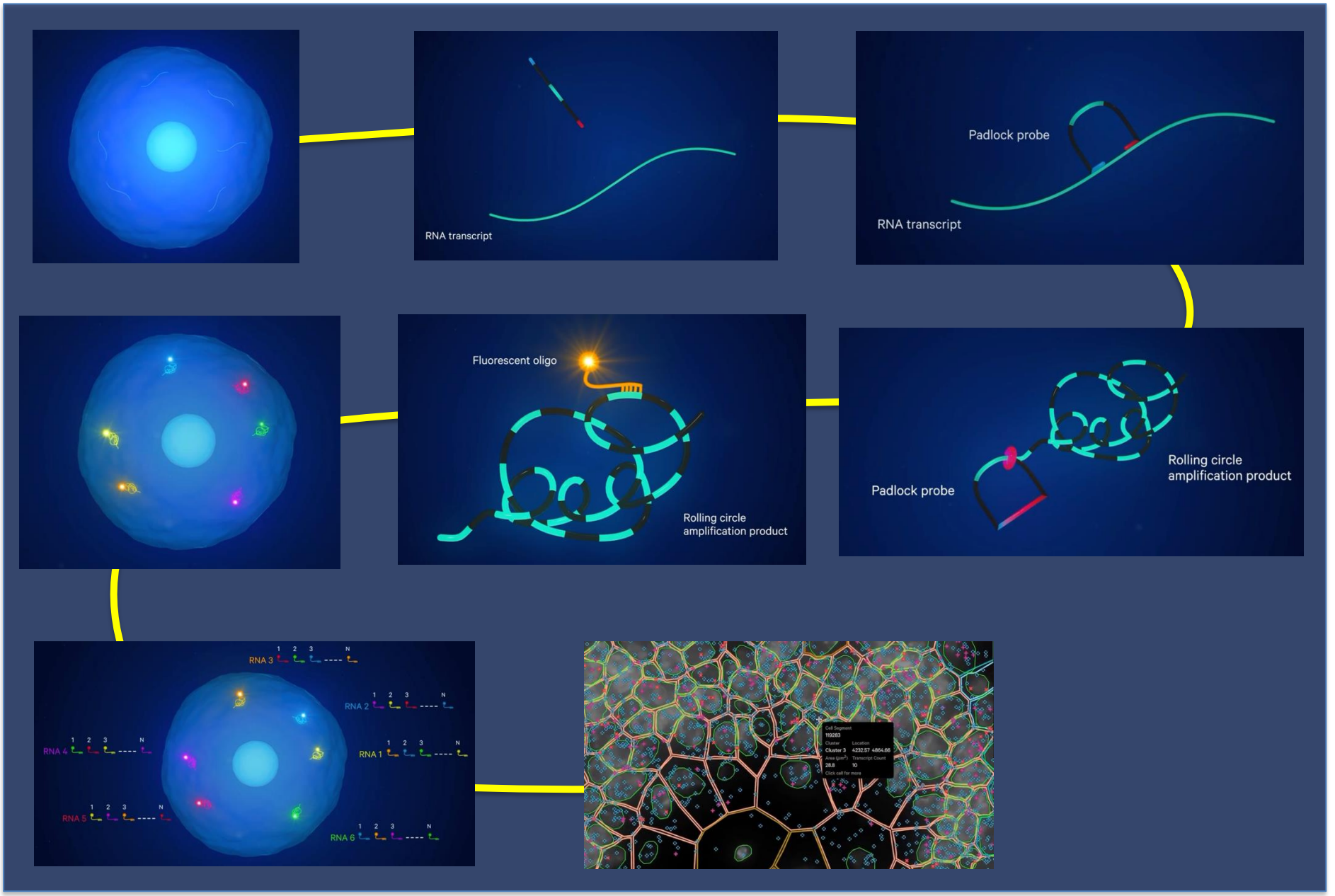


- Different fluorescently labeled probes (ie. beacons) are hybridized in each round.
- Combinations of colors -> Transcript ID

https://vizgen.com/technology

Movie: https://www.youtube.com/watch?v=O0QekKSscjA

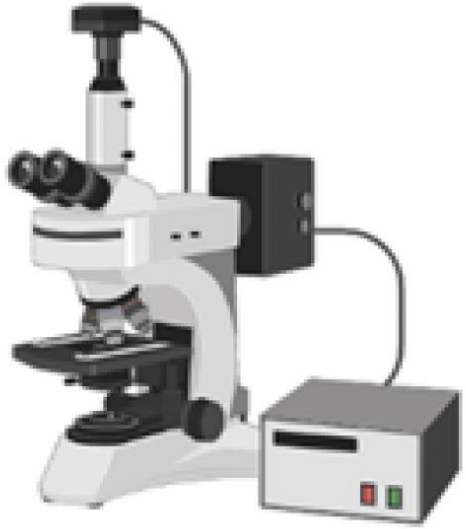
10X Genomics Xenium – 100s to 1000s of Targeted RNAs visualized at subcellular resolution



https://www.10xgenomics.com/videos/s3lqk4sivj?autoplay=true

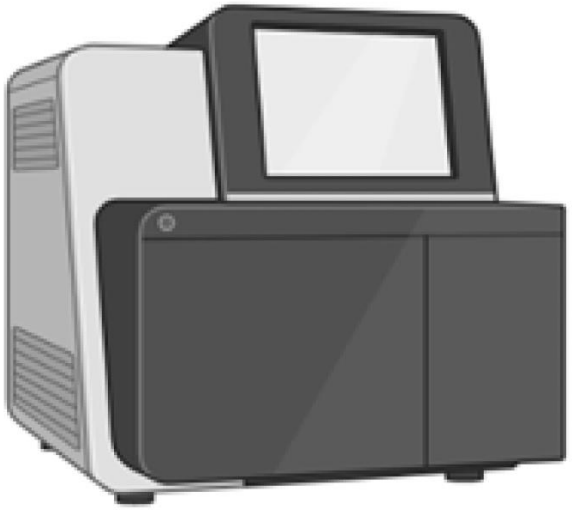
Classes of Spatial Transcriptomics

Imaging Readout

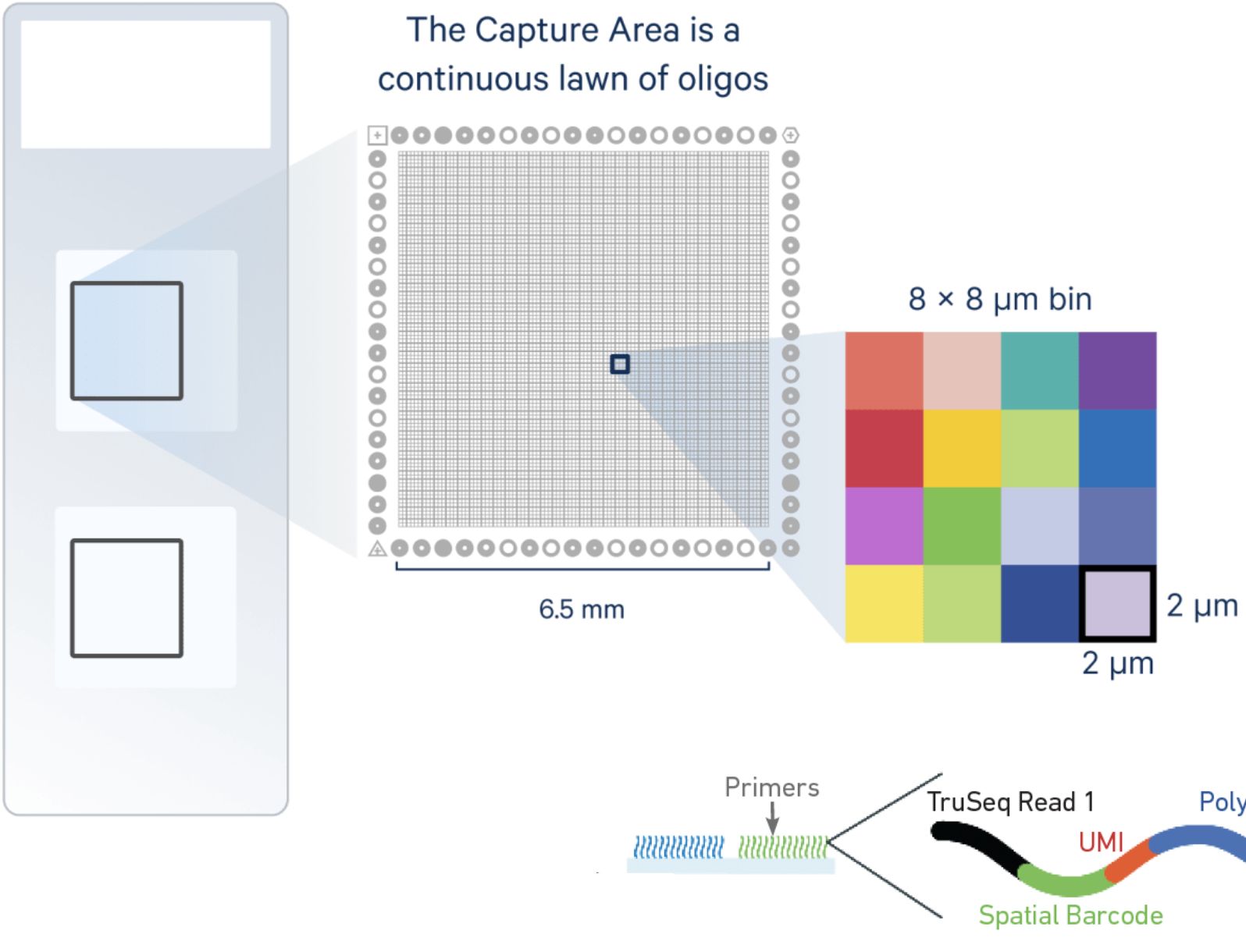


Based on In Situ Hybridization (ISH) and fluorescent tags

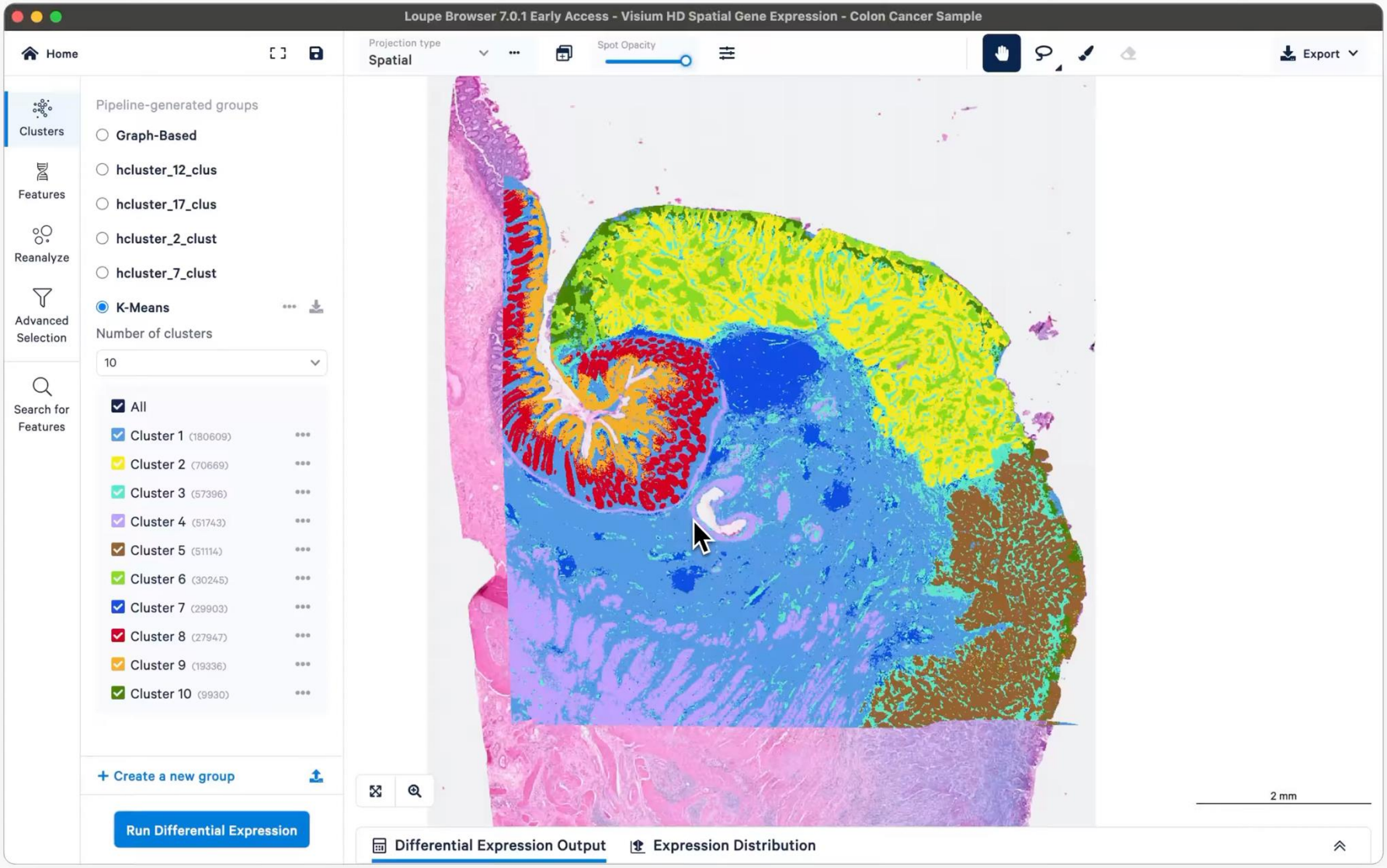
Sequencing Readout



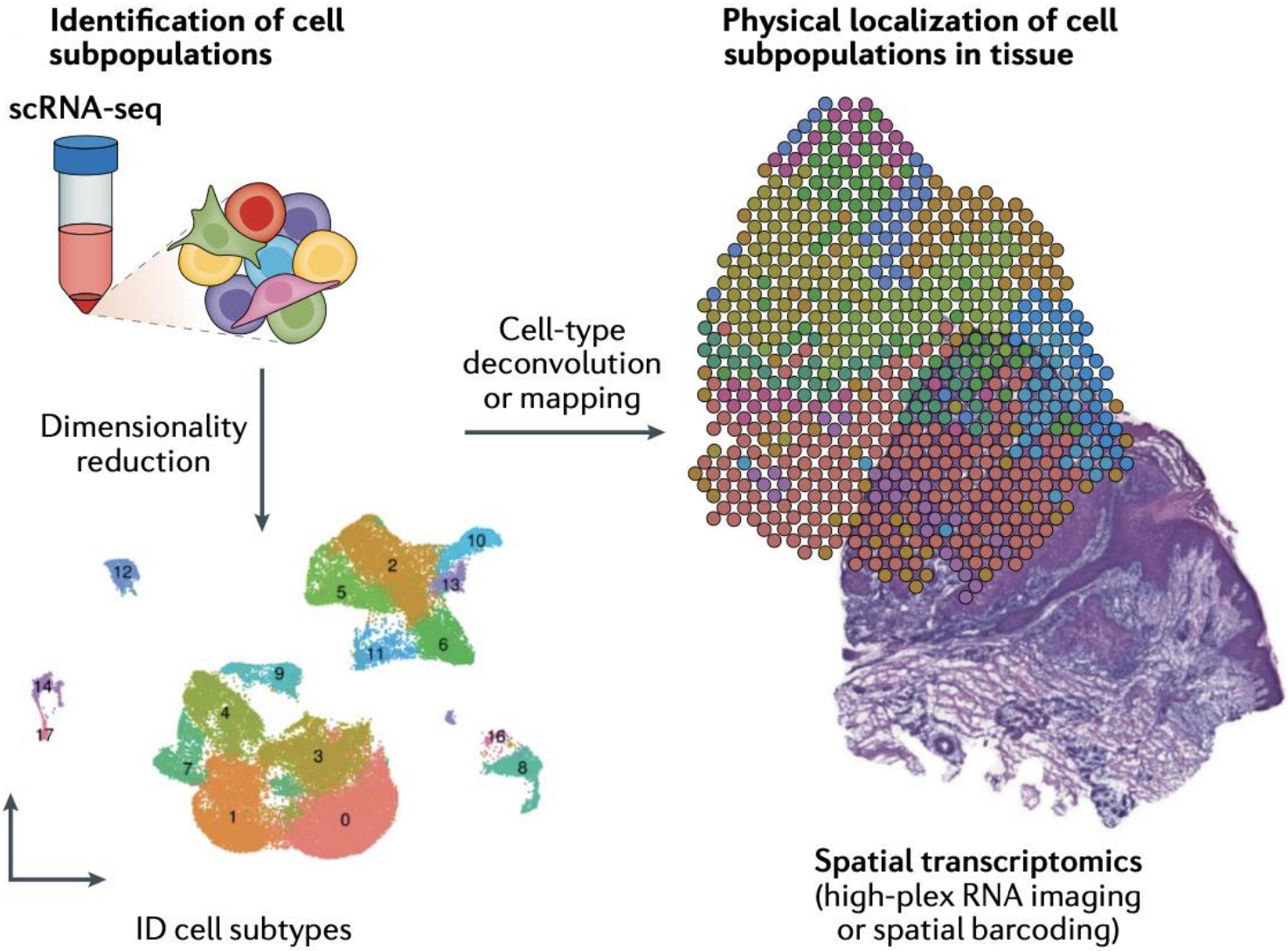
Spatial RNA-seq – 10X Visium HD



Spatial RNA-seq – 10X Visium HD

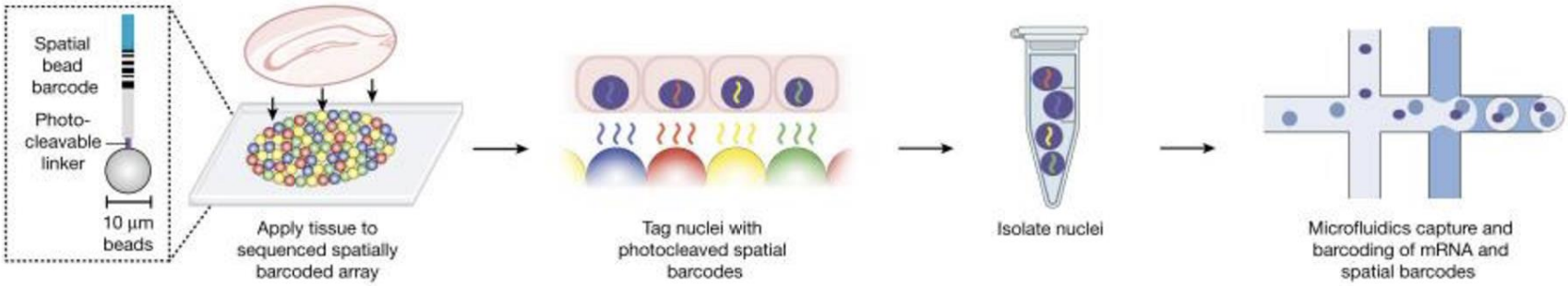


Integration of Single Cell and Spatial Transcriptomes

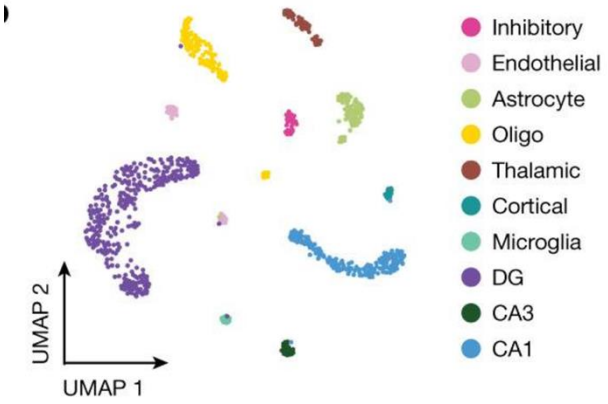


Longo, NRG, 2021

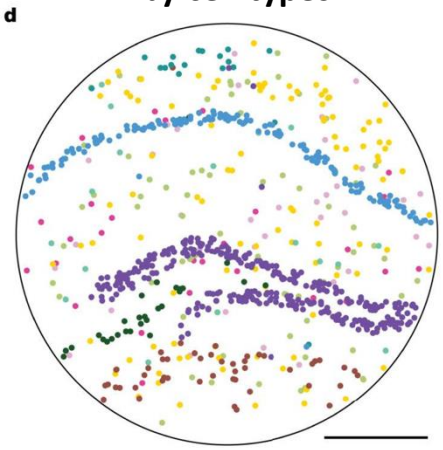
Slide-Tags: integrated single nuclei and spatial transcriptomics



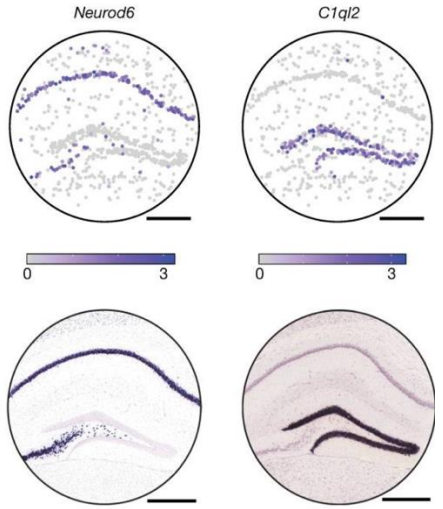
UMAP and cell types, expression-based clustering



Cells plotted according to spatial coordinates, colored by cell types



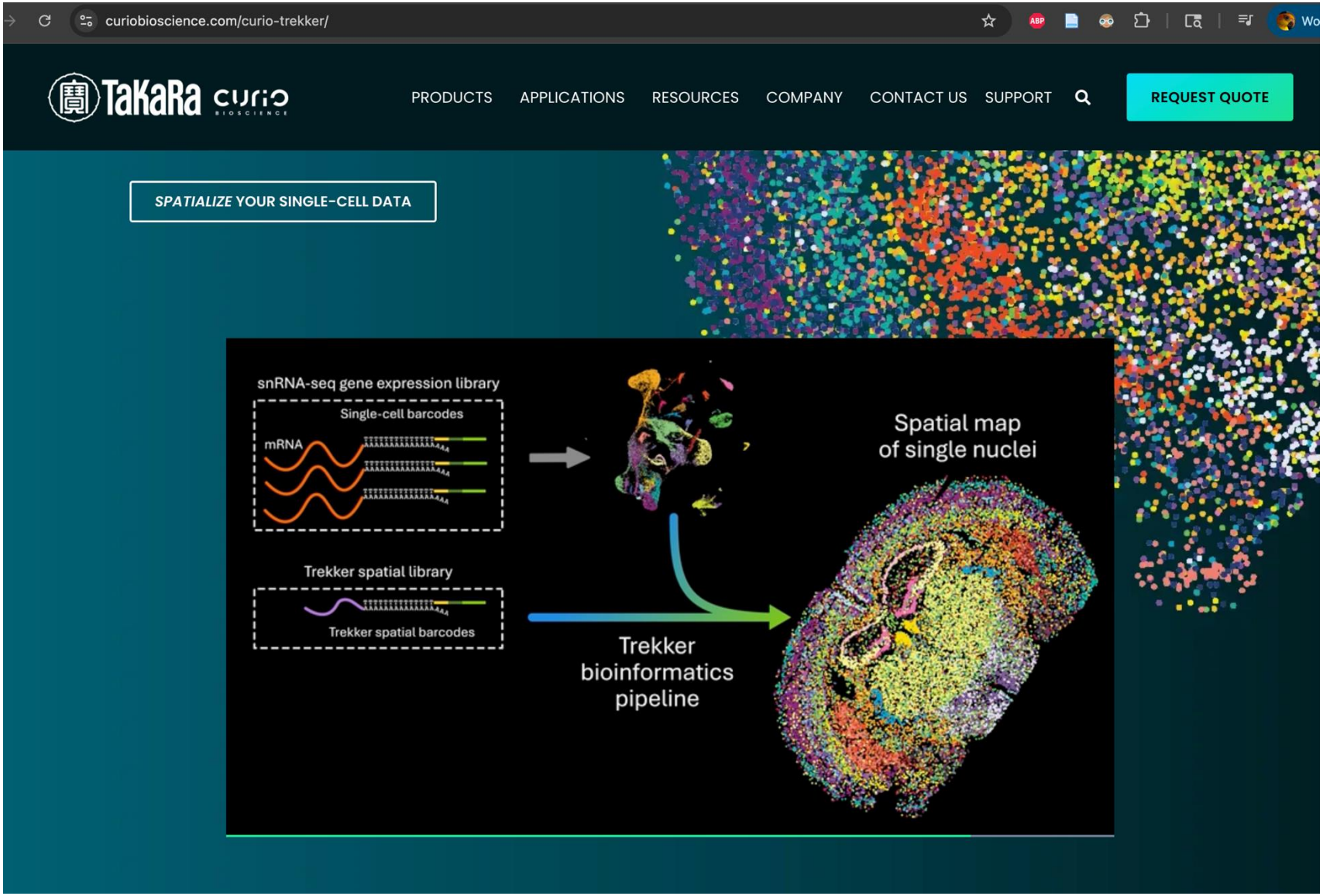
Spatial Expression of Marker Genes



In situ hybridization (Allen Mouse Brain Atlas)

Russell et al., Nature 2023, PMID: 38093010

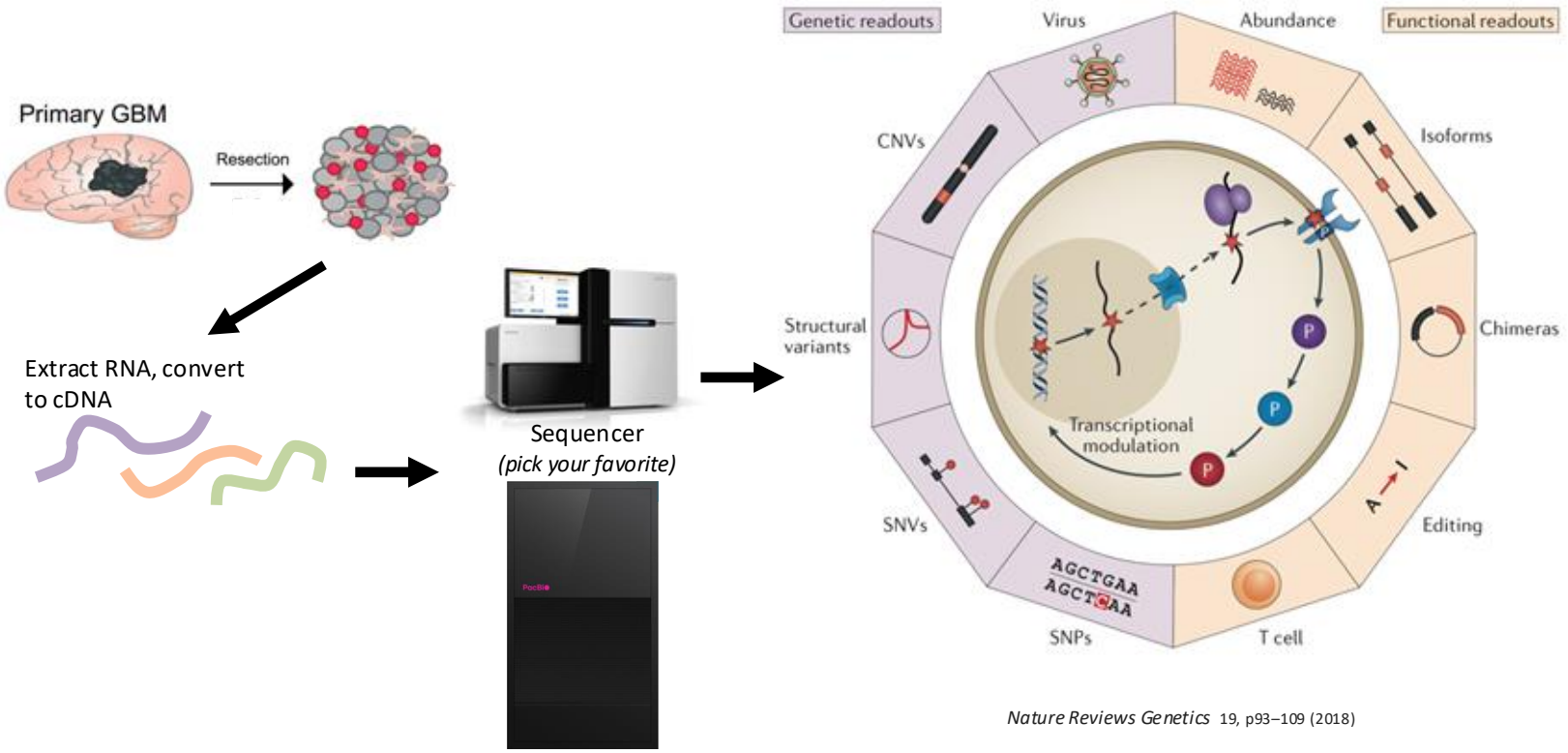
Slide-Tags commercialized as ‘Trekker’ by Curio Bioscience



Video: https://curiobioscience.com/curio-trekker/

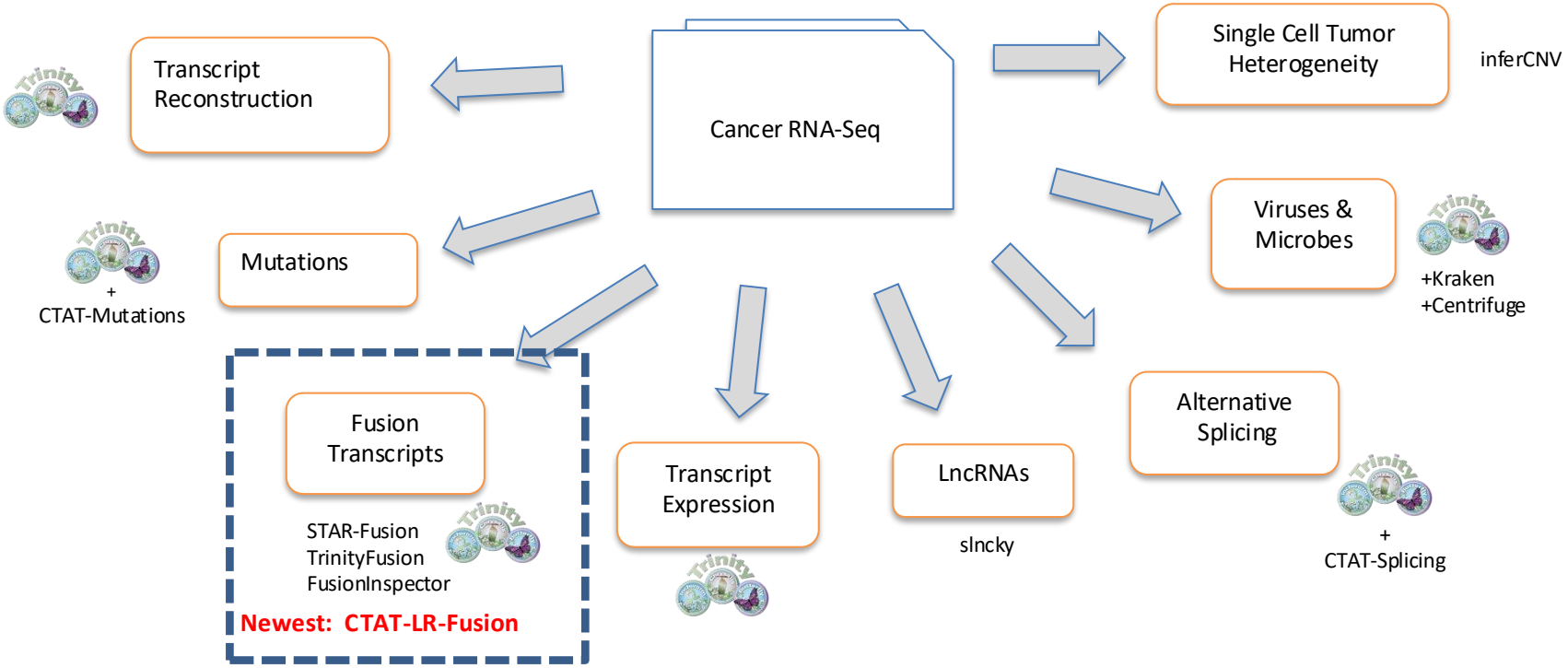
Part 9. Applications in Cancer Transcriptomics

RNA-Seq Empowers Transcriptome Studies of Cancer

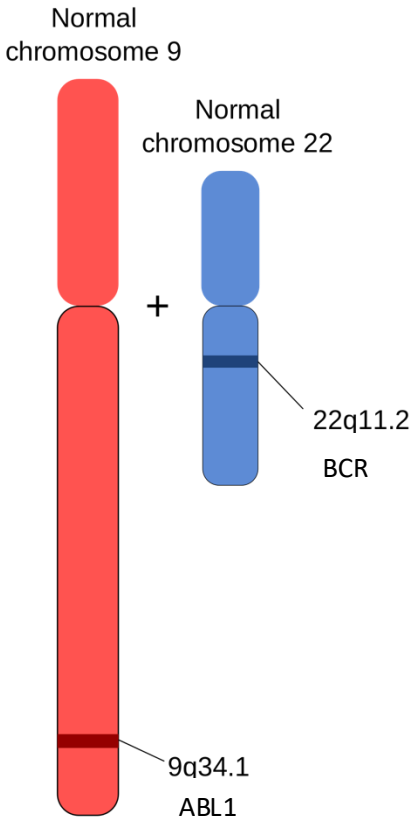


Nature Reviews Genetics 19, p93–109 (2018)

Cancer Transcriptome Analysis Toolkit (CTAT)



Chromosomal Translocations Can Lead to Oncogenic Fusion Transcripts



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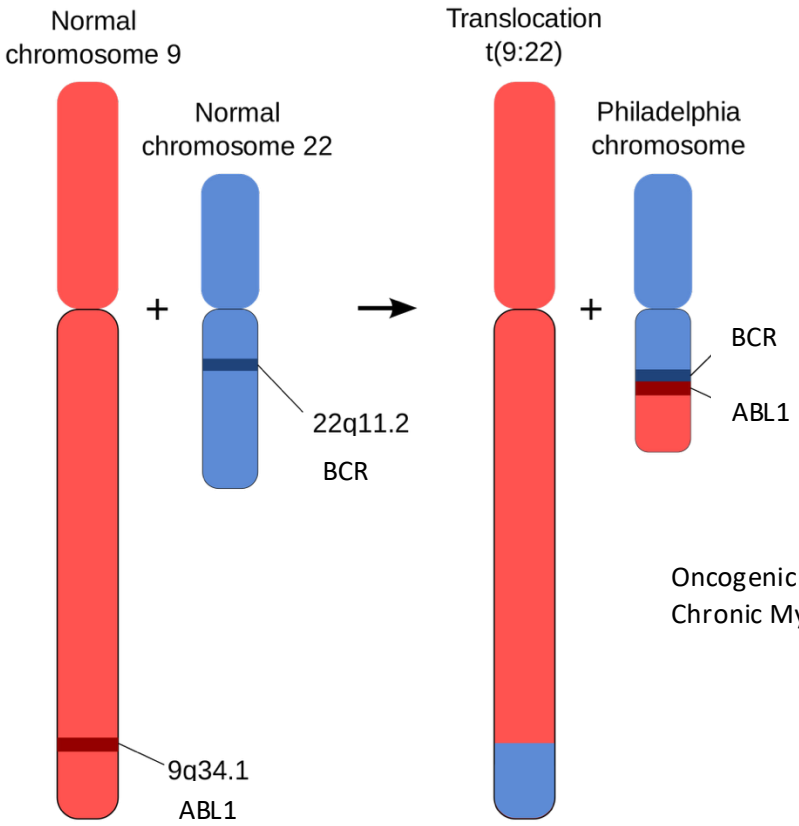
Chromosomal Translocations Can Lead to Oncogenic Fusion Transcripts



Can detect BCR::ABL1 via its fusion transcript:

Oncogenic driver BCR::ABL1 fusion found in 95% of Chronic Myelogenous Leukemia (CML) patients

BROAD CLINICAL LABS

Diagnostics and Therapeutics Involving Oncogenic Fusion Transcripts in Cancer

BCR-ABL1 (Philadelphia chromosome)
- Chronic Myelogenous Leukemia (CML) cases (95% of cases)
- Treatable with tyrosine kinase inhibitors

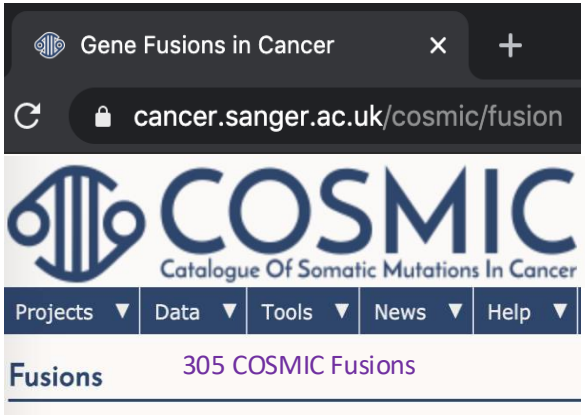
SS18—SSX
- Synovial sarcoma (~100% of cases)

TMPRSS2-ERG
- Prostate cancers (50% of cases)

EML4-ALK
- Non small cell lung carcinoma (4% of cases)
- anaplastic lymphoma kinase (ALK) inhibitors improve patient outcome

DNAJB1-PRKACA
- Fibrolamellar hepatocellular carcinoma (FL-HCC), 100% of cases, but a rare cancer.

FGFR3-TACC3
- ~8% of glioblastoma patients



BROAD CLINICAL LABS

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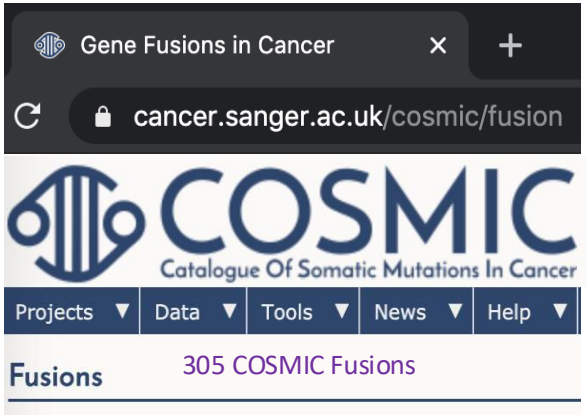

BROAD CLINICAL LABS

General Approaches to Fusion Transcript Discovery

Paired-end Illumina RNA-Seq

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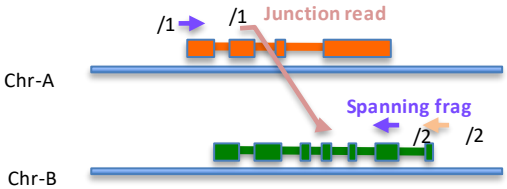
General Approaches to Fusion Transcript Discovery

Paired-end Illumina RNA-Seq

Align *reads* to the genome,
Identify discordant pairs and junction/split reads.



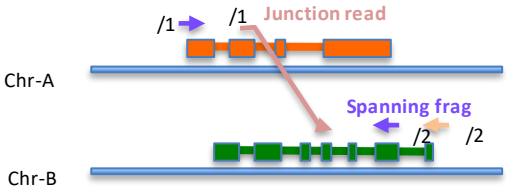
BROAD CLINICAL LABS

General Approaches to Fusion Transcript Discovery

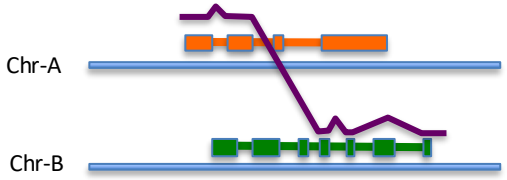
Paired-end Illumina RNA-Seq

De novo RNA-seq assembly

Align *reads* to the genome,
Identify discordant pairs and junction/split reads.



Align *transcripts* to genome,
Identify Fusion Transcripts

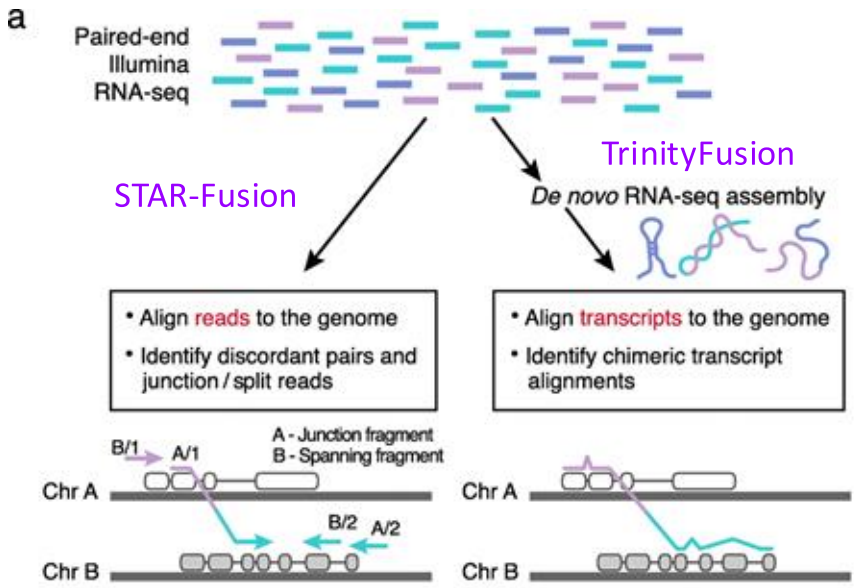


BROAD CLINICAL LABS

Our Prior Work on Fusion Detection, Benchmarking, and Analysis via Illumina RNA-seq

Accuracy assessment of fusion transcript detection via read-mapping and de novo fusion transcript assembly-based methods

Genome Biology volume 20, Article number: 213 (2019)



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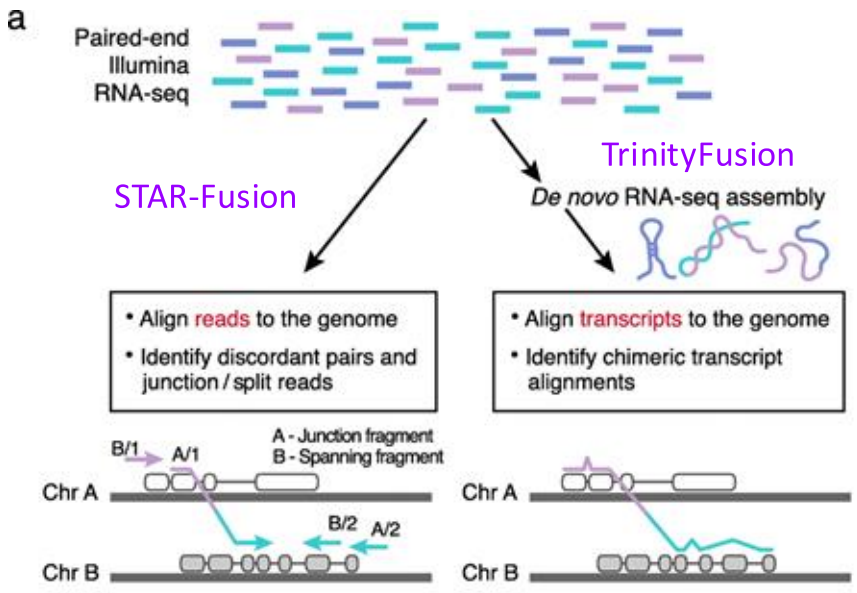
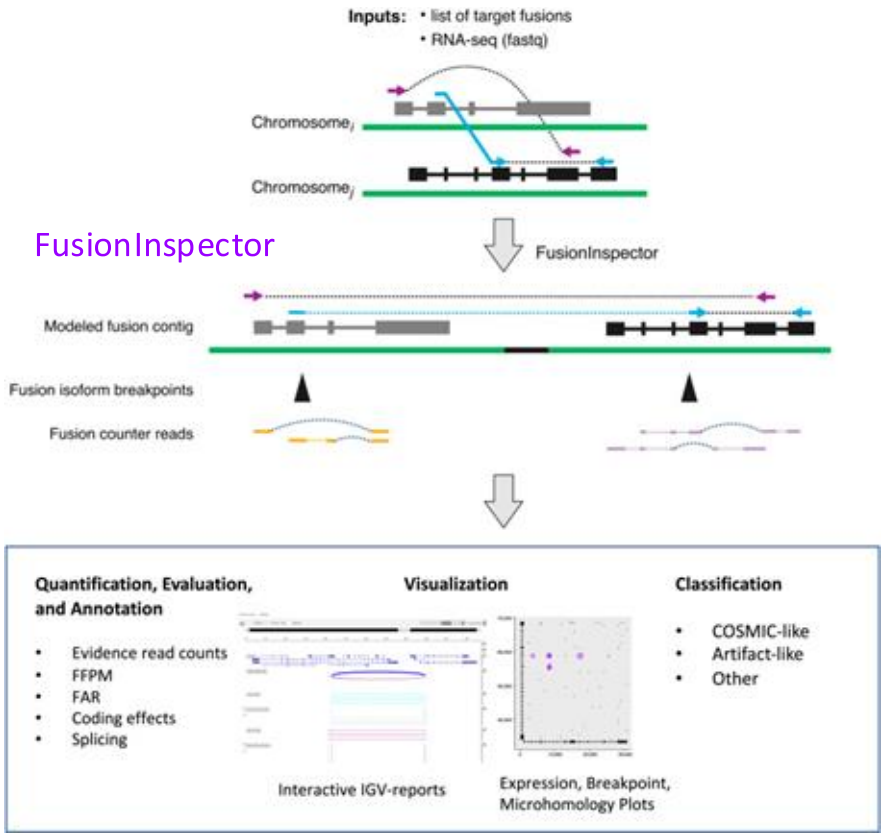


Targeted in silico characterization of fusion transcripts in tumor and normal tissues via FusionInspector

Cell Rep Methods. 2023 May 8;3(5):100467.

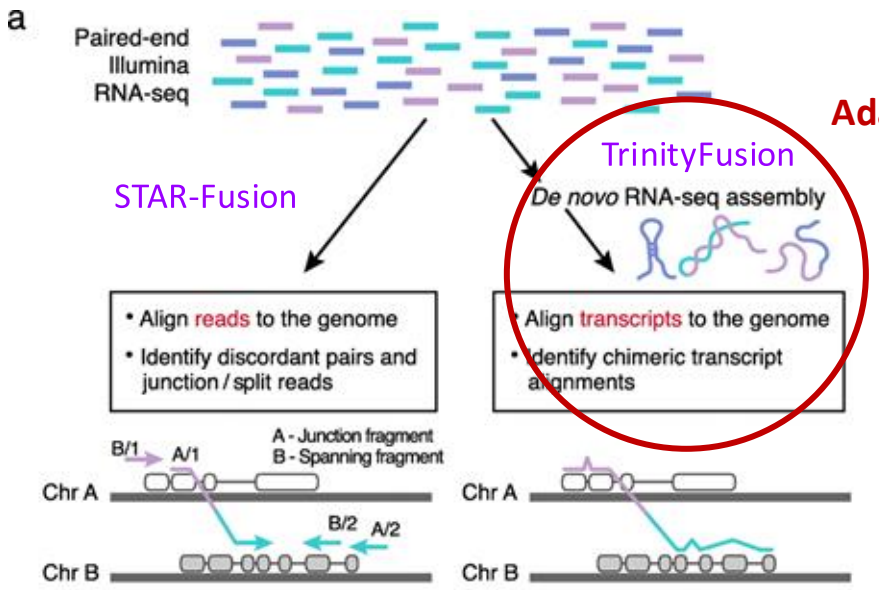


BROAD CLINICAL LABS

Adapting TrinityFusion and FusionInspector to Long Read Fusion Detection

Accuracy assessment of fusion transcript detection via read-mapping and de novo fusion transcript assembly-based methods

Genome Biology volume 20, Article number: 213 (2019)



Adapt to Long Reads

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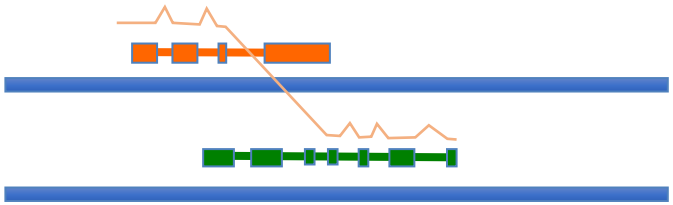
Cell Rep Methods. 2023 May 8;3(5):100467.

BROAD CLINICAL LABS

New Addition to our **C**ancer **T**ranscriptome **A**nalysis **T**oolkit: **CTAT-LR-fusion**

(borrows general approach from TrinityFusion and FusionInspector, adapted for LR)

(1) **Quickly Identify Fusion Candidate (ctat-minimap2)** *4x faster*



TrinityFusion-like

BROAD CLINICAL LABS

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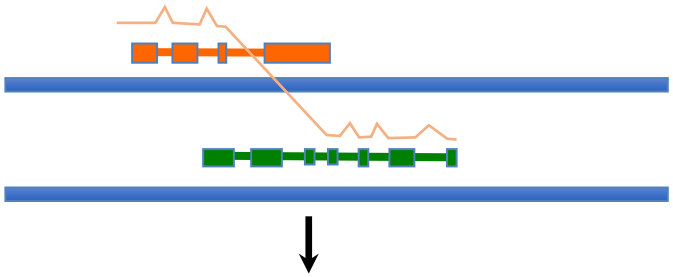
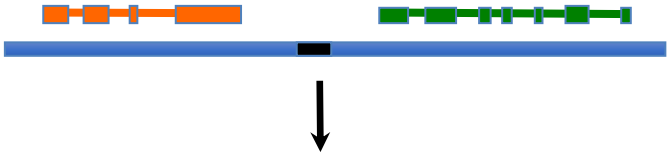


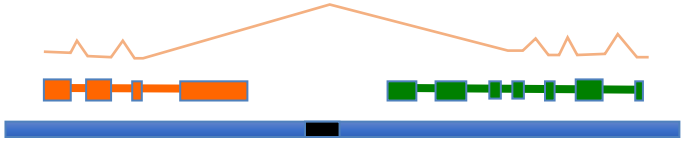
TrinityFusion-like

Make mini-fusion contigs

(2)



Rigorous minimap2-alignment, capture precise breakpoints



FusionInspector-like

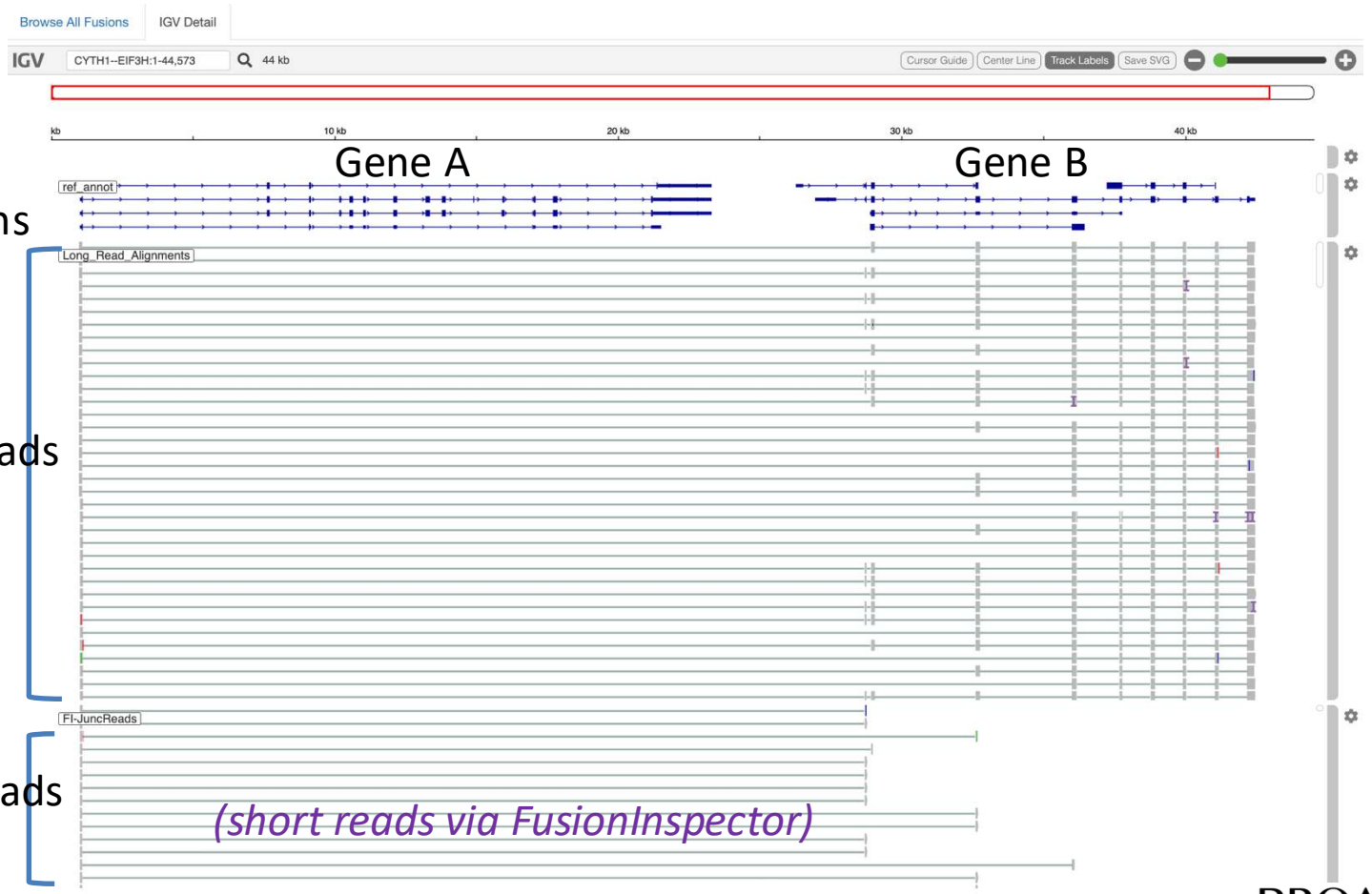
BROAD CLINICAL LABS

CTAT-LR-Fusion Interactive Reports for Visualization and Analysis

Ref. Annotations

Long Fusion reads (PacBio)

Short Fusion reads (Illumina)



BROAD CLINICAL LABS

Single Cell MAS-Iso-seq Applied to T-cell Enriched Melanoma Patient Sample

Long and Short scRNA-seq

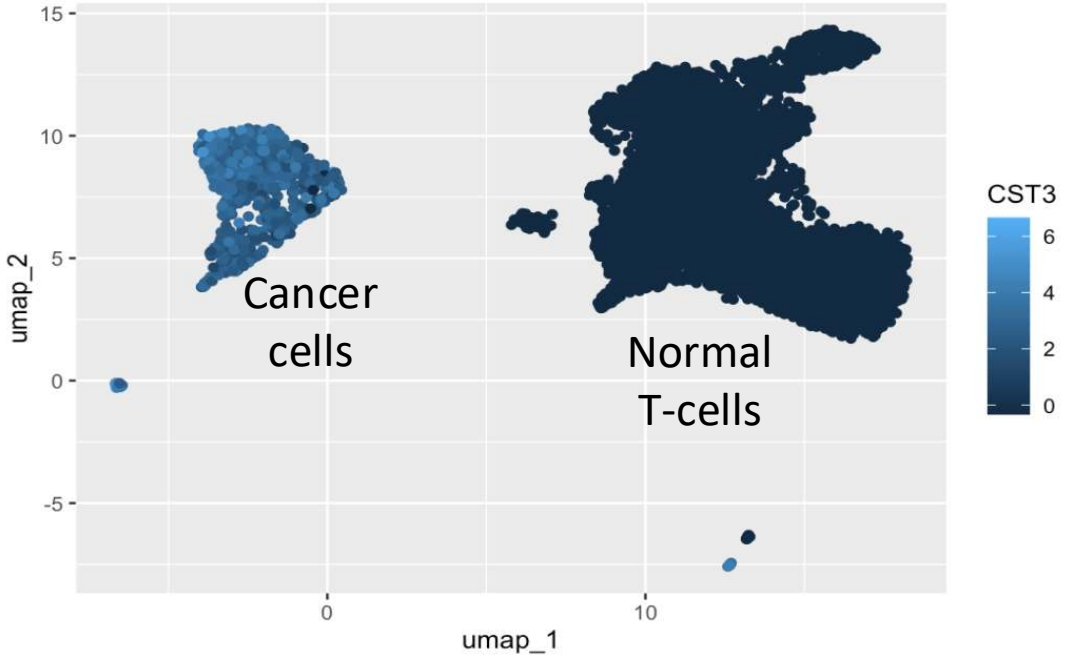
~ 20M PacBio MAS-Iso-seq reads
~ 200M Illumina 10x 3’ reads

~ 7k Total cells (10% cancer cells)

Brief Communication | Published: 08 June 2023

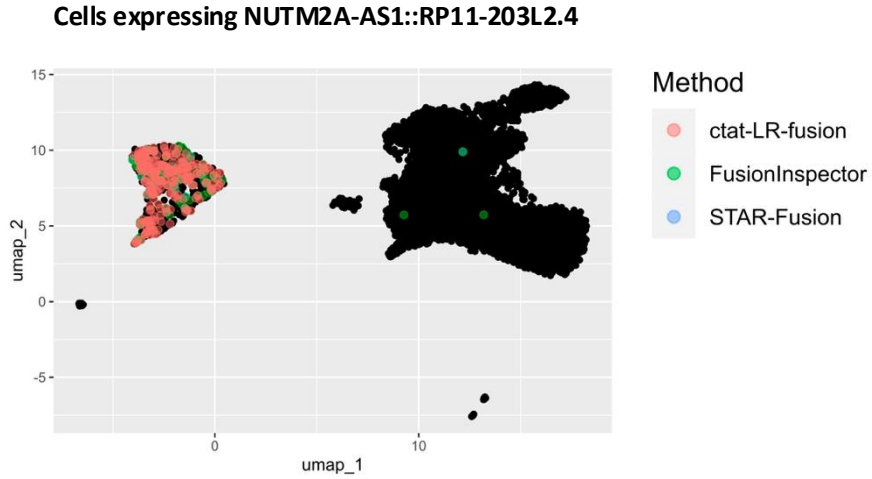
High-throughput RNA isoform sequencing using programmed cDNA concatenation

Aziz M. Al’Khafaji, Jonathan T. Smith, Kiran V. Garimella, Mehrtash Babadi, Victoria Popic, Moshe Sade-Feldman, Michael Gatzen, Siranush Sarkizova, Marc A. Schwartz, Emily M. Blaum, Allyson Day, Maura Costello, Tera Bowers, Stacey Gabriel, Eric Banks, Anthony A. Philippakis, Genevieve M. Boland, Paul C. Blainey & Nir Hacohen



BROAD CLINICAL LABS

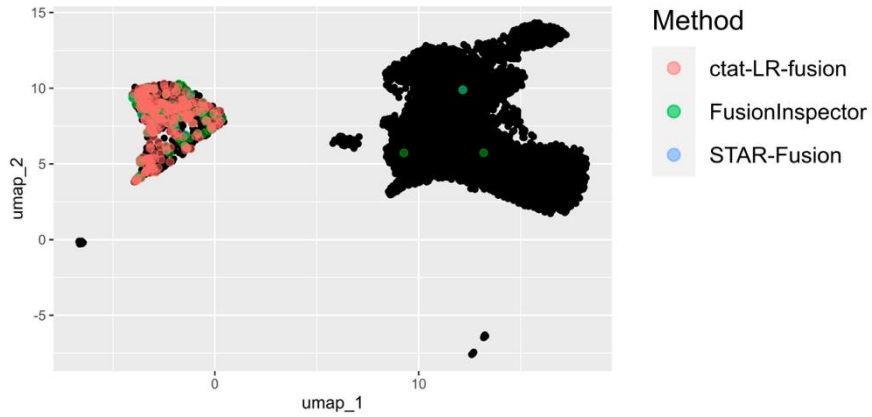
Single Tumor-specific Fusion Transcript Detected: NUTM2A-AS1 (Oncogene) :: RP11-203L2.4



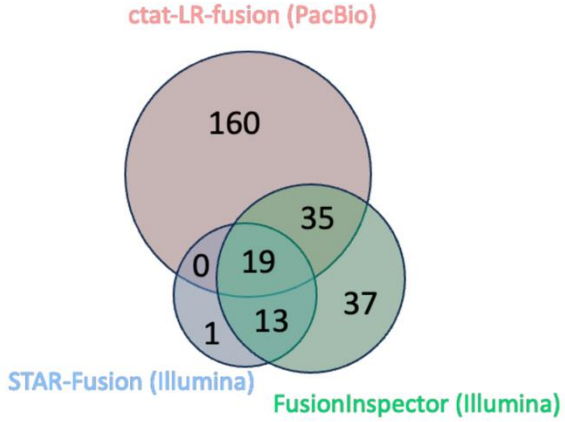
BROAD CLINICAL LABS

Single Tumor-specific Fusion Transcript Detected: NUTM2A-AS1 (Oncogene) :: RP11-203L2.4

Cells expressing NUTM2A-AS1::RP11-203L2.4



Cells Identified with NUTM2A-AS1::RP11-203L2.4



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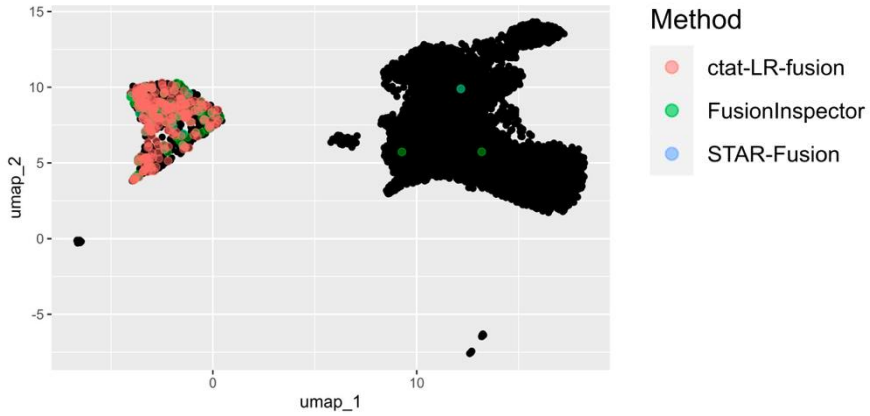

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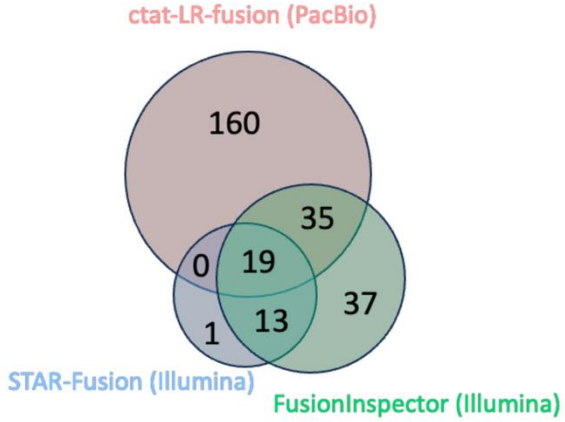
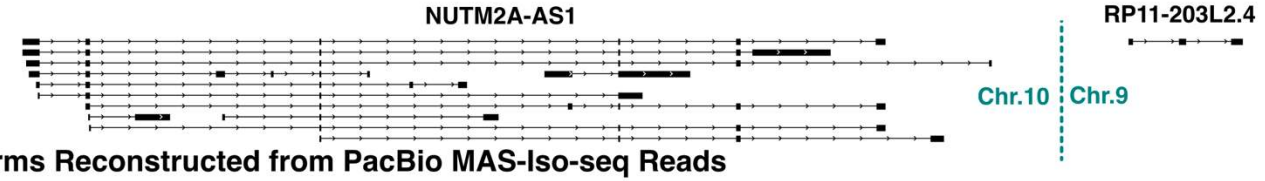
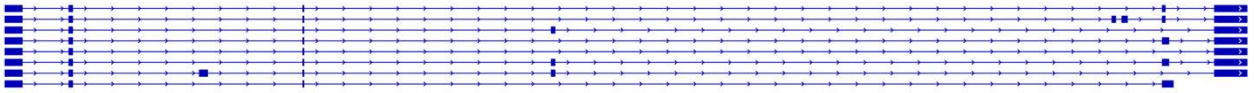


Reference Isoform Structures



Isoforms Reconstructed from PacBio MAS-Iso-seq Reads



8 full-length fusion isoforms

Fusion Isoform Junctions Detected from Short Illumina Reads

5 sets of isoform breakpoints

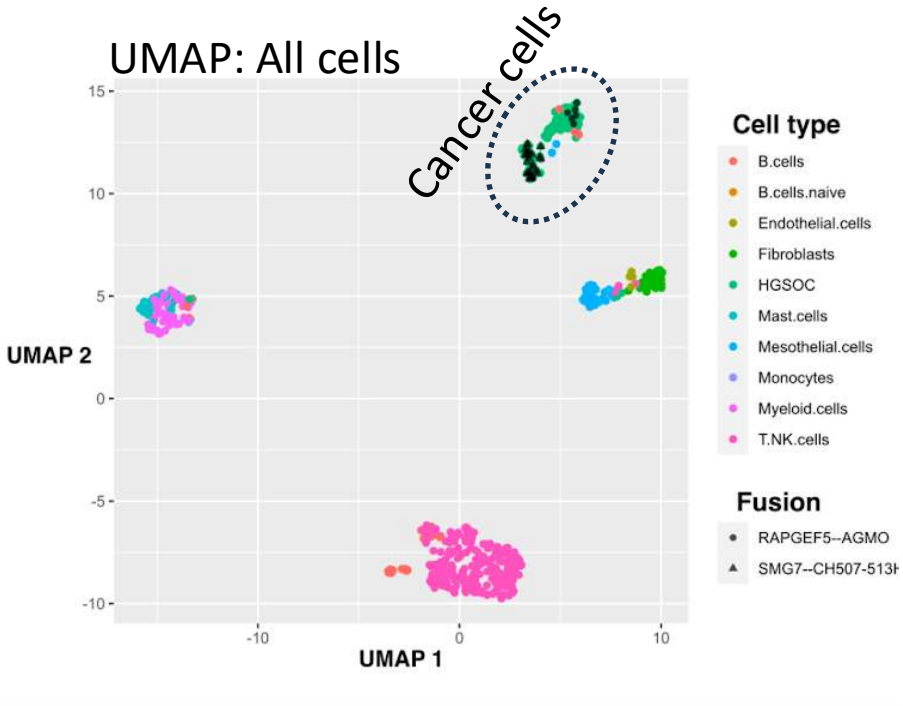
BROAD CLINICAL LABS

Detection of Fusion Transcripts in High Grade Serous Ovarian Cancer via Long Read Isoform Sequencing

Detection of isoforms and genomic alterations by high-throughput full-length single-cell RNA sequencing in ovarian cancer

Arthur Dondi, Ulrike Lischetti, Francis Jacob, Franziska Singer, Nico Borgsmüller, Ricardo Coelho, Tumor Profiler Consortium, Viola Heinzelmann-Schwarz, Christian Beisel & Niko Beerenwinkel

Nature Communications **14**, Article number: 7780 (2023) | Cite this article



Patient 1:

- 54M PacBio Isoform reads
- 35M Illumina 10x 3’ reads
- ~500 cells (20% cancer)
- 4 cancer-specific fusions detected

BROAD CLINICAL LABS

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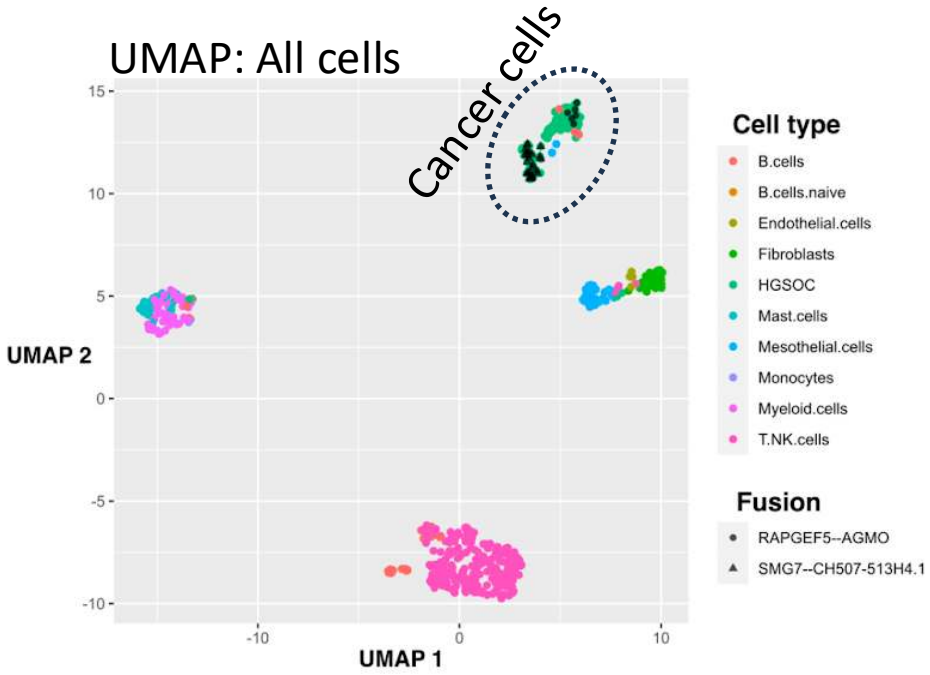
UMAP: All cells



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- 35M Illumina 10x 3' reads
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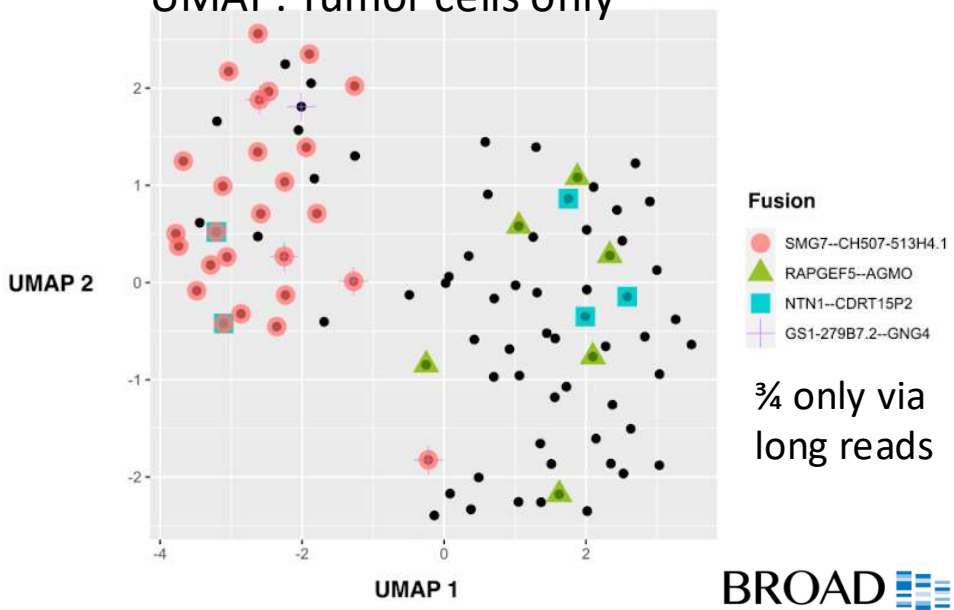
- 4 cancer-specific fusions detected

UMAP: Tumor cells only



¾ only via long reads

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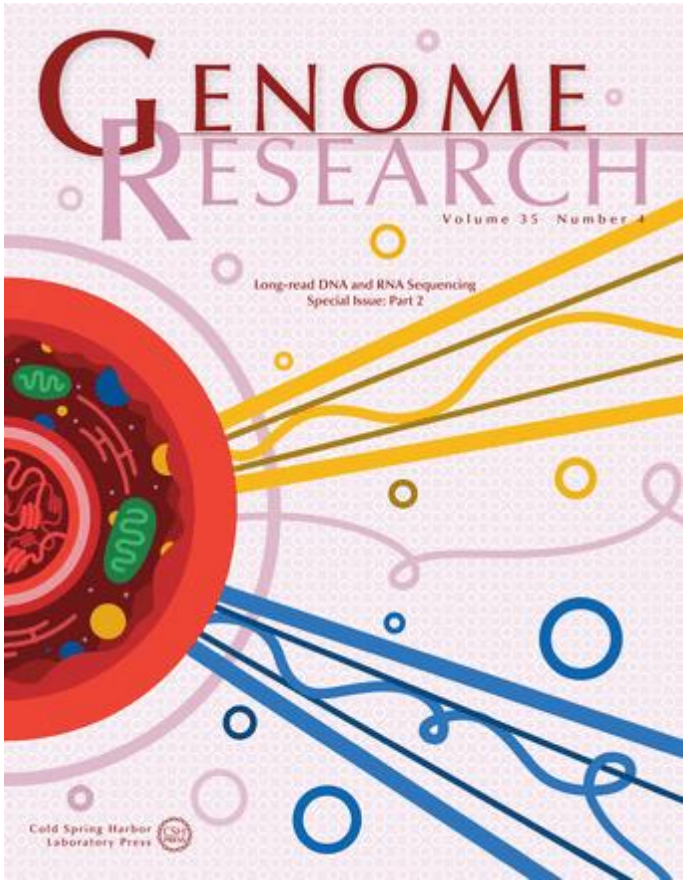


April, 2025

Accurate fusion transcript identification from long- and short-read isoform sequencing at bulk or single-cell resolution

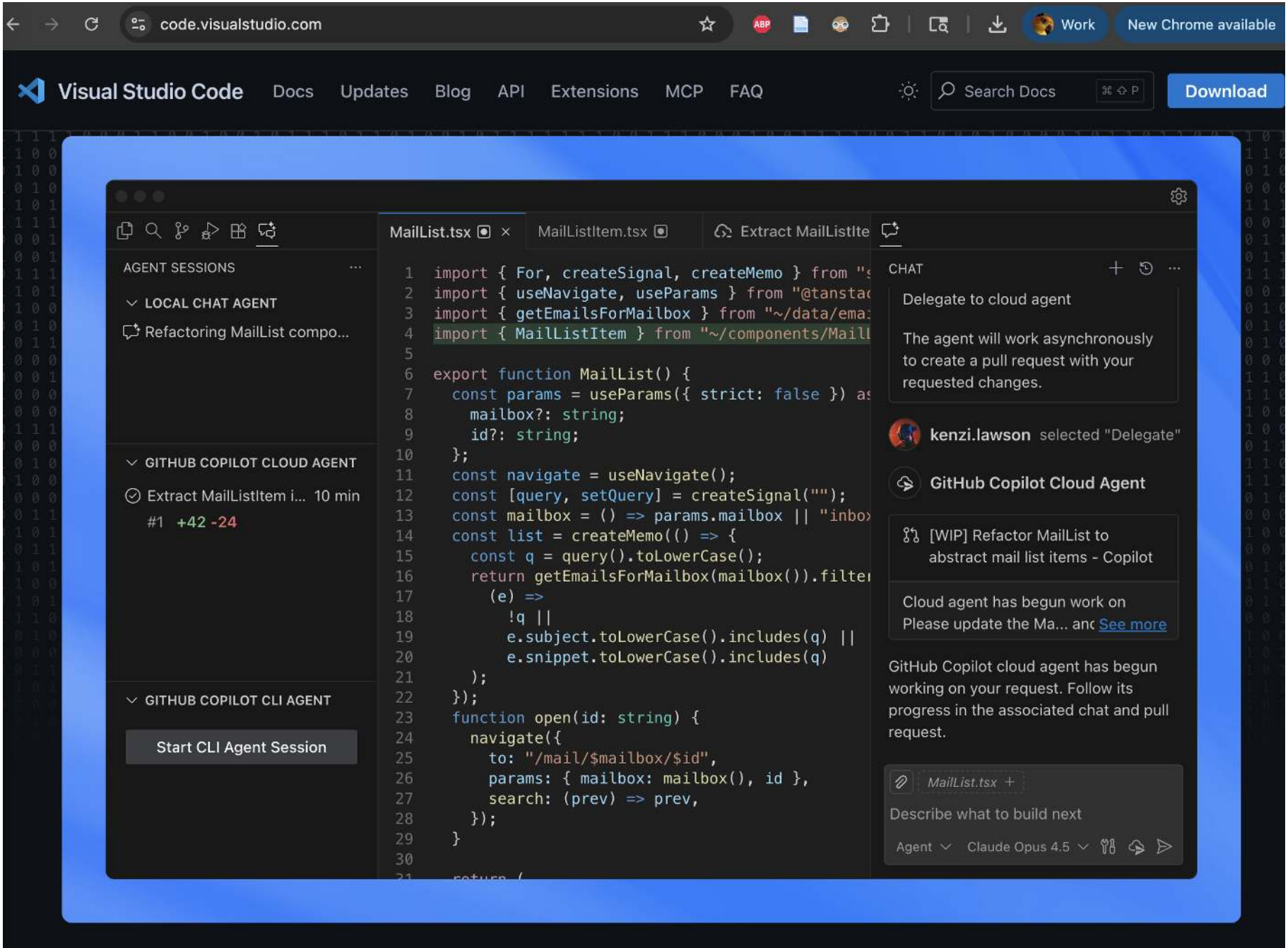
Qian Qin[1], Victoria Popic[1], Kirsty Wienand[1], Houlin Yu[1], Emily White[1], Akanksha Khorgade[1], Asa Shin[1], Christophe Georgescu[1], Catarina D. Campbell[1], Arthur Dondi[2,3], Niko Beerenwinkel[2,3], Francisca Vazquez[1], Aziz M. Al'Khafaji[1] and Brian J. Haas[1]

In Summary

- Many applications for RNA-seq, technology continues to evolve.
- Analysis can involve reference genomes or be genome-free via de novo transcriptome assembly – Trinity can help.
- Quantification involves counting reads and considering read-mapping uncertainty
- Long reads now available for applications previously limited to short reads, involve far less read mapping uncertainty, and enable isoform rather than gene expression analyses.
- Single cell and spatial transcriptomics studies are revolutionizing our understanding of tissue complexity, diversity of cell types, and cellular interactions - particularly in studies of cancer.
- Massive resources being built - whole organism cell atlases and high-resolution spatial maps, and new software tools and algorithms developed for leveraging long reads in bulk, single cell, and spatial studies.

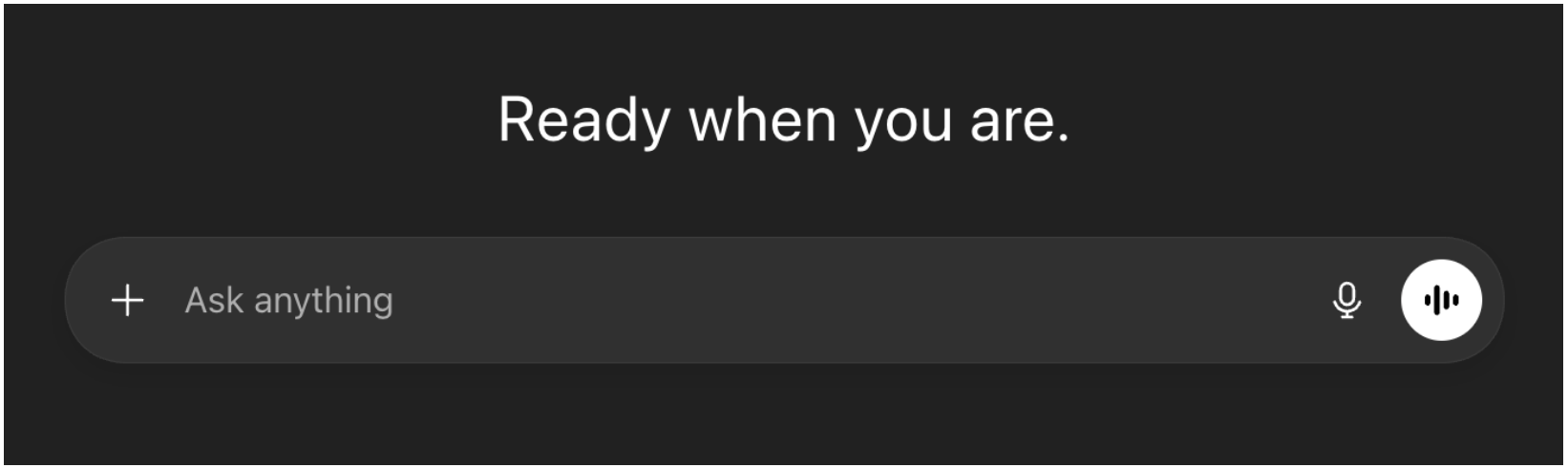

AI

Favorite tools for leveraging AI - Coding



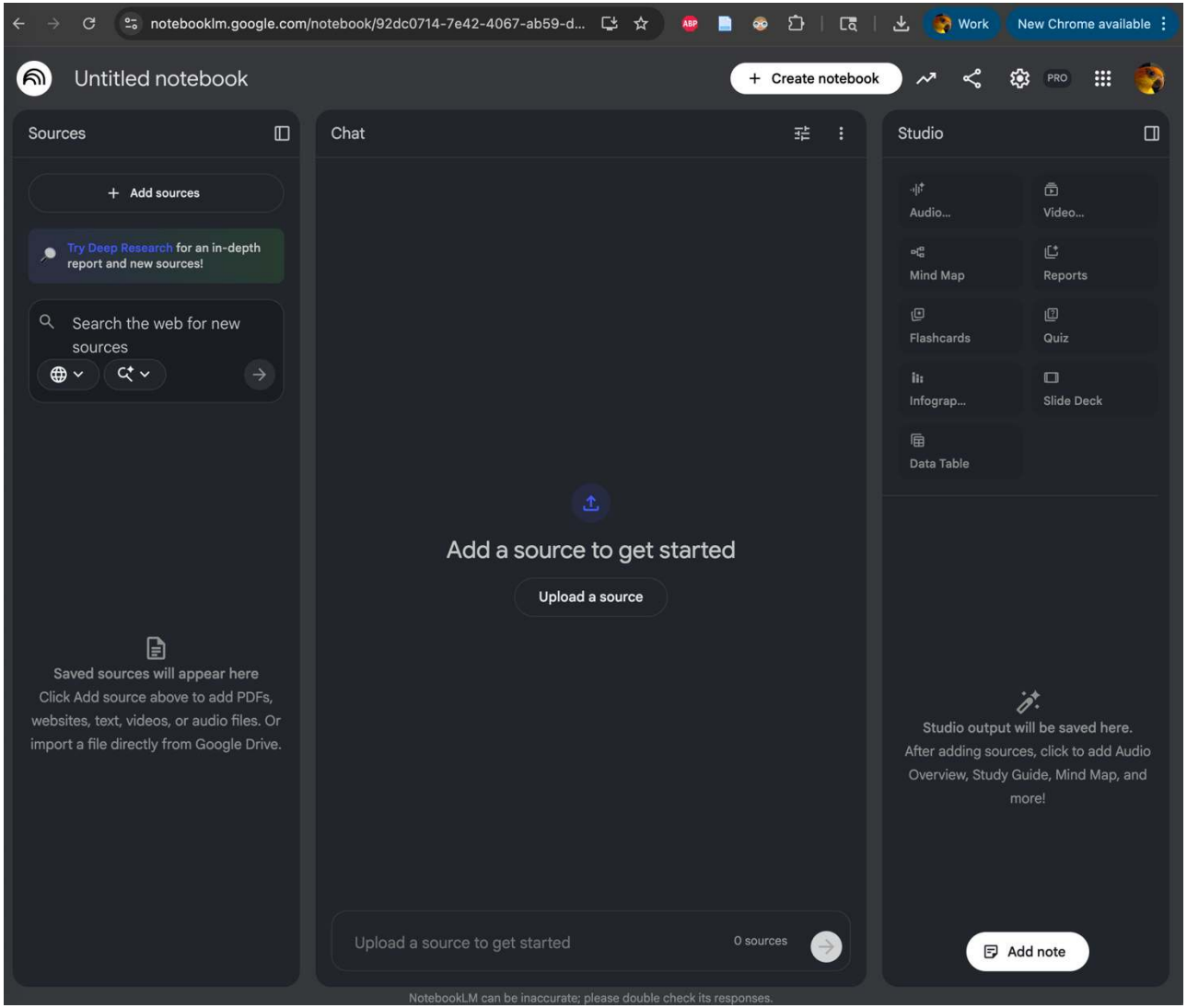
Favorite tools for leveraging AI – Learning and Writing

- ChatGPT
- Claude.io
- Gemini



Favorite tools for leveraging AI – Learning and Writing

NotebookLM



- Upload papers
- Auto-slide decks
- Makes podcasts!

Image made by Gemini nano banana