The Krumlov
Intro to Trinity RNA-seq

Brian Haas
Broad Institute

Workshop on Genomics, Cesky Krumlov, May 2023

Intro to Brian Haas

Education and Career History

BS,MS Molecular Bio
DNA Repair
SUNY Albany

1991-1999

→

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

Bioinformatics Analyst & Engineer

MS. Computer Science / Johns Hopkins

→

BROAD
INSTITUTE

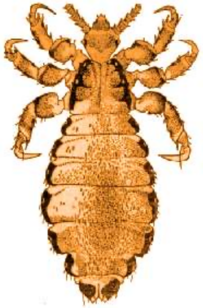
Cambridge, Massachusetts, USA

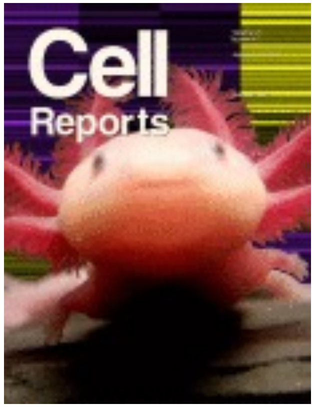
2007-current

Computational Biologist / Manager / PI

Ph.D. Bioinformatics / Boston University

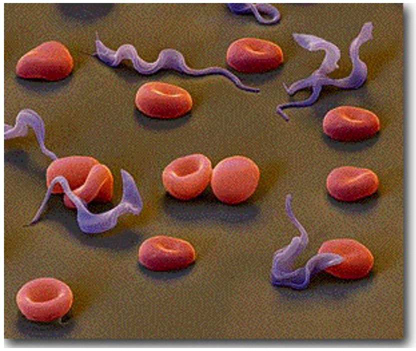
Annotation and Analysis for Diverse Genomes and Transcriptomes

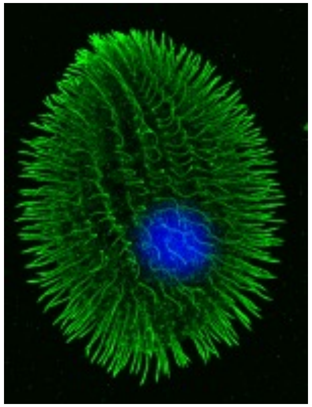


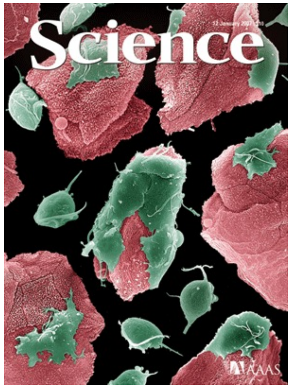


nature
biotechnology
VOLUME 28 NUMBER 9 SEPTEMBER 2010
THE SCIENCE AND BUSINESS OF BIOTECHNOLOGY
Castor bean genome
Synthetic biology meets antibody screening
Dissecting GPCR signaling
BIOPHARMACEUTICAL Benchmarks

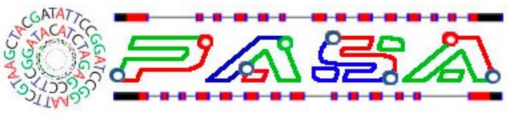
nature
GENE THERAPY
Mitochondrial DNA replacement in primates
CLIMATE CHANGE
HEALTH CARE
BLIGHTED HARVEST
Genome sequence unearths roots of potato pathogen's adaptability







My Favorite Activity – Bioinformatics Tool Development and Application



NAR, 2003

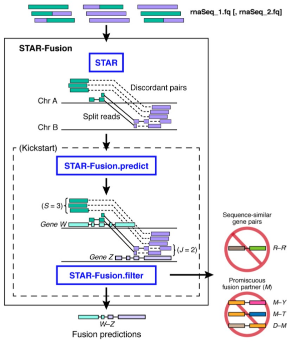
Bioinformatics, 2004

EVidenceModeler
Genome Biology, 2008

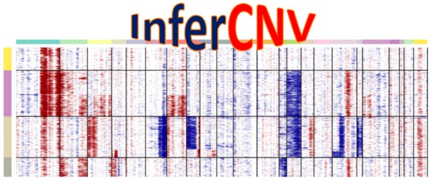
BROAD
INSTITUTE

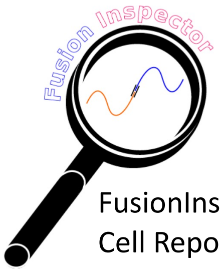
Chimera Slayer
Genome Research, 2011

Nature Biotech, 2011
Nature Protocols, 2013



STAR-Fusion
Genome Biology, 2019

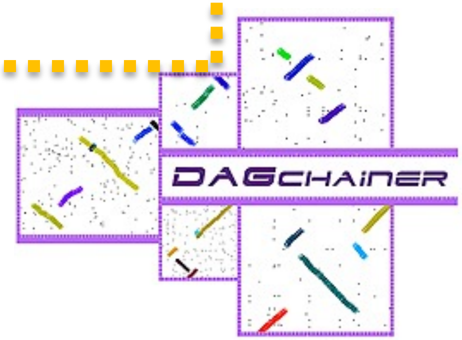




FusionInspector
Cell Reports Methods, 2023

My Favorite Activity – Bioinformatics Tool Development and Application

NAR, 2003



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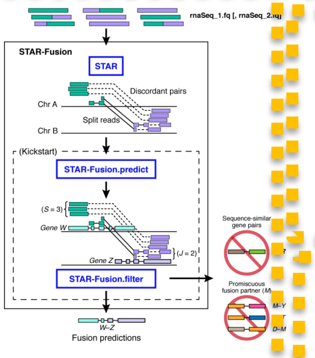
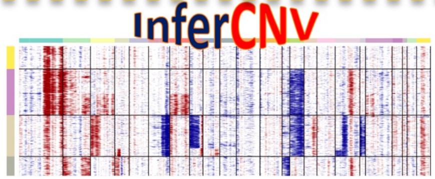
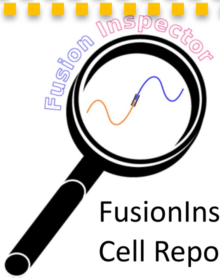


STAR-Fusion
Genome Biology, 2019

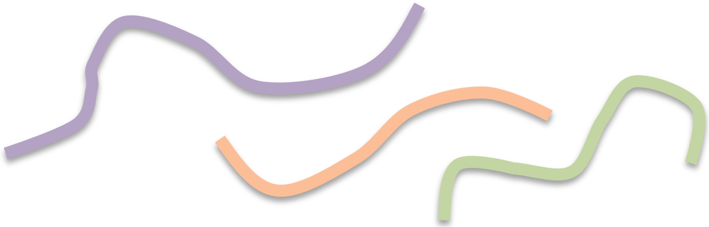




FusionInspector
Cell Reports Methods, 2023

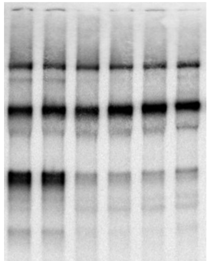
Biological Investigations Empowered by Transcriptomics

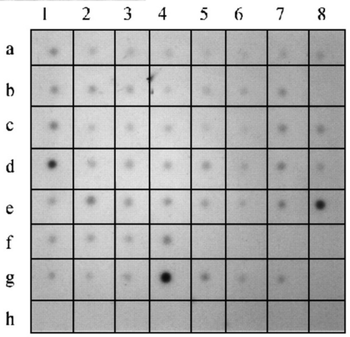
Extract RNA,
... some protocol for processing, ...



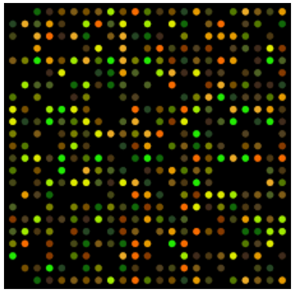
Analysis Method
(pick your favorite)

Northern

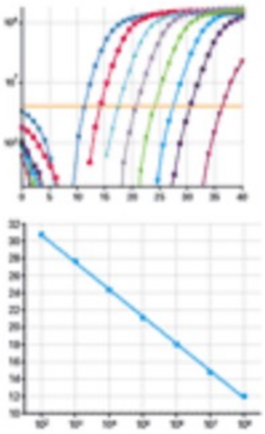




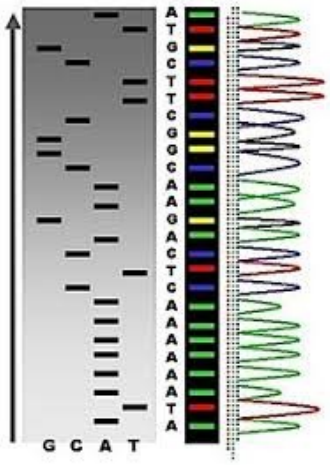
Dot Blot



Microarray



qRT-PCR



Sanger Sequencing

Other...

Minion

MinION MkI: portable, real time biological analyses

MinION

Historical Timeline to Modern Transcriptomics (from 1970)

Reverse Transcription (1970)

Northern Blot
Sanger Sequencing
(1977)

Expressed Sequence Tags (1992)

cDNA microarrays (1995)

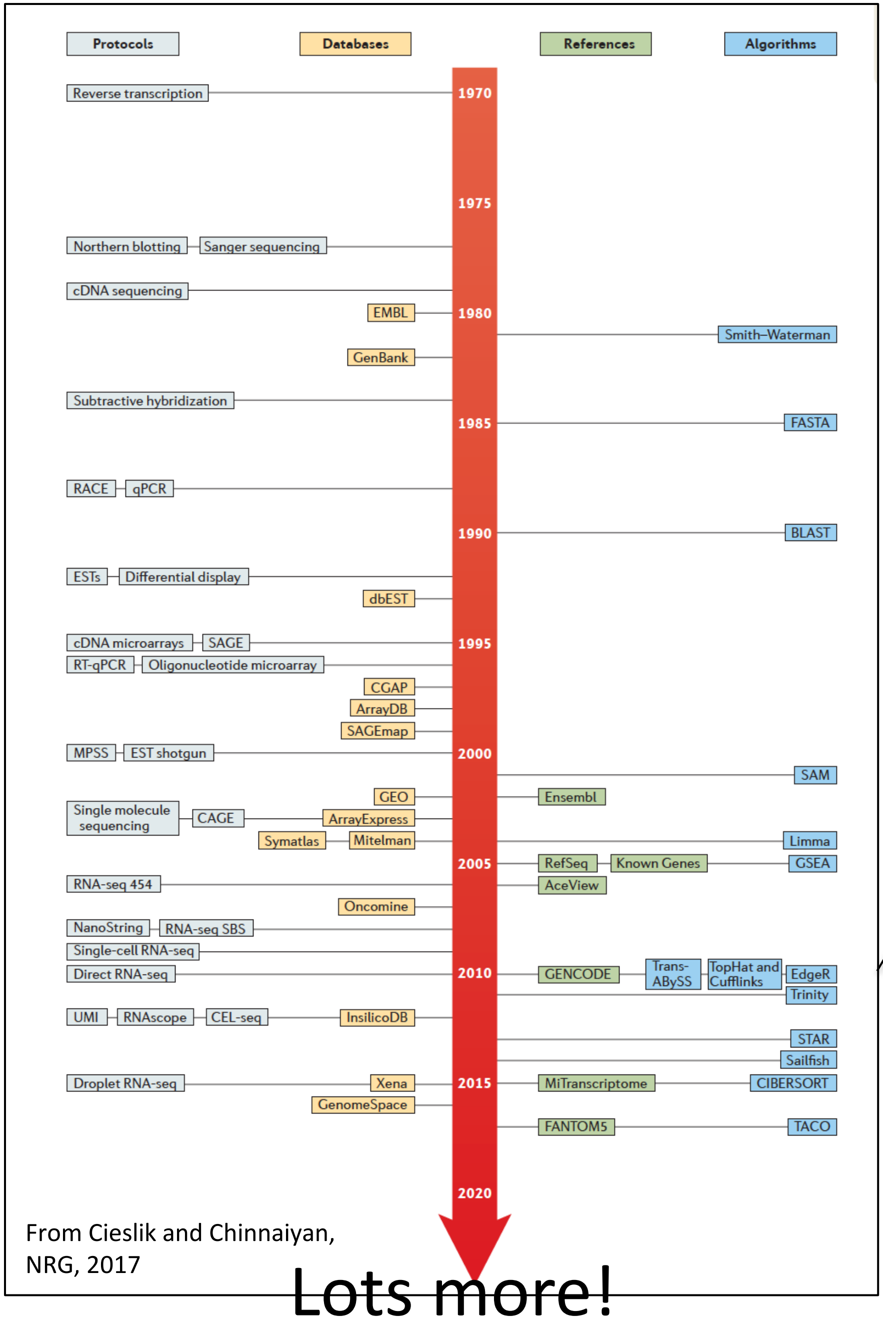
RNA-Seq (2006-2008)

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)

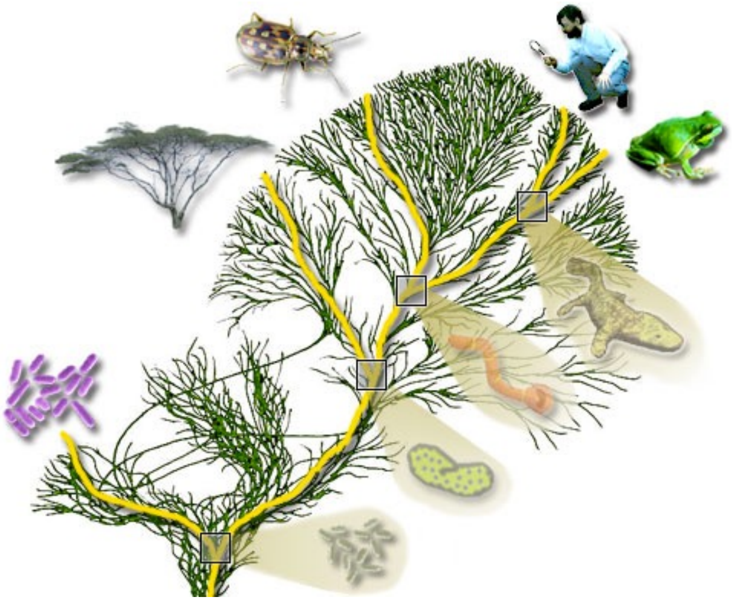
SAMtools (2009)
Tophat/Cufflinks (2010)

RSEM

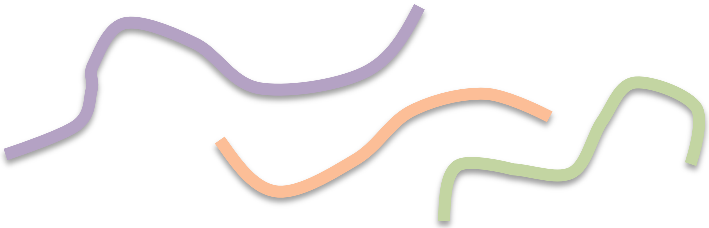
(2011)

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

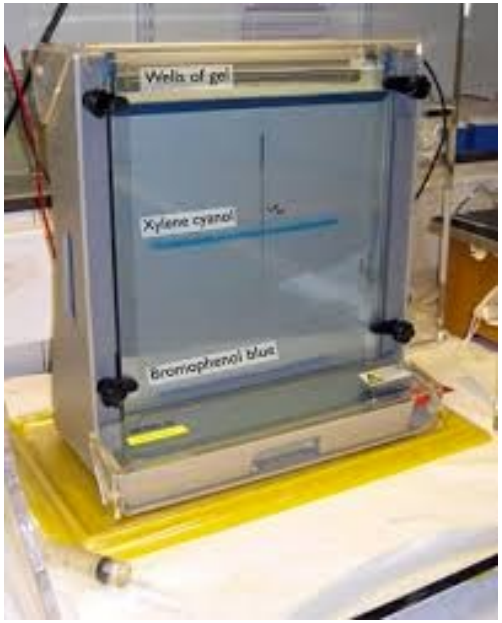


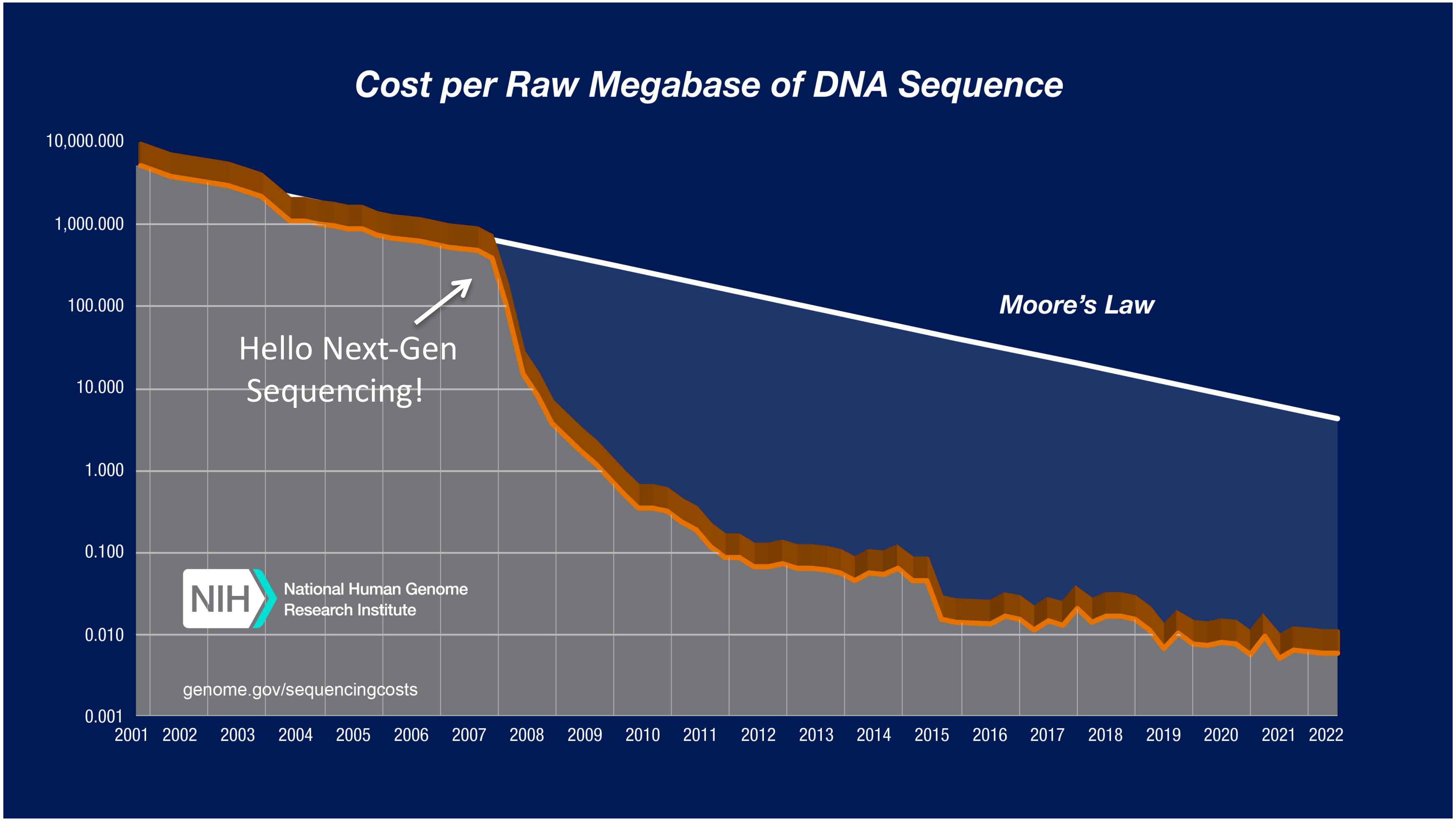
Next-gen Sequencer
(pick your favorite)

Millions to Billions of Reads

Personal Reflections...

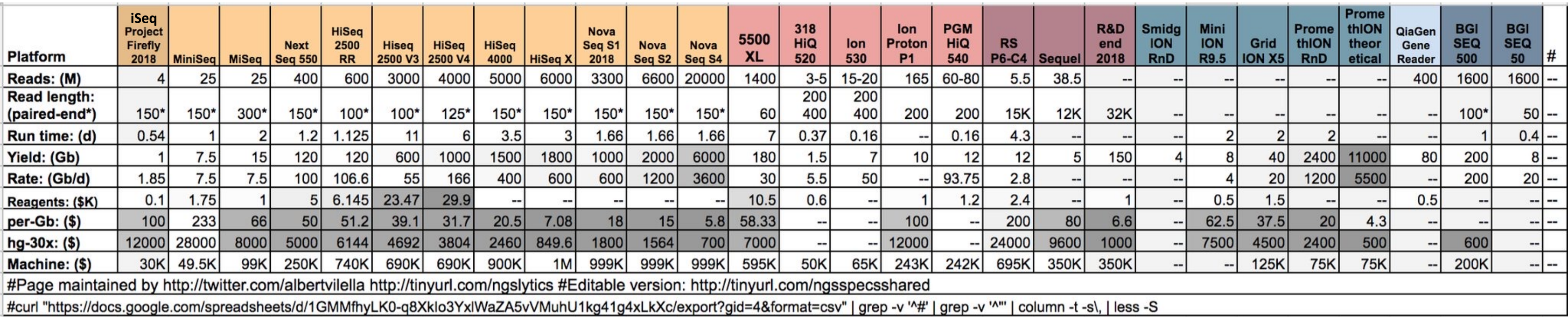
Circa 1995





From https://www.genome.gov/about-genomics/fact-sheets/DNA-Sequencing-Costs-Data

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	--	--

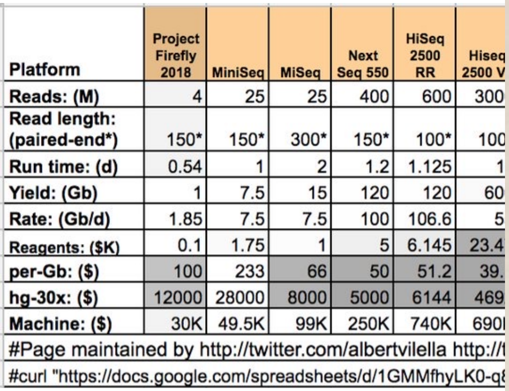
```
#Page maintained by http://twitter.com/albertvilella http://tinyurl.com/ngslytics #Editable version: http://tinyurl.com/ngsspecsshared
#curl "https://docs.google.com/spreadsheets/d/1GMMfhyLK0-q8Xklo3YxlWaZA5vVMuhU1kg41g4xLkXc/export?gid=4&format=csv" | grep -v '^#' | grep -v '^"' | column -t -s\, | less -S
```

Stats circa 2018

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

*Not all shown at scale

Generating RNA-Seq: *How to Choose?*

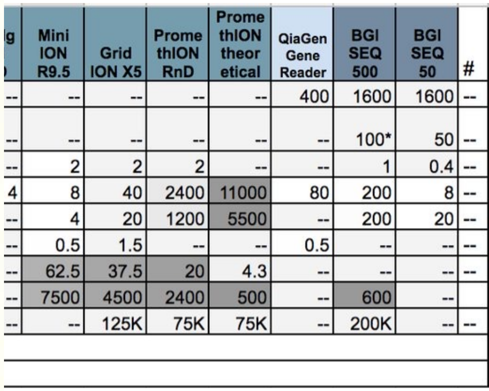


Platform	Project Firefly 2018	MiniSeq	MiSeq	Next Seq 550	HiSeq 2500 RR	Hiseq 2500 V
Reads: (M)	4	25	25	400	600	300
Read length: (paired-end*)	150*	150*	300*	150*	100*	100
Run time: (d)	0.54	1	2	1.2	1.125	1
Yield: (Gb)	1	7.5	15	120	120	60
Rate: (Gb/d)	1.85	7.5	7.5	100	106.6	5
Reagents: ($K)	0.1	1.75	1	5	6.145	23.4
per-Gb: ($)	100	233	66	50	51.2	39.
hg-30x: ($)	12000	28000	8000	5000	6144	469
Machine: ($)	30K	49.5K	99K	250K	740K	690I

#Page maintained by http://twitter.com/albertvilella http://t

#curl "https://docs.google.com/spreadsheets/d/1GMMfhyLK0-q8

"What I especially like about this baby is this little drawer where I can keep my lunch."



g)	Mini ION R9.5	Grid ION X5	Prome thION RnD	Prome thION theor etical	QiaGen Gene Reader	BGI SEQ 500	BGI SEQ 50	#
--	--	--	--	--	400	1600	1600	--
--	--	--	--	--	--	100*	50	--
--	2	2	2	--	--	1	0.4	--
4	8	40	2400	11000	80	200	8	--
--	4	20	1200	5500	--	200	20	--
--	0.5	1.5	--	--	0.5	--	--	--
--	62.5	37.5	20	4.3	--	--	--	--
--	7500	4500	2400	500	--	600	--	
--	--	125K	75K	75K	--	200K	--	--

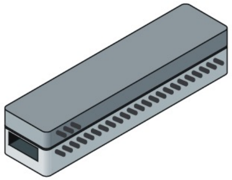
Thx Joshua Levin, for the cartoon. ☺

Maybe something more portable?

Today's Most Popular Sequencing Technologies

Illumina

Pacific Biosciences

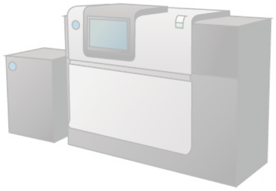


Oxford Nanopore

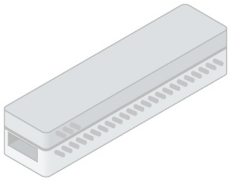
Images from "RNA sequencing: the teenage years"
Rory Stark, Marta Grzelak & James Hadfield
Nature Reviews Genetics volume 20, pages631–656(2019)

Today's Most Popular Sequencing Technologies

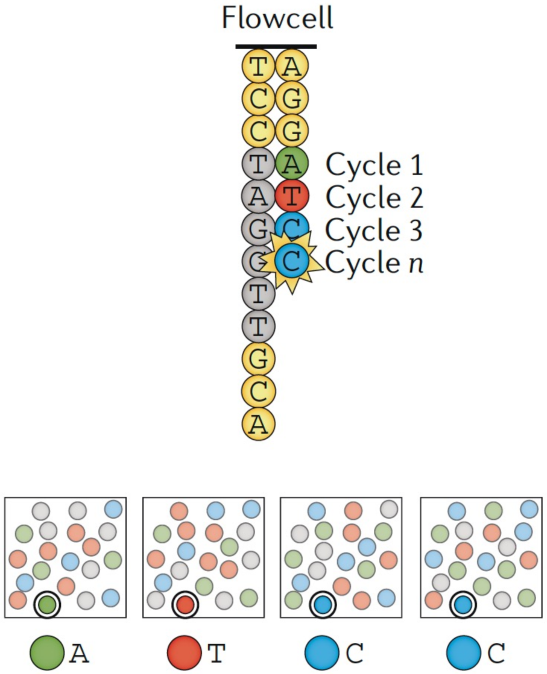
Illumina



Pacific Biosciences



Oxford Nanopore

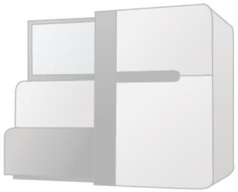


Hundreds of millions to billions of highly accurate but shorter reads.

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

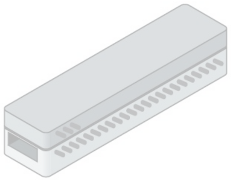
Video at: https://youtu.be/fCd6B5HRaZ8

Today's Most Popular Sequencing Technologies

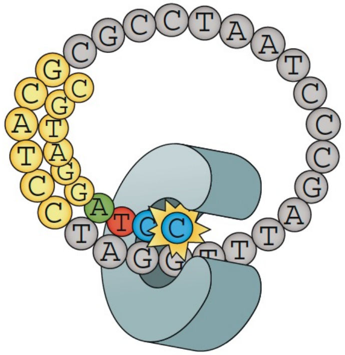


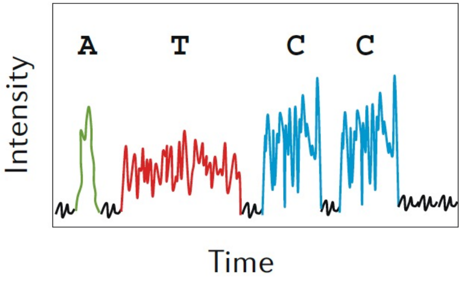
Illumina

Pacific Biosciences



Oxford Nanopore



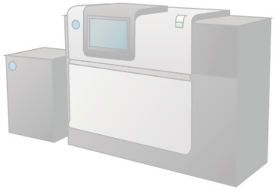


Limited sequencing depth, but highly accurate full-length single molecule reads.

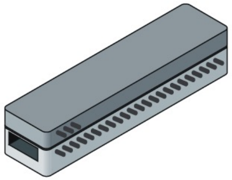
Video at: https://www.youtube.com/watch?v=_lD8JyAbwEo

Today’s Most Popular Sequencing Technologies

Illumina



Pacific Biosciences



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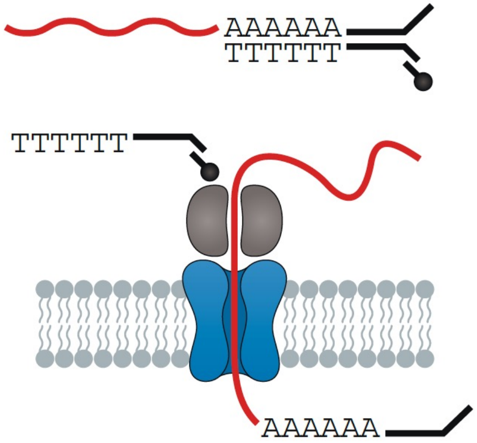
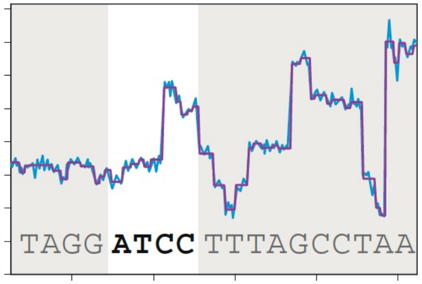




Video:
https://nanoporetech.com/how-it-works#fullVideo&modal=fullVideo

Limited sequencing depth, and moderate-to-highly accurate full-length single molecule reads.

Can do direct RNA sequencing! and find evidence for methylation

A Plethora of Biological Sequence Analyses Enabled by RNA-Seq

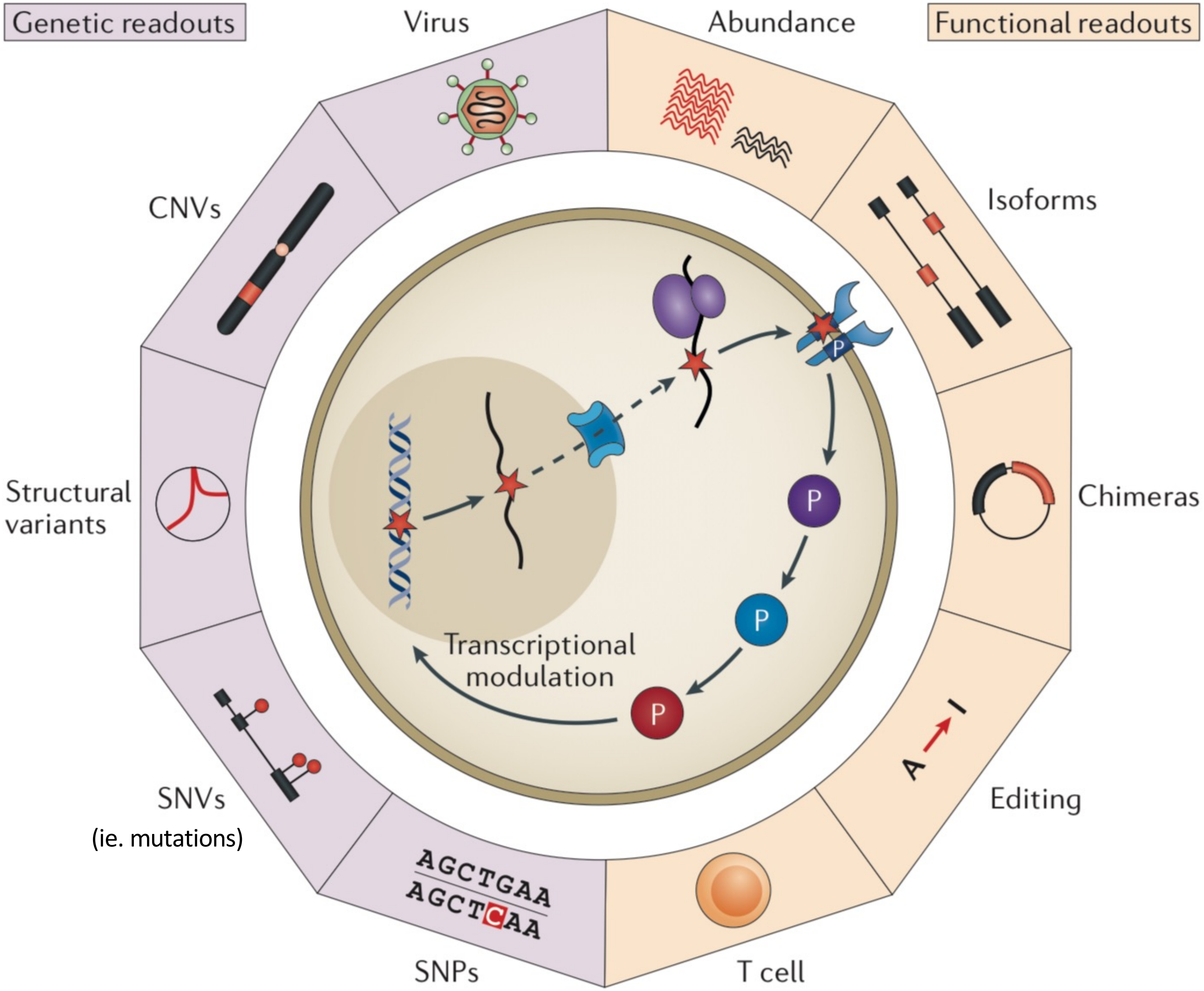
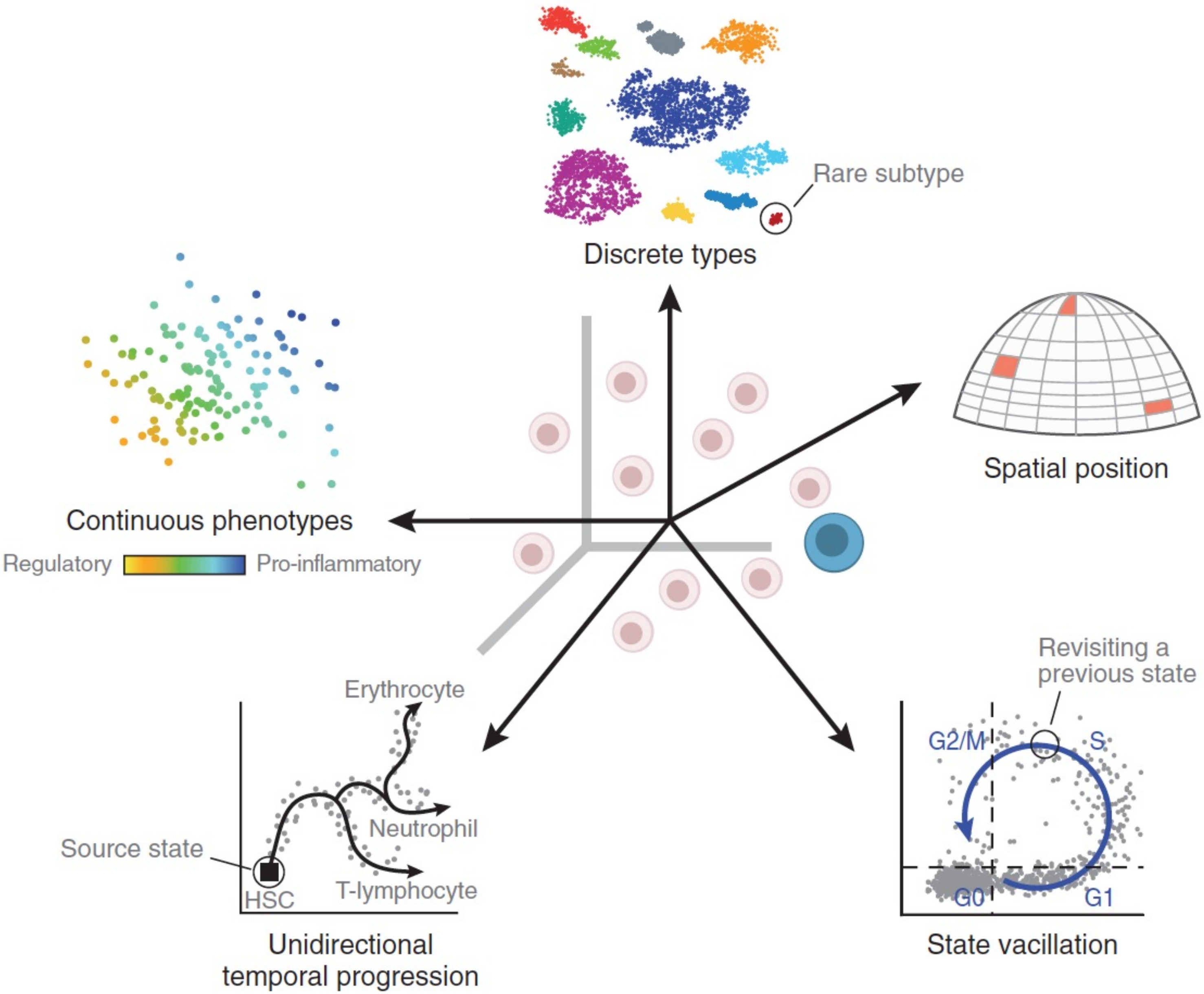


Figure 2 | **Transcriptome profiling for genetic causes and functional phenotypic readouts.**

From Cieslik and Chinnaiyan, NRG, 2017

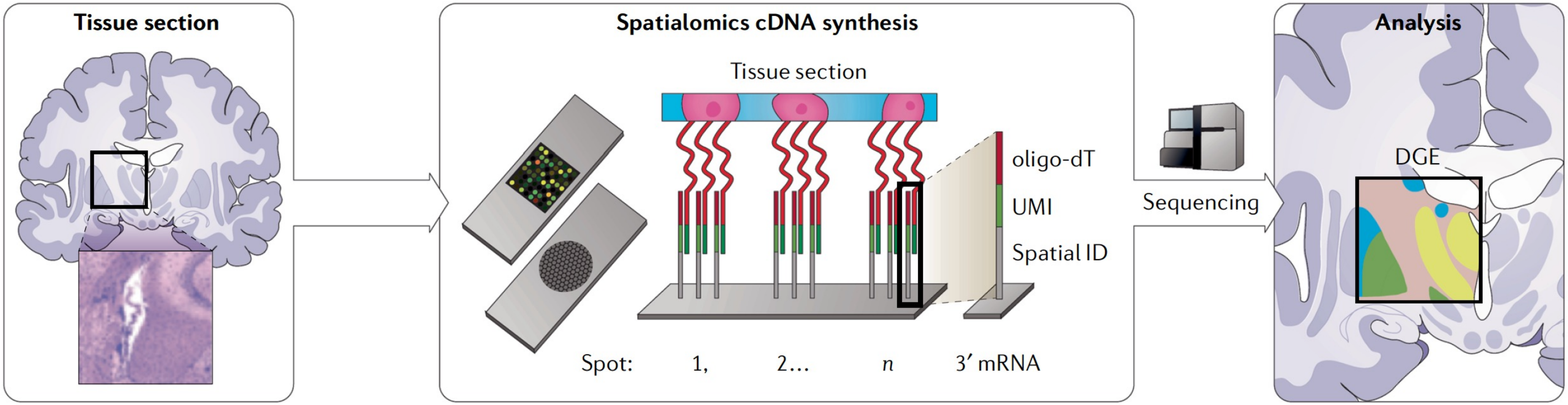
RNA-Seq is Empowering Discovery at Single Cell Resolution



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

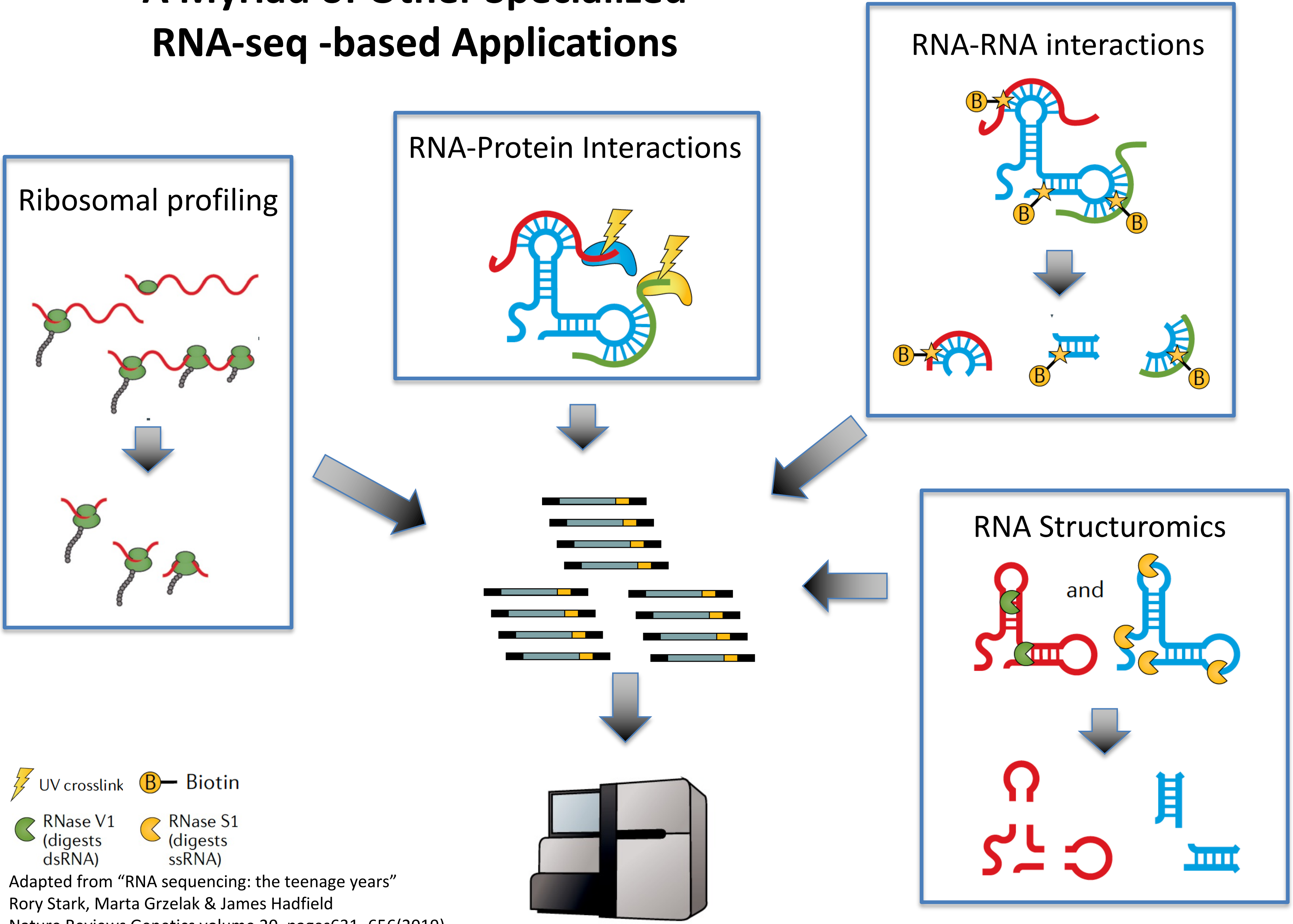
RNA-Sequencing as your lens towards biological discovery

⚡ 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

UV crosslink

B— Biotin

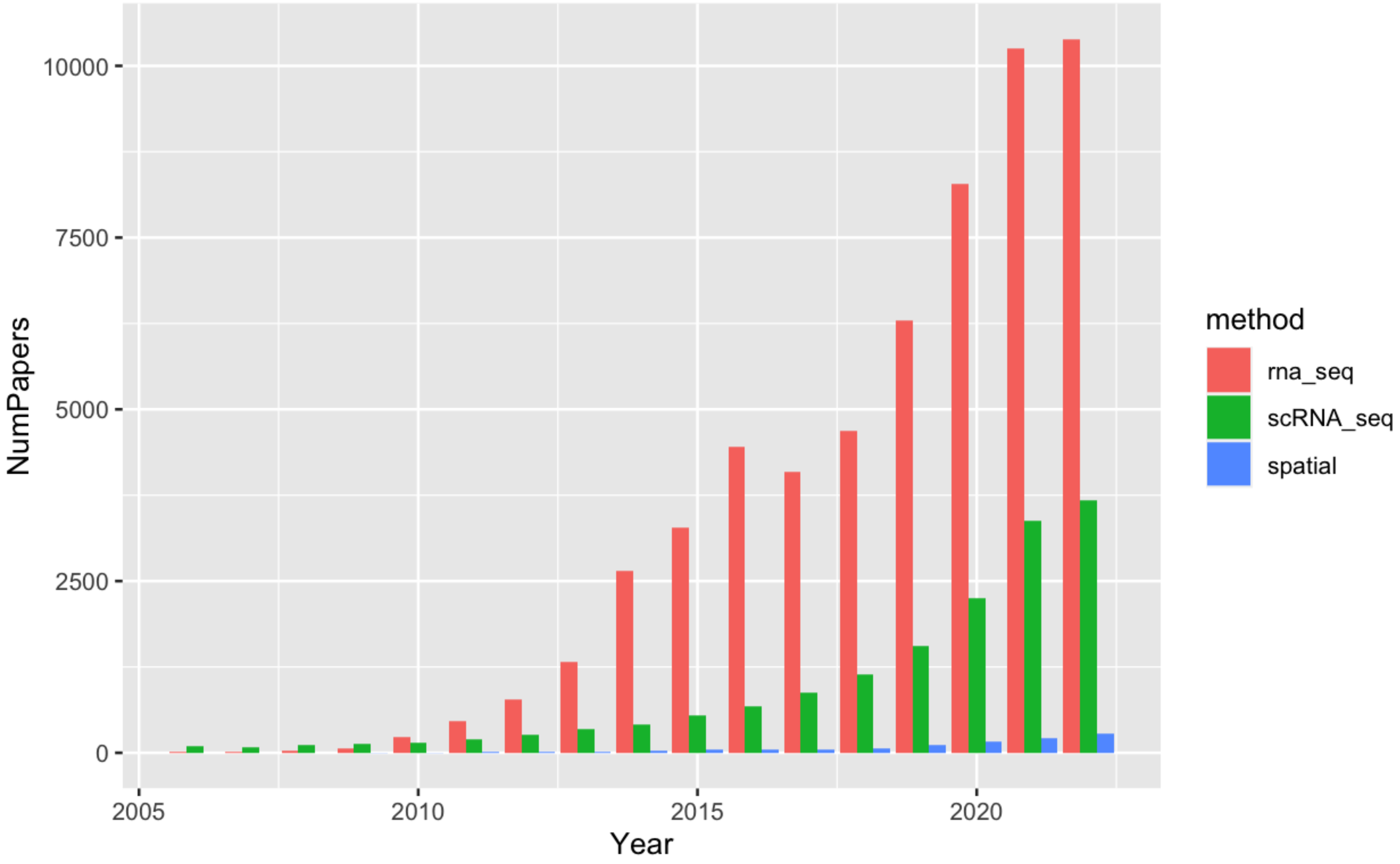
RNase V1 (digests dsRNA)

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)

RNA-seq Publication Trend

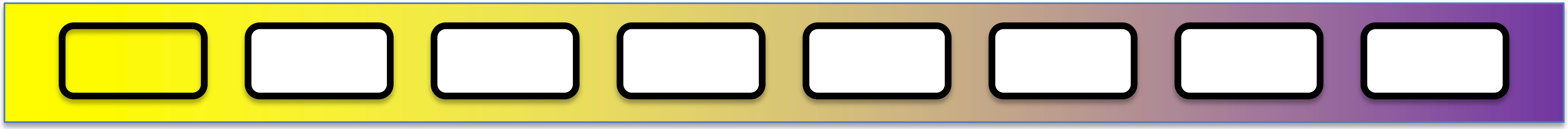
Paper Counts from PubMed



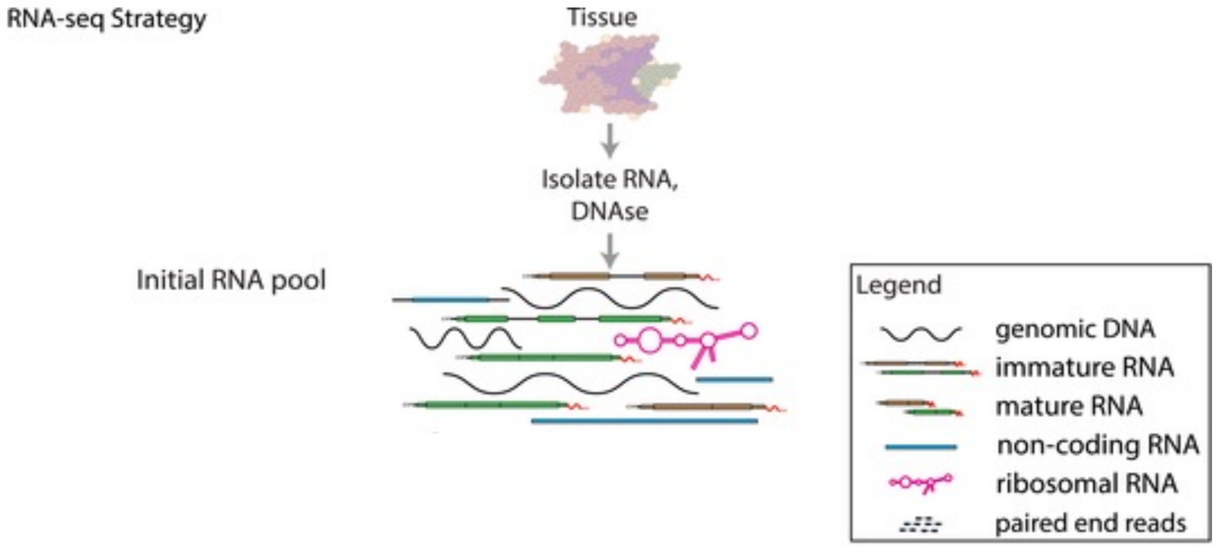
Transcriptomics Lecture Overview

1. Overview of RNA-Seq
2. Transcript reconstruction methods
3. Trinity de novo assembly
4. Transcriptome quality assessment
5. Latest advances for RNA-seq
6. Short lab activity – running Trinity

Part 1. Overview of RNA-Seq



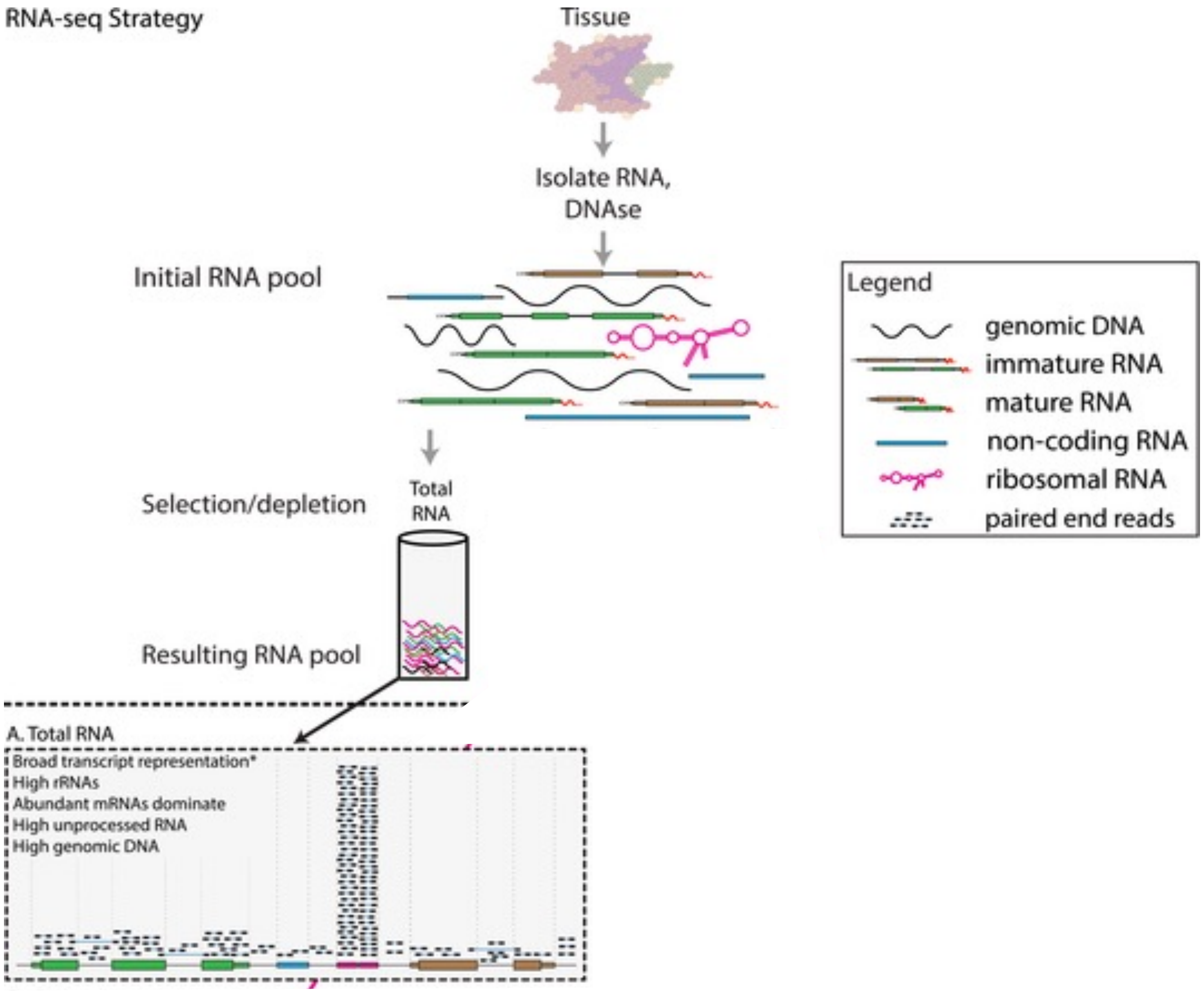
RNA-seq library enrichment strategies that influence interpretation and analysis.



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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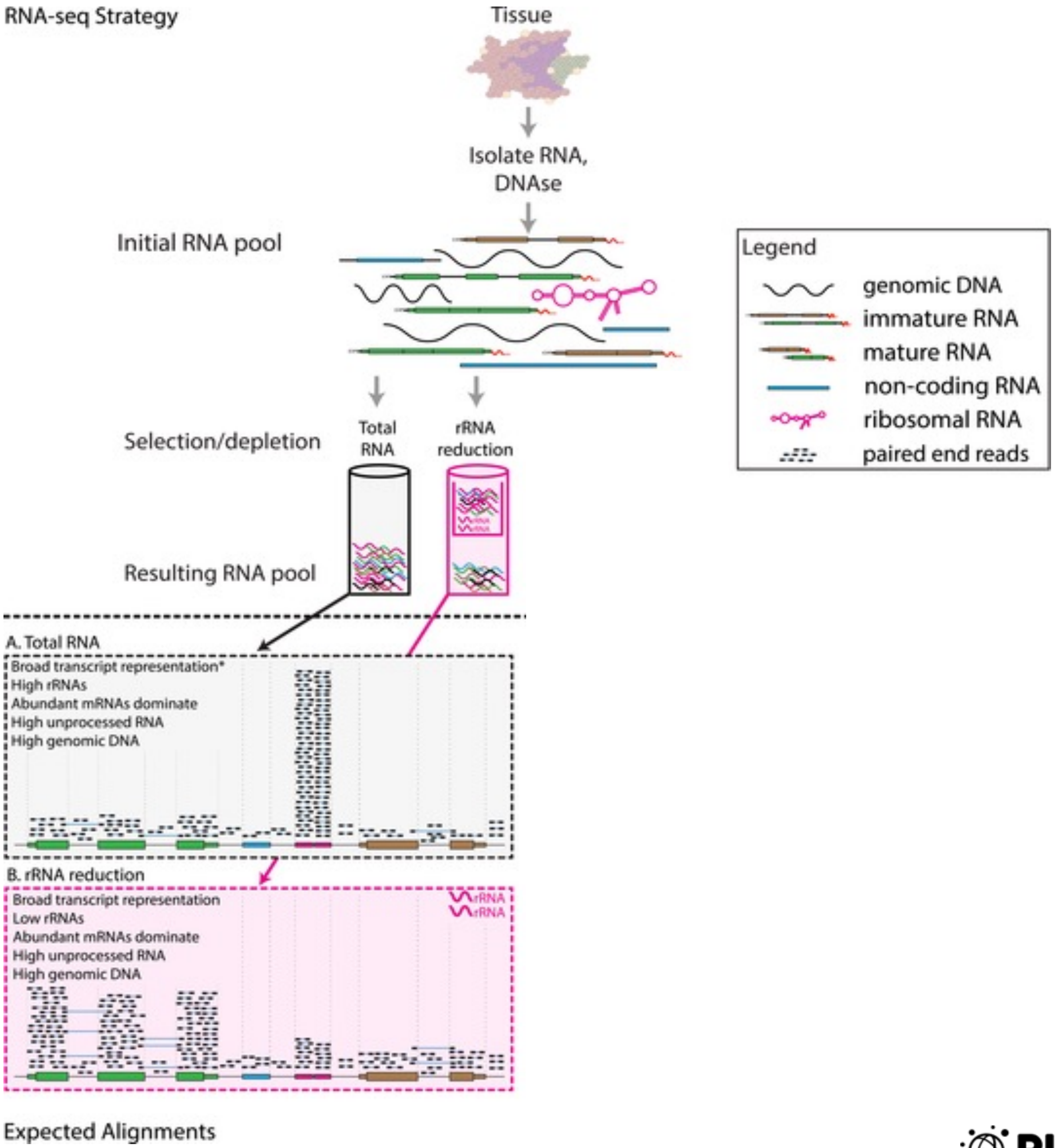

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PLOS COMPUTATIONAL BIOLOGY

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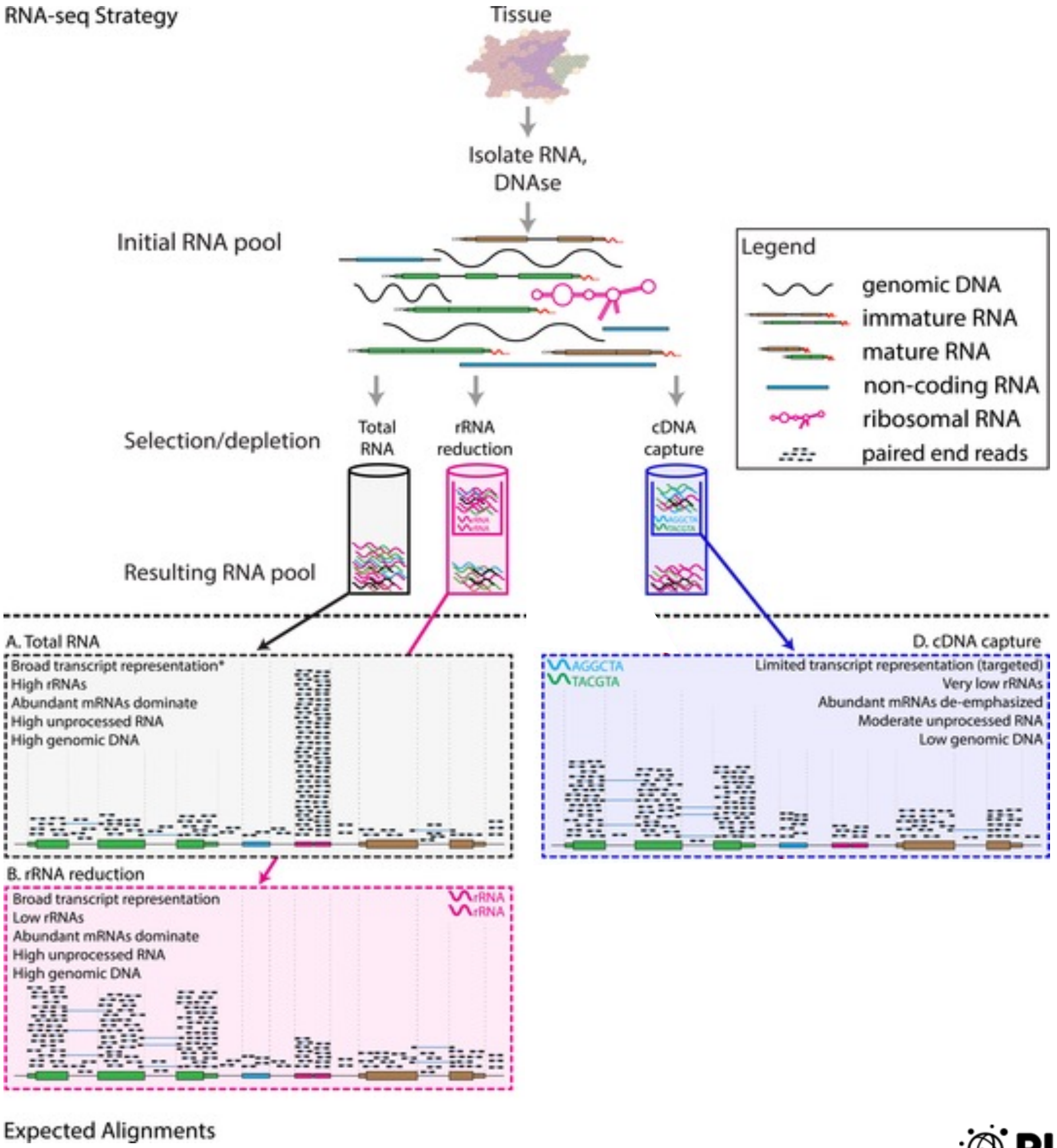

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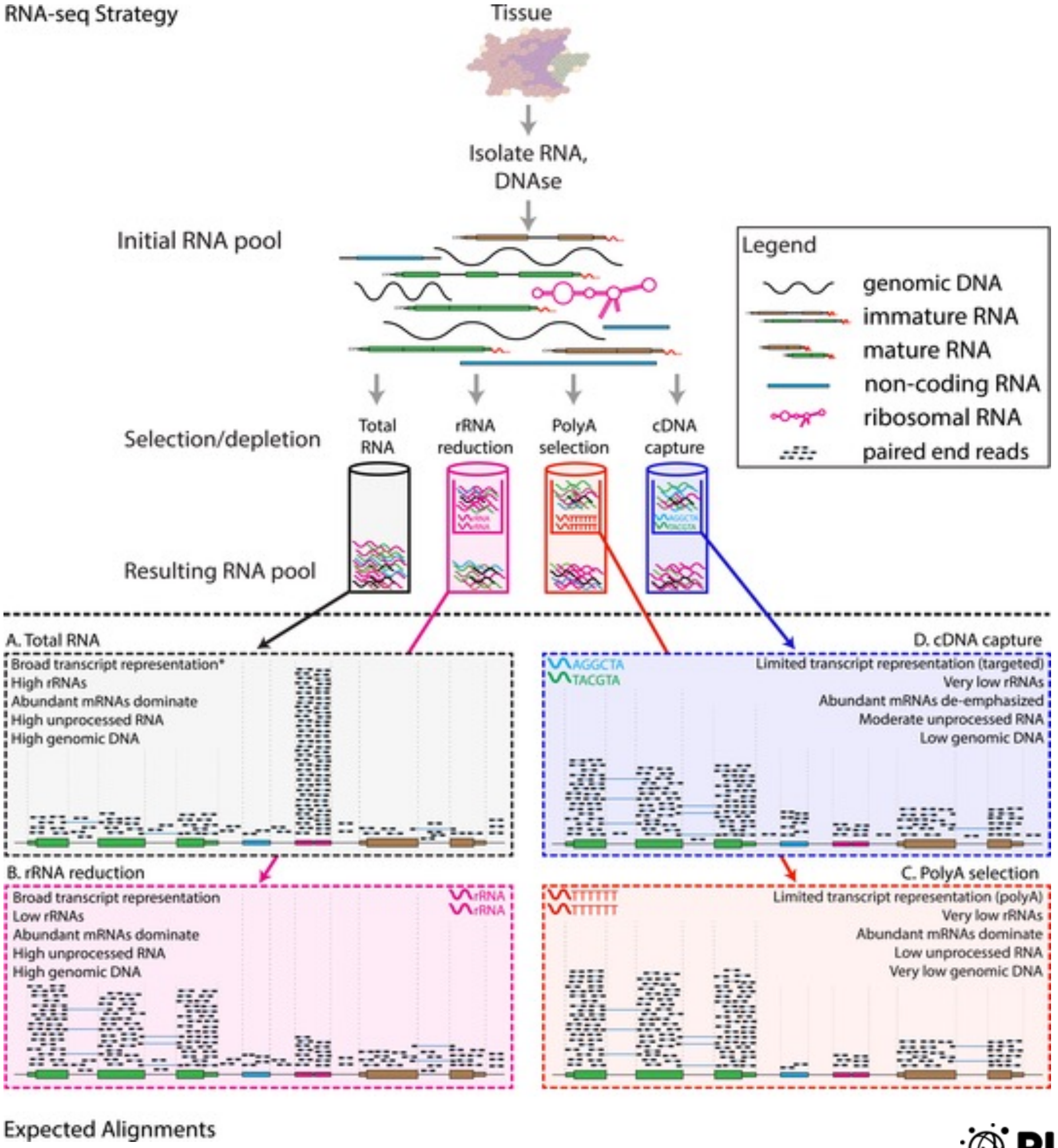

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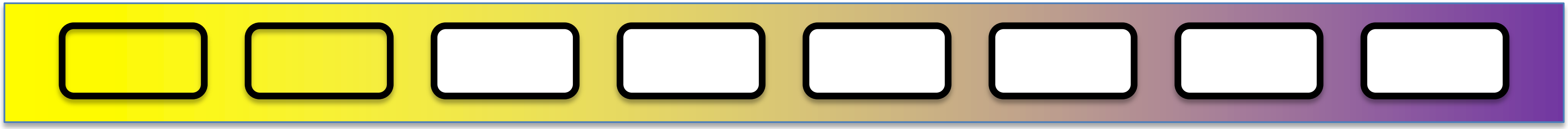
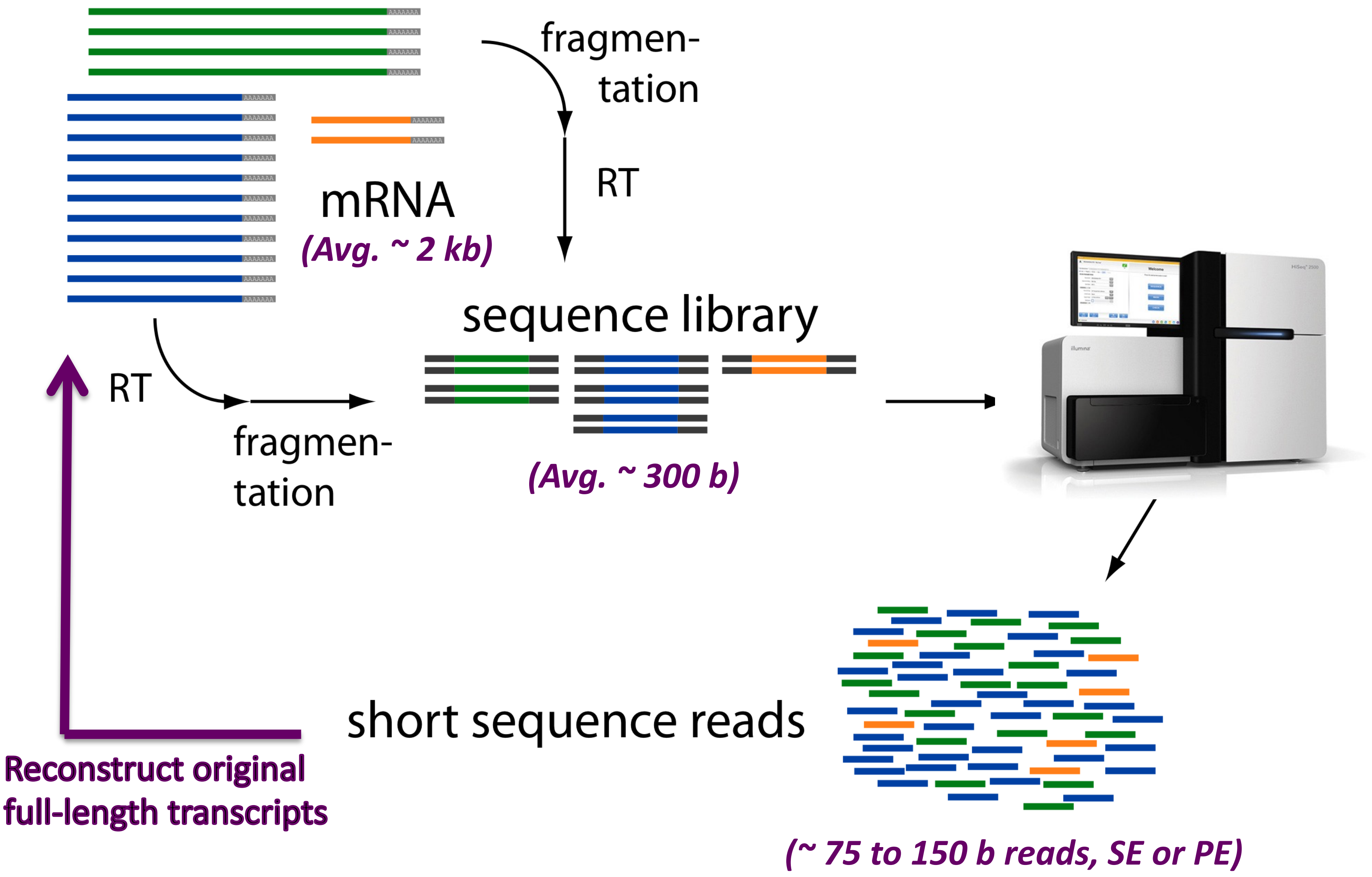
PLOS | COMPUTATIONAL BIOLOGY

Part 2. Transcript Reconstruction Methods



RNA-Seq Challenge: Transcript Reconstruction



mRNA

(Avg. ~ 2 kb)

fragmentation

RT

RT

fragmentation

sequence library

(Avg. ~ 300 b)

short sequence reads

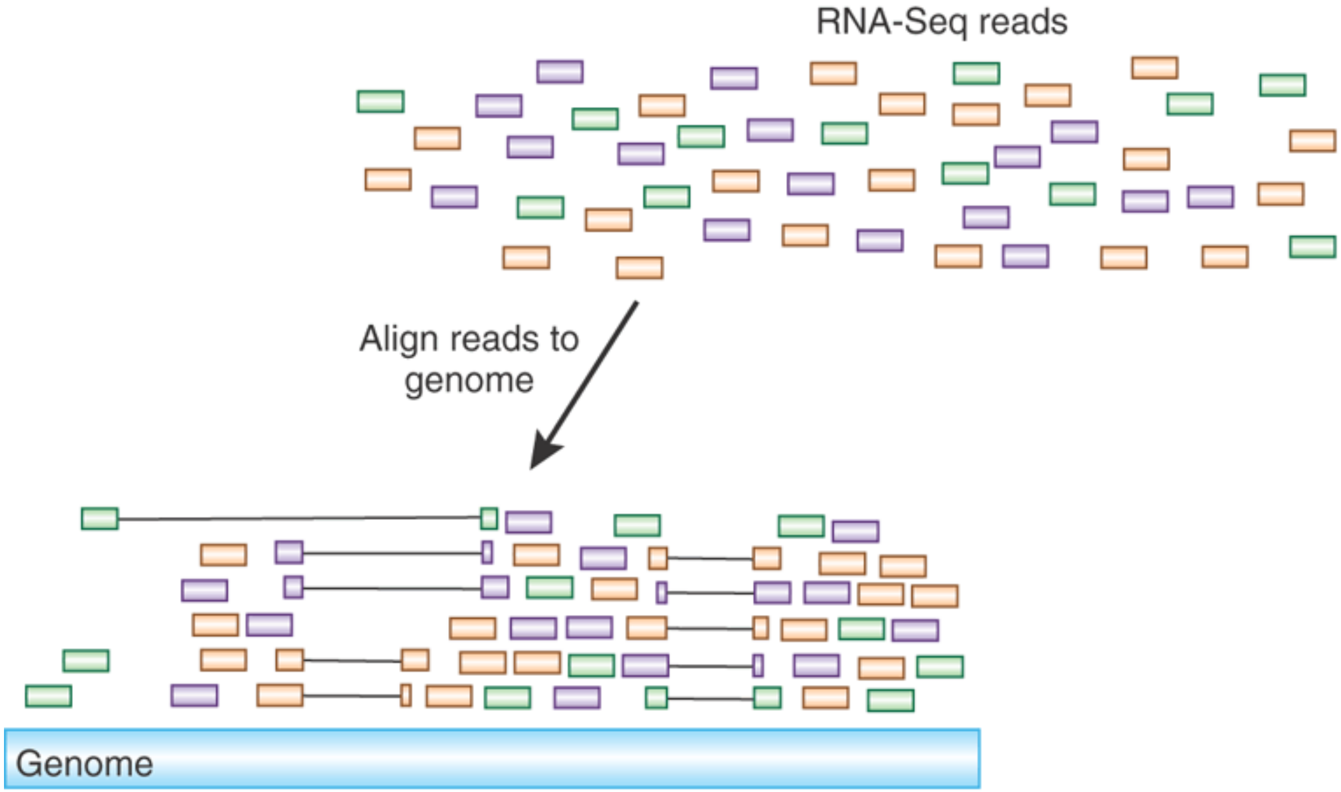
(~ 75 to 150 b reads, SE or PE)

Reconstruct original full-length transcripts

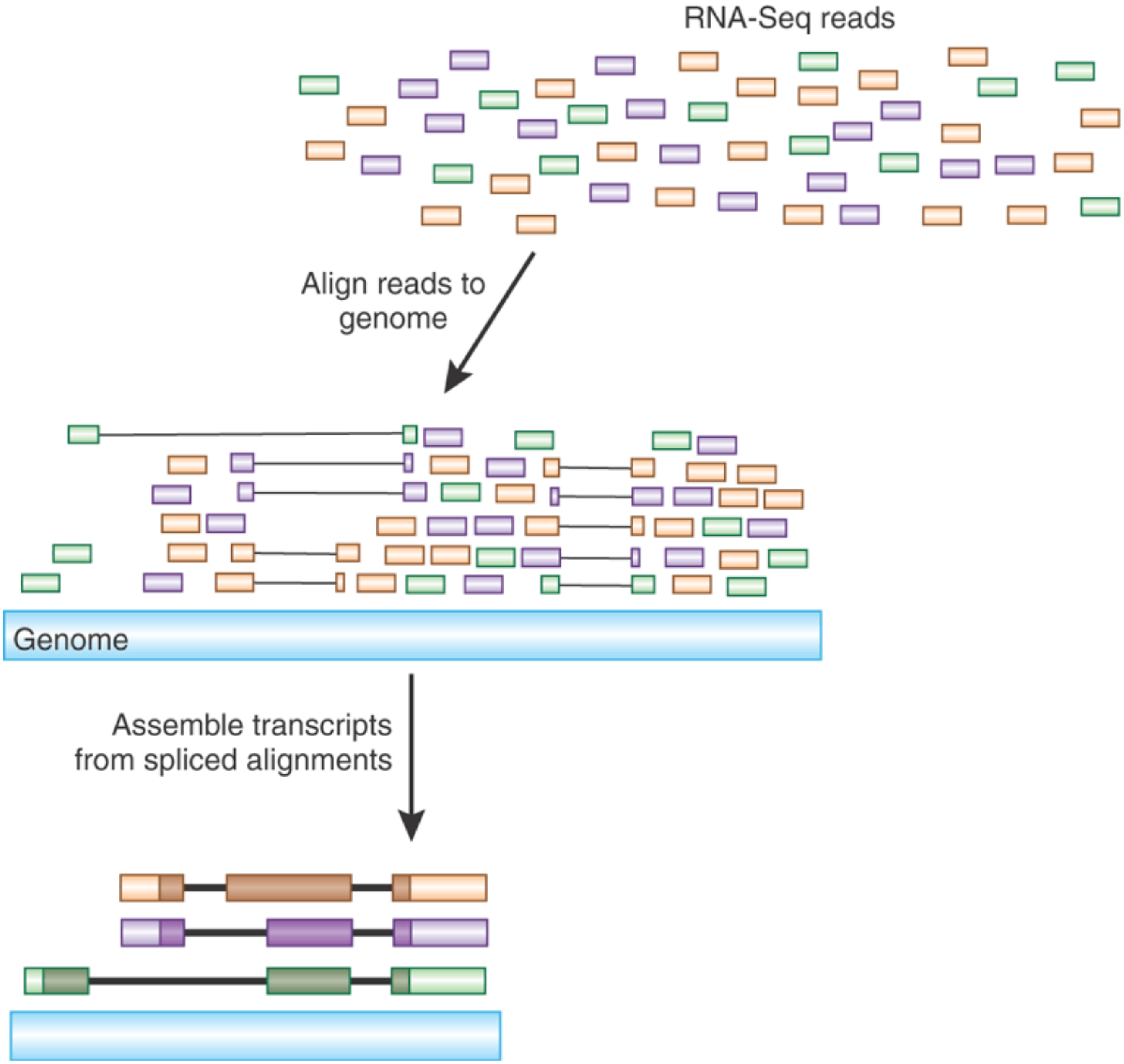
Adapted from: http://www2.fml.tuebingen.mpg.de/raetsch/members/research/transcriptomics.html

Transcript Reconstruction from RNA-Seq Reads

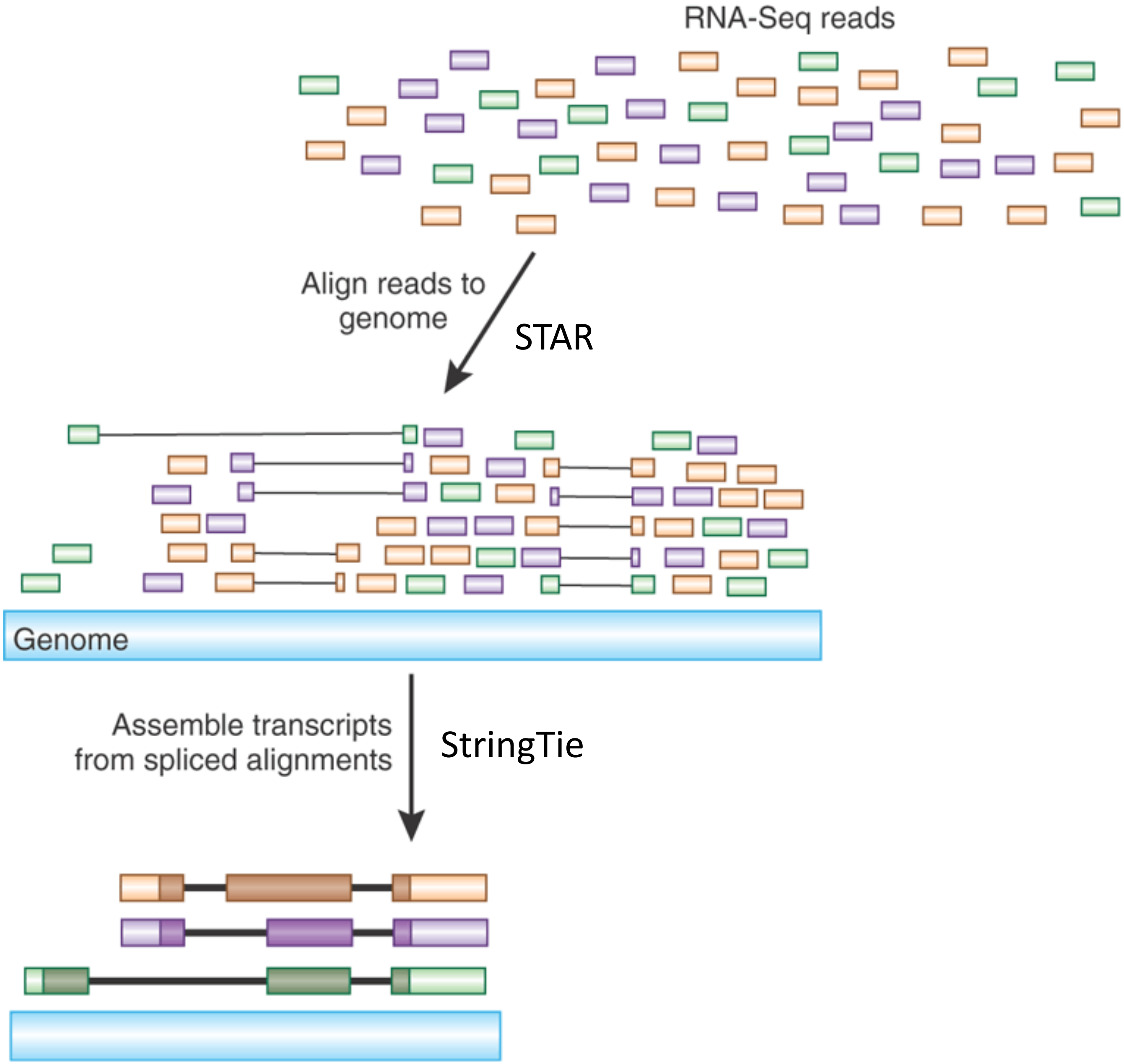
Transcript Reconstruction from RNA-Seq Reads



Transcript Reconstruction from RNA-Seq Reads



Transcript Reconstruction from RNA-Seq Reads



RNA-Seq reads

Align reads to genome

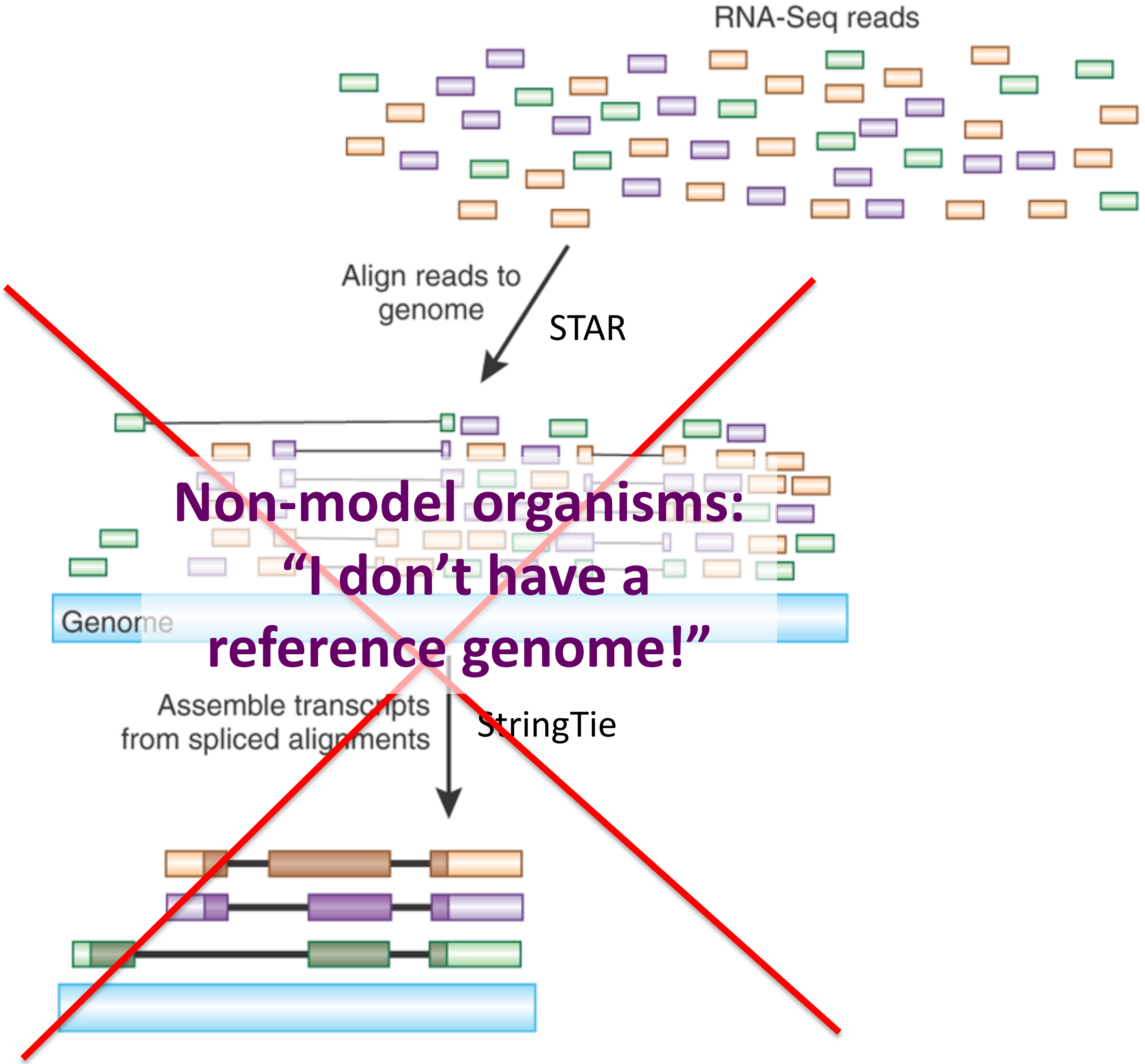
STAR

Genome

Assemble transcripts from spliced alignments

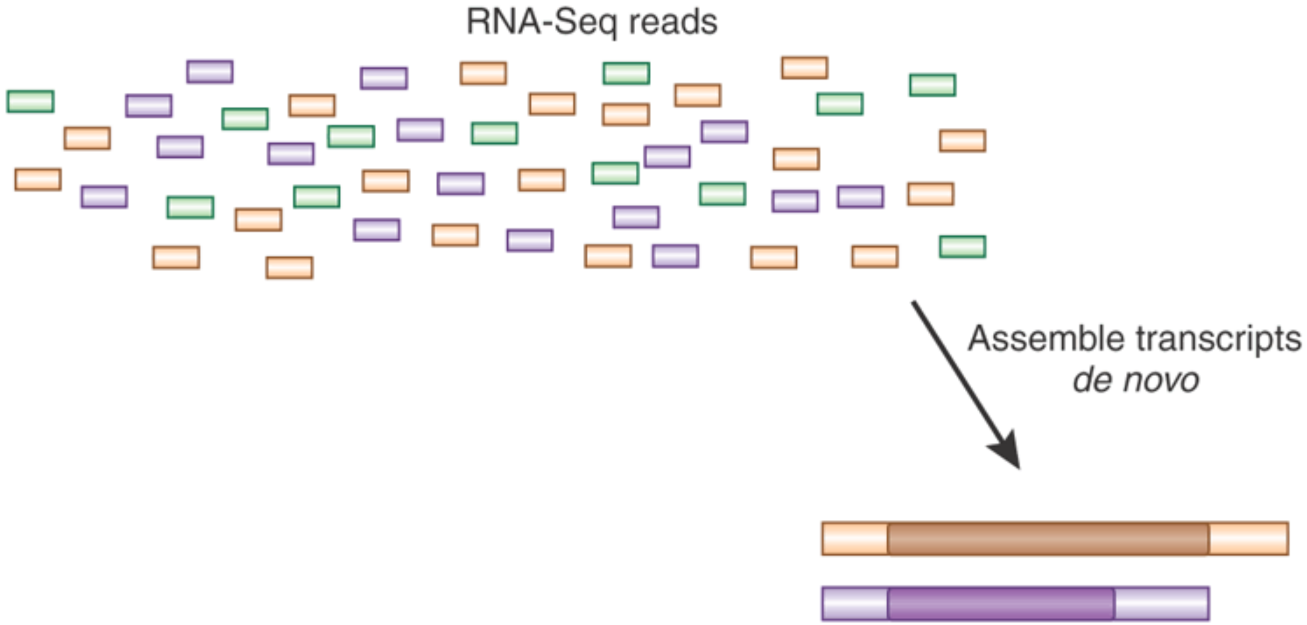
StringTie

Transcript Reconstruction from RNA-Seq Reads

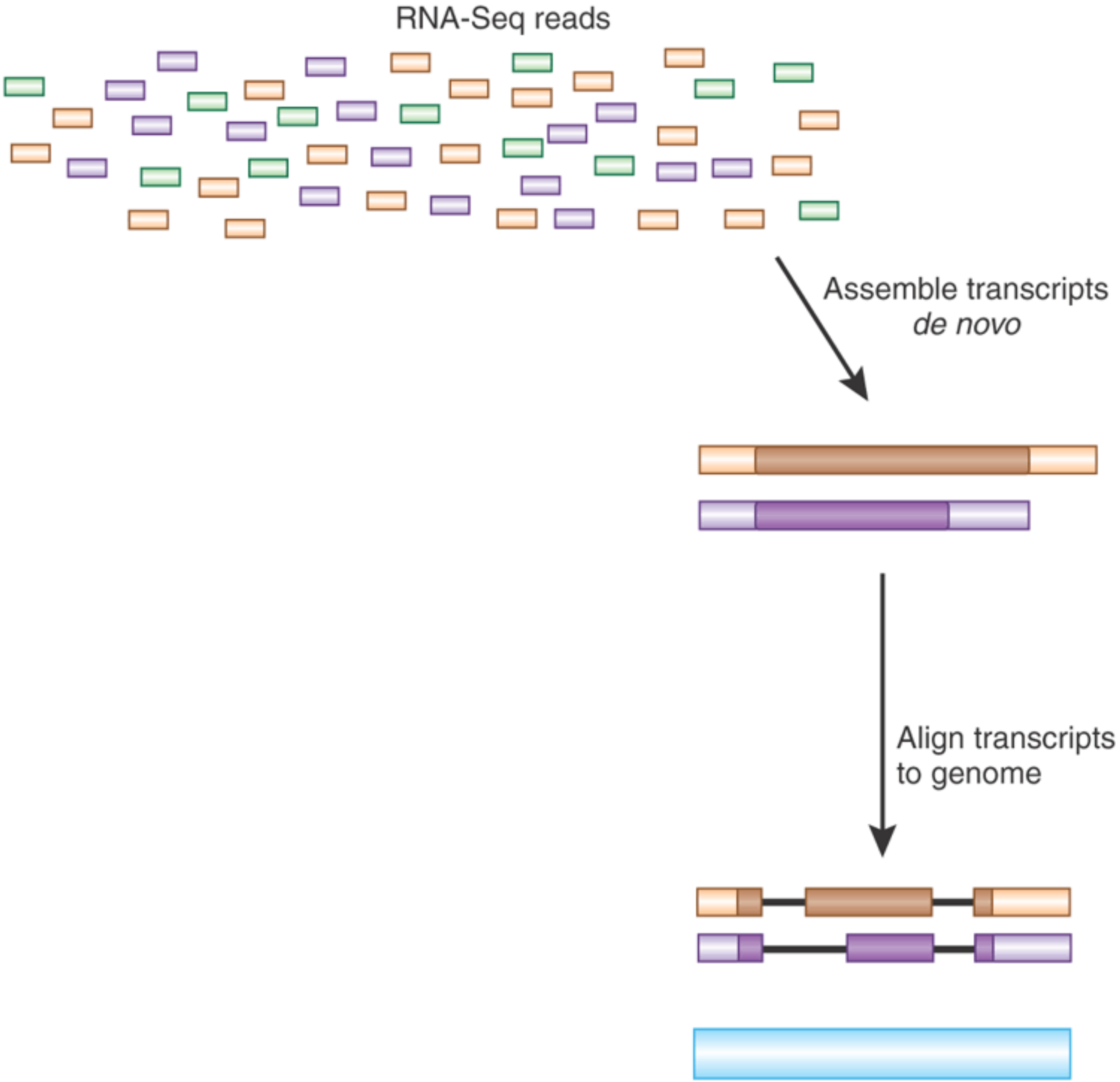


Non-model organisms: "I don't have a reference genome!"

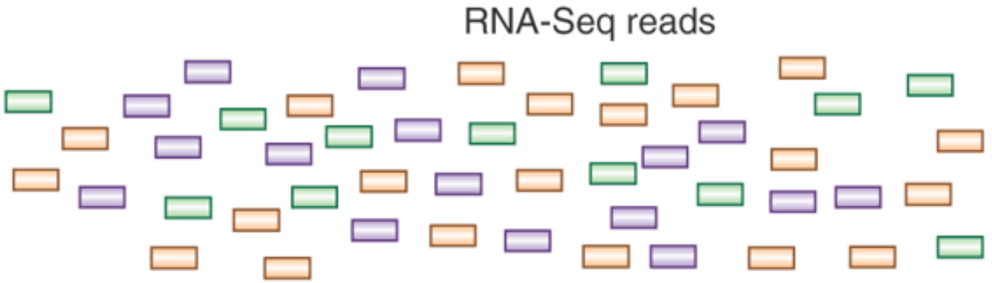
Transcript Reconstruction from RNA-Seq Reads



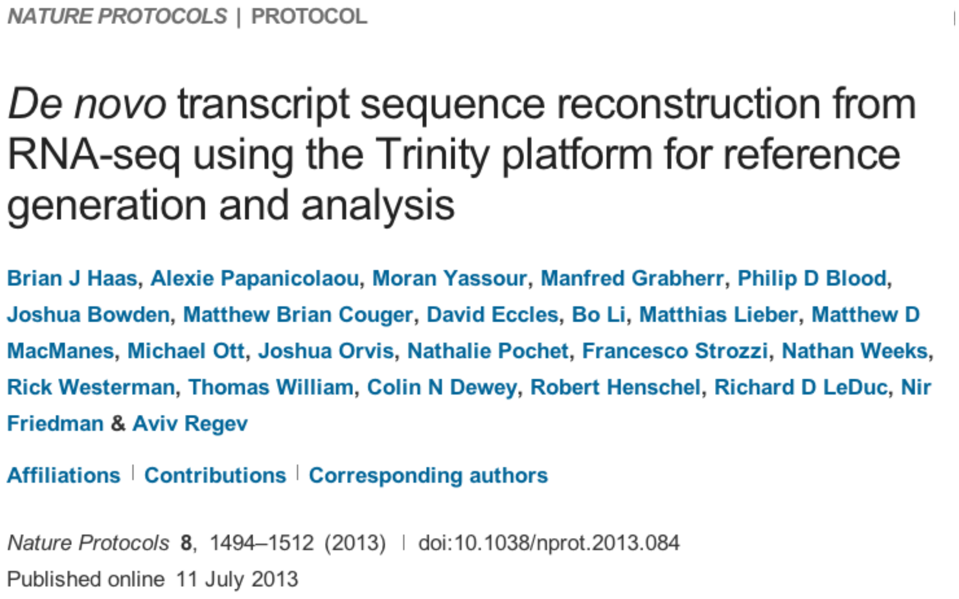
Transcript Reconstruction from RNA-Seq Reads



Transcript Reconstruction from RNA-Seq Reads



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



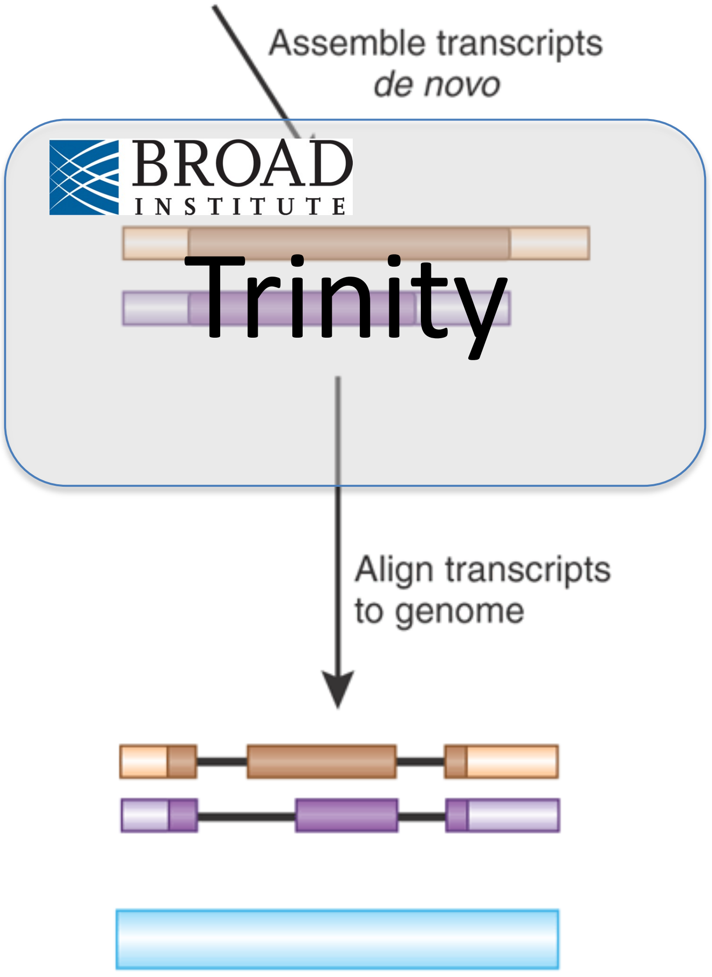
NATURE PROTOCOLS | PROTOCOL

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

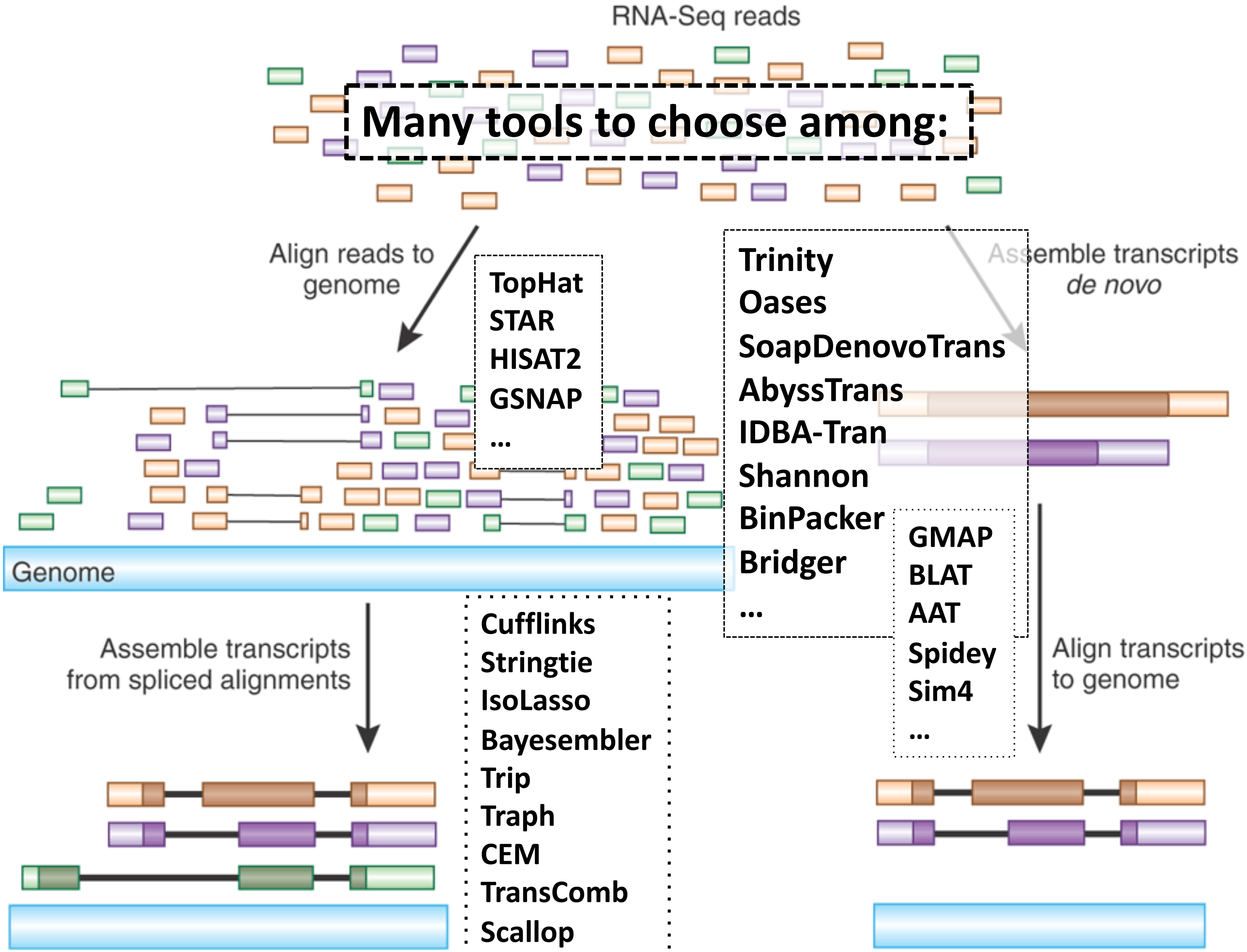
Brian J Haas, Alexie Papanicolaou, Moran Yassour, Manfred Grabherr, Philip D Blood, Joshua Bowden, Matthew Brian Couger, David Eccles, Bo Li, Matthias Lieber, Matthew D MacManes, Michael Ott, Joshua Orvis, Nathalie Pochet, Francesco Strozzi, Nathan Weeks, Rick Westerman, Thomas William, Colin N Dewey,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



Transcript Reconstruction from RNA-Seq Reads


RNA-Seq reads
Align reads to genome
Assemble transcripts *de novo*
Genome
Assemble transcripts from spliced alignments
Align transcripts to genome

Many tools to choose among:

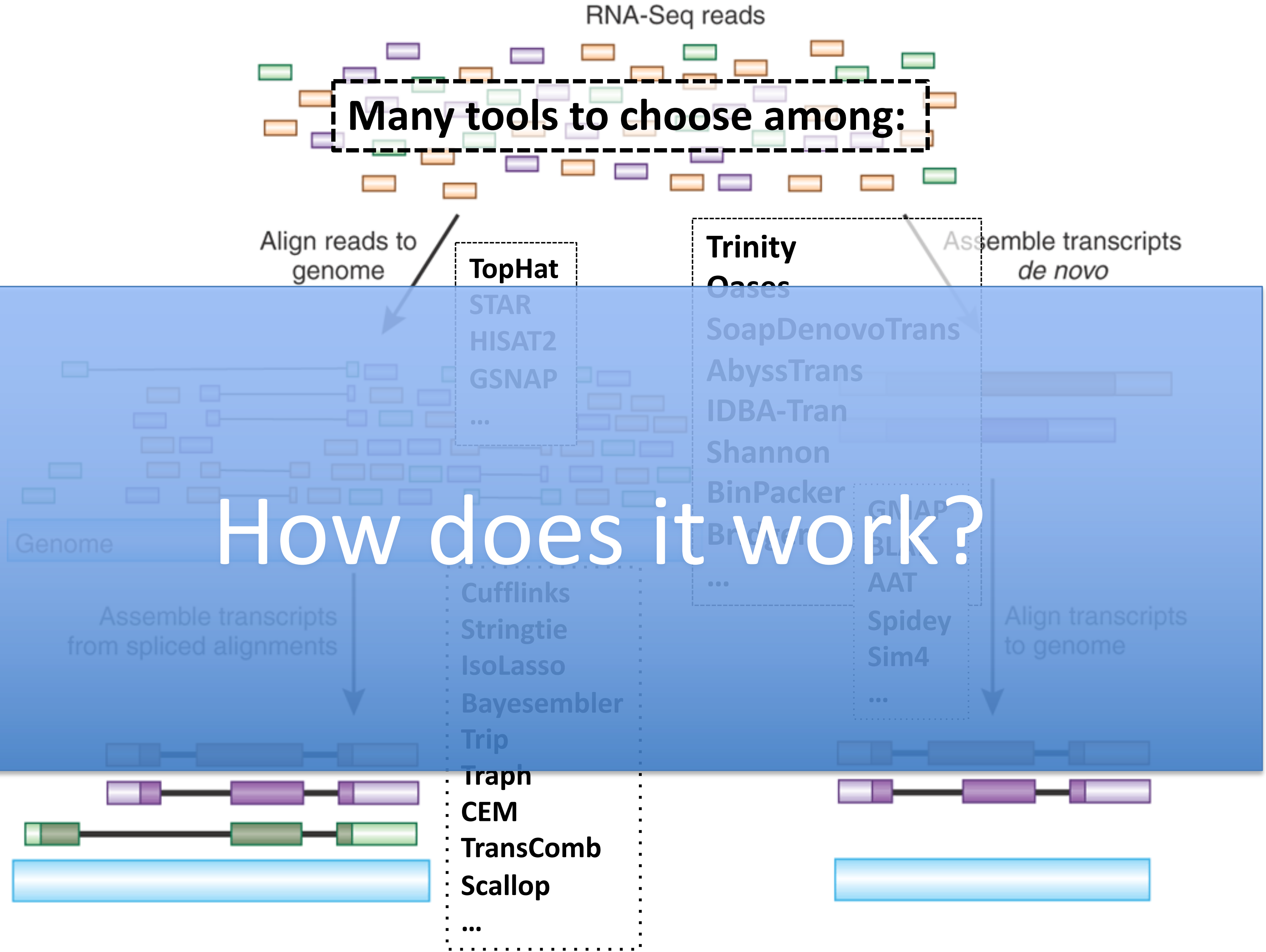
TopHat
STAR
HISAT2
GSNAP
…

Trinity
Oases
SoapDenovoTrans
AbyssTrans
IDBA-Tran
Shannon
BinPacker
Bridger
…

Cufflinks
Stringtie
IsoLasso
Bayesembler
Trip
Traph
CEM
TransComb
Scallop
…

GMAP
BLAT
AAT
Spidey
Sim4
…

Transcript Reconstruction from RNA-Seq Reads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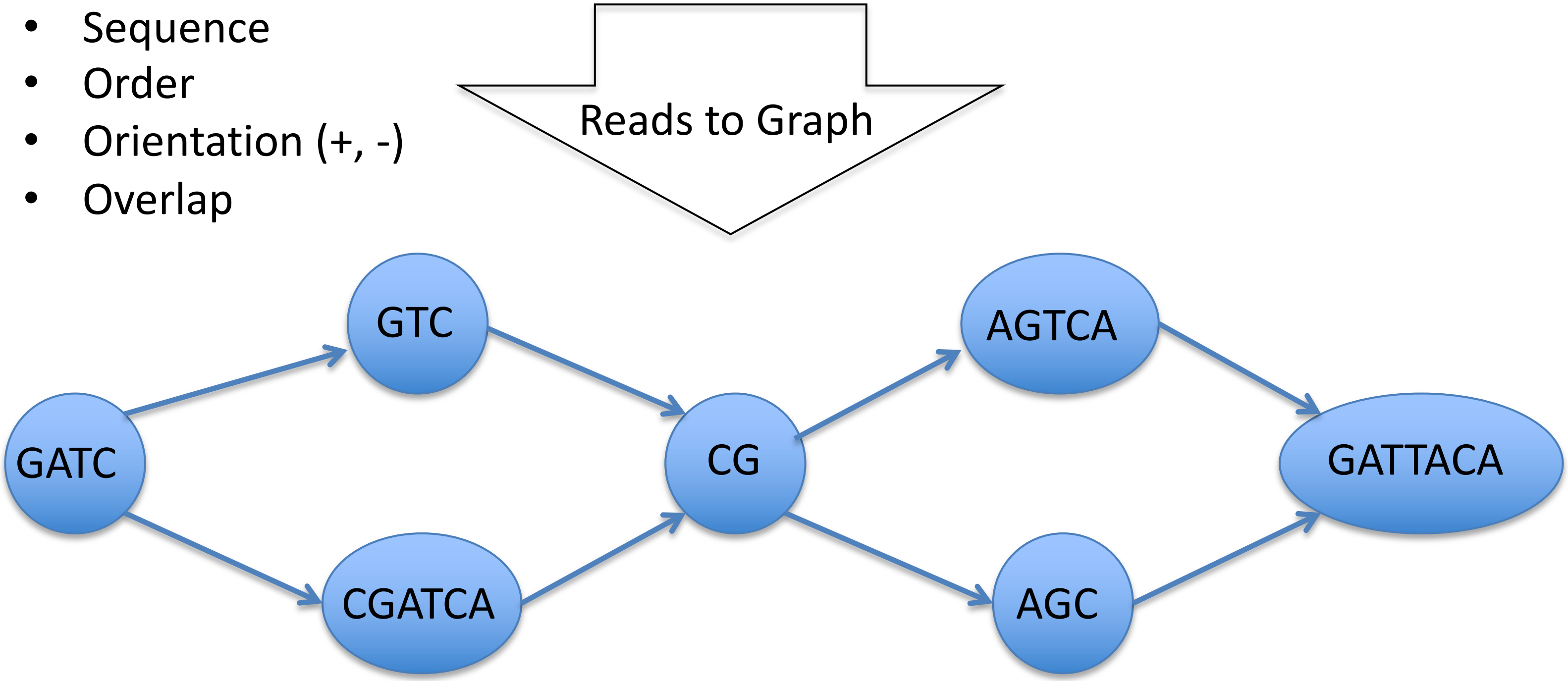
Scallop
…

GMAP
BLAT
AAT
Spidey
Sim4
…

How does it work?

Graph Data Structures Commonly Used For Assembly

- Sequence
- Order
- Orientation (+, -)
- Overlap

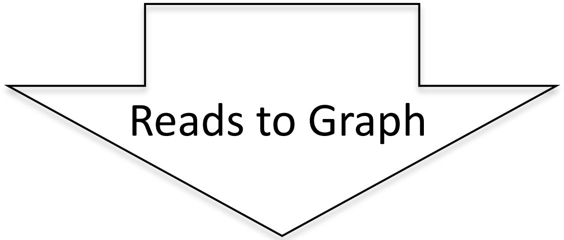


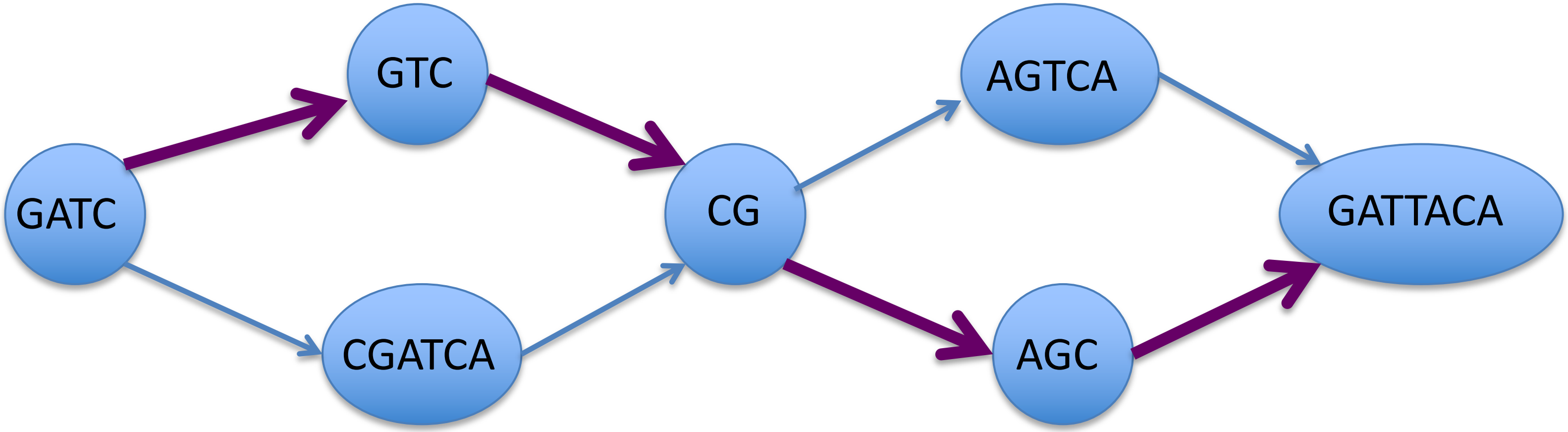
Nodes = sequence (+/-)
Edges = order, overlap

Graph Data Structures Commonly Used For Assembly

RNA-Seq reads

- Sequence
- Order
- Orientation (+, -)
- Overlap



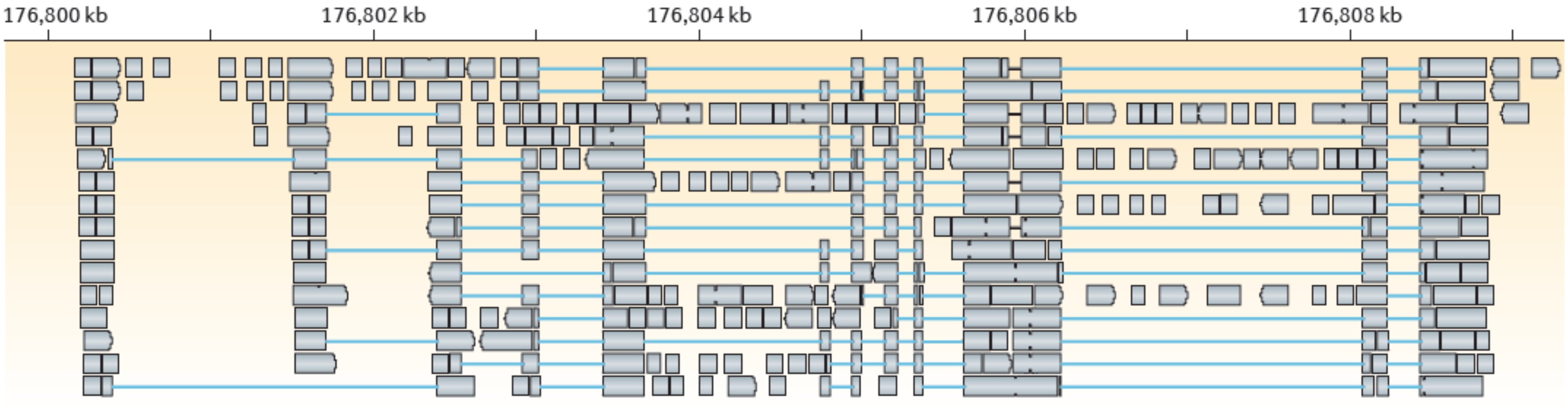


GATCGTCCGAGCGATTACA

Nodes = sequence (+/-)
Edges = order, overlap

Genome-Guided Transcript Reconstruction

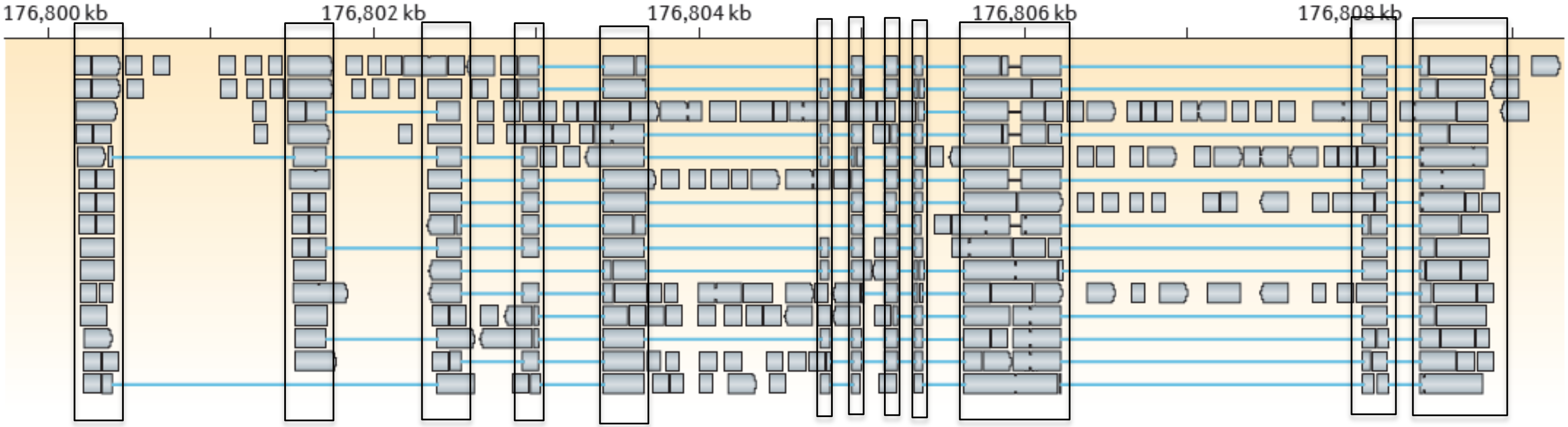
Splice-align reads to the genome



From Martin & Wang. Nature Reviews in Genetics. 2011

Genome-Guided Transcript Reconstruction

Splice-align reads to the genome

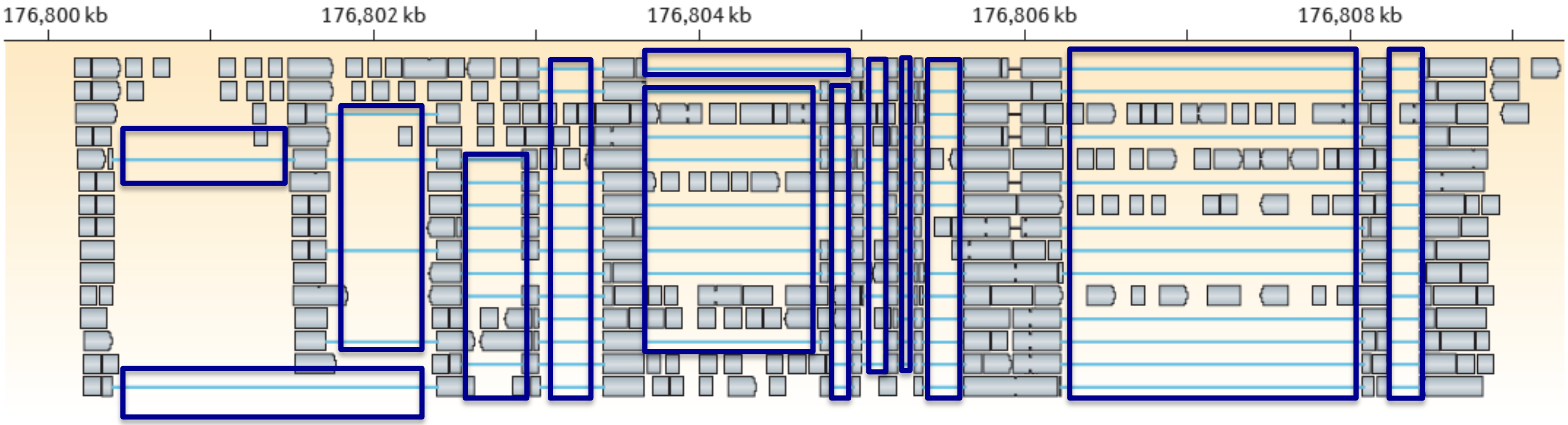


Alignment segment piles => exon regions

From Martin & Wang. Nature Reviews in Genetics. 2011

Genome-Guided Transcript Reconstruction

Splice-align reads to the genome

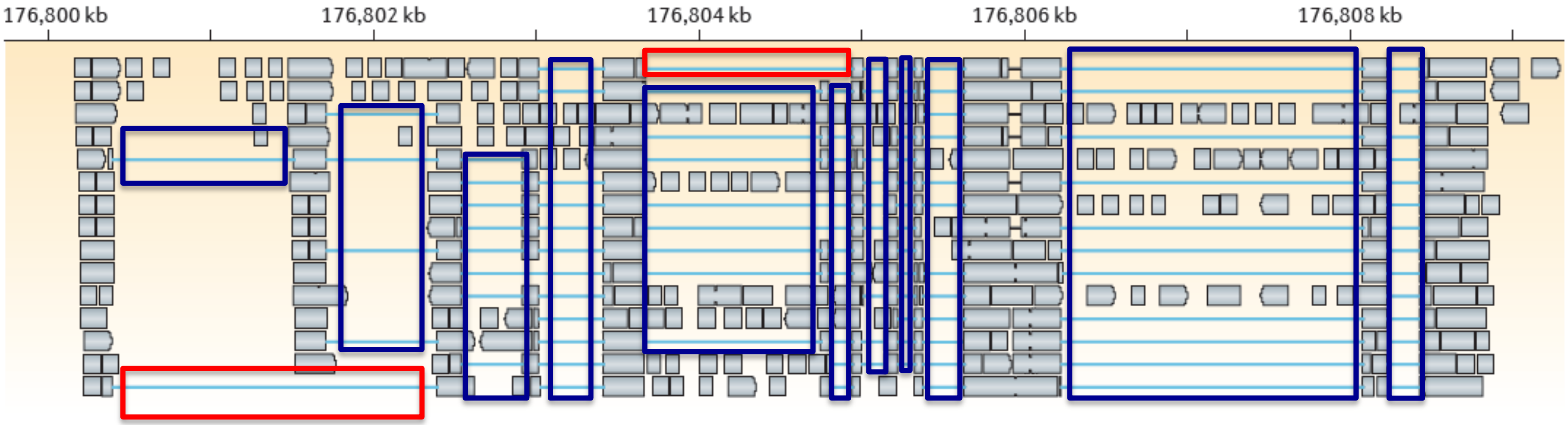


Large alignment gaps => introns

From Martin & Wang. Nature Reviews in Genetics. 2011

Genome-Guided Transcript Reconstruction

Splice-align reads to the genome

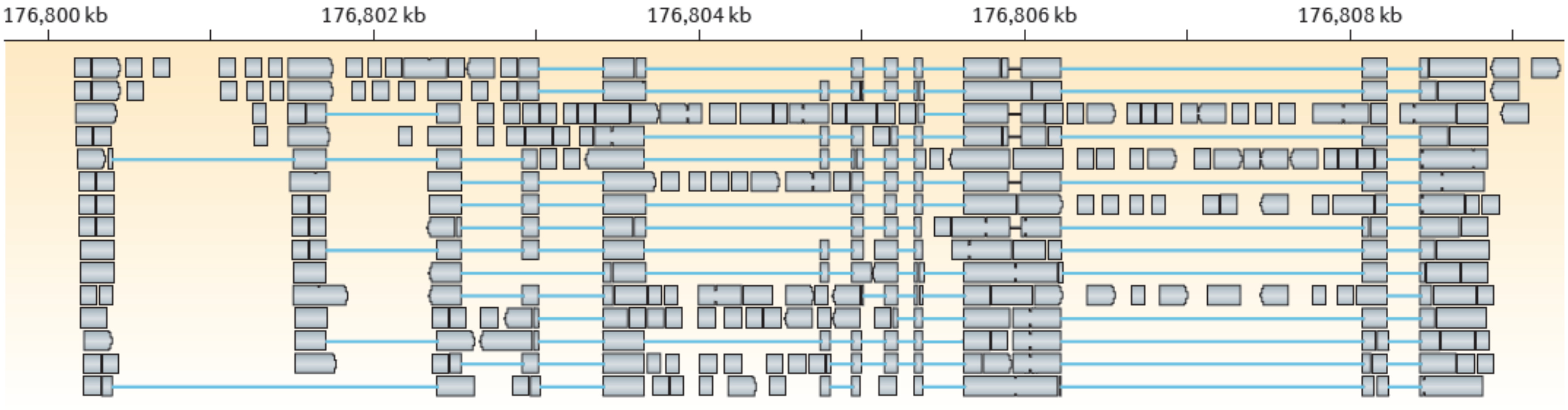


Overlapping but different introns = evidence of alternative splicing

From Martin & Wang. Nature Reviews in Genetics. 2011

Genome-Guided Transcript Reconstruction

Splice-align reads to the genome



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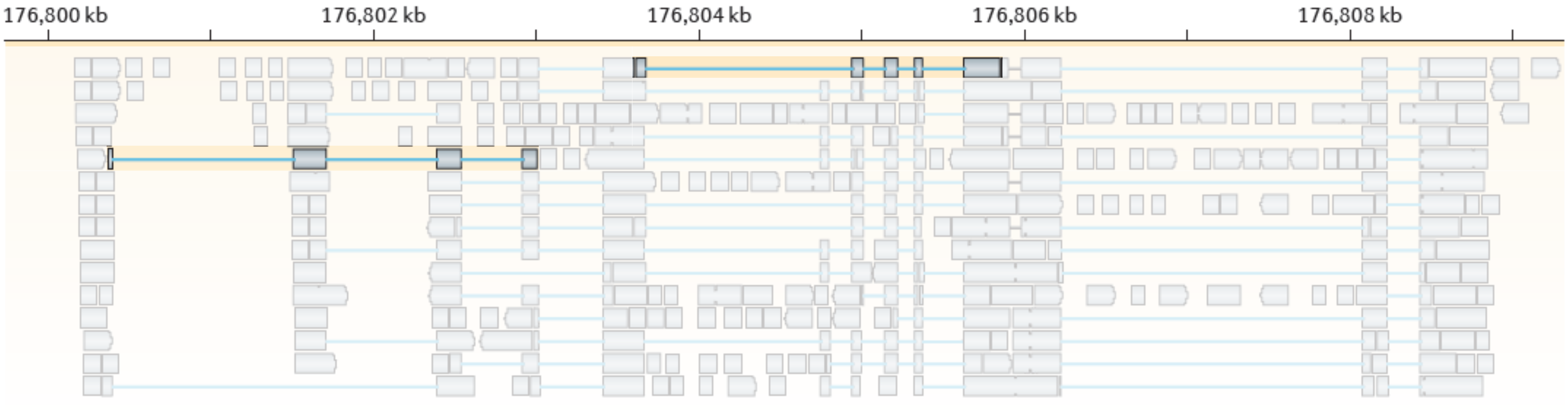
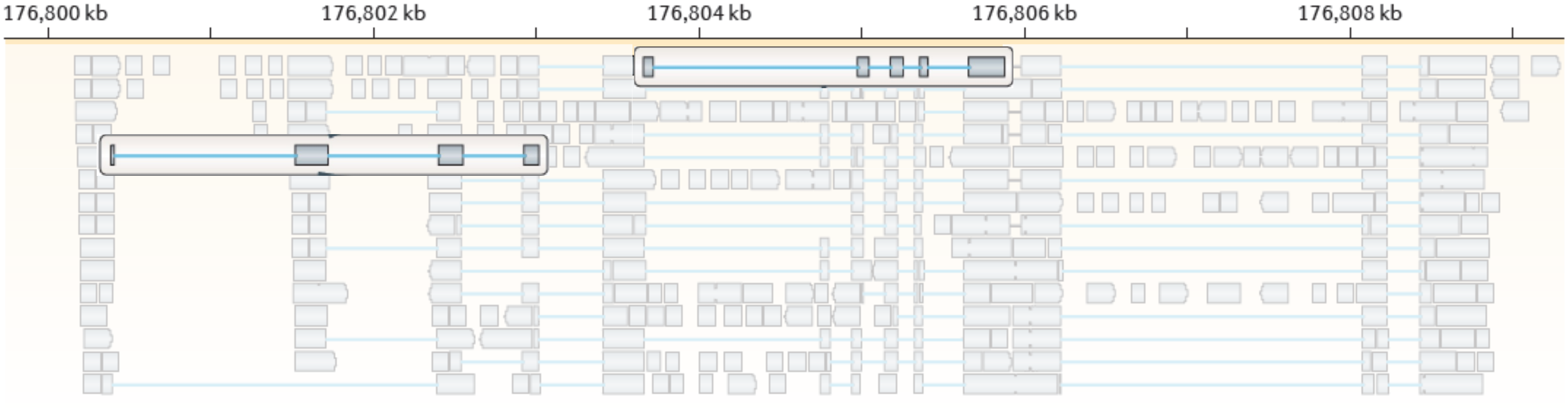


Individual reads can yield multiple exon and intron segments (splice patterns)

From Martin & Wang. Nature Reviews in Genetics. 2011

Genome-Guided Transcript Reconstruction

Splice-align reads to the genome

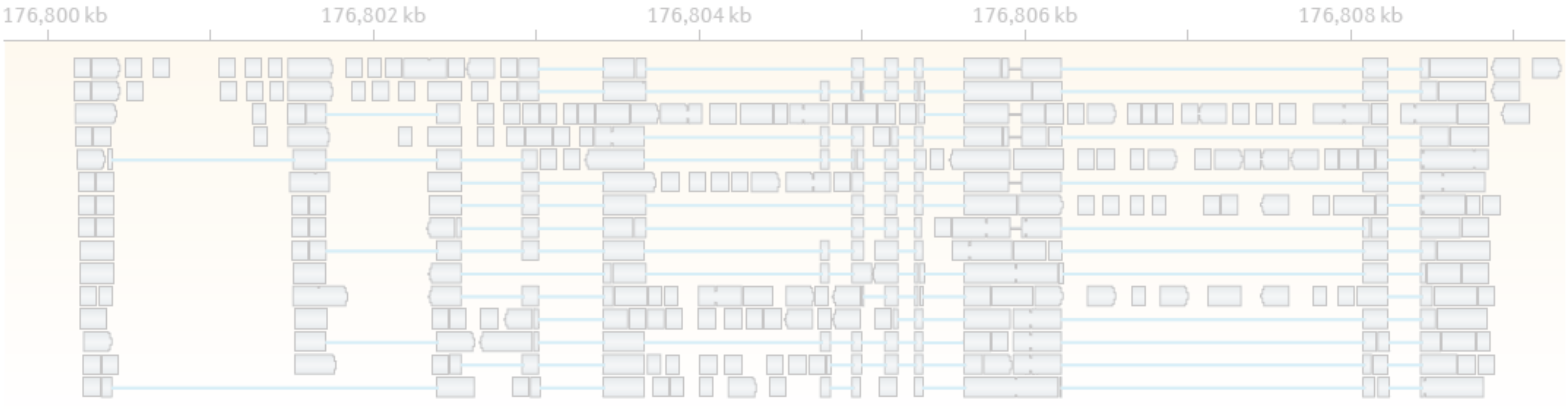


Nodes = unique splice patterns

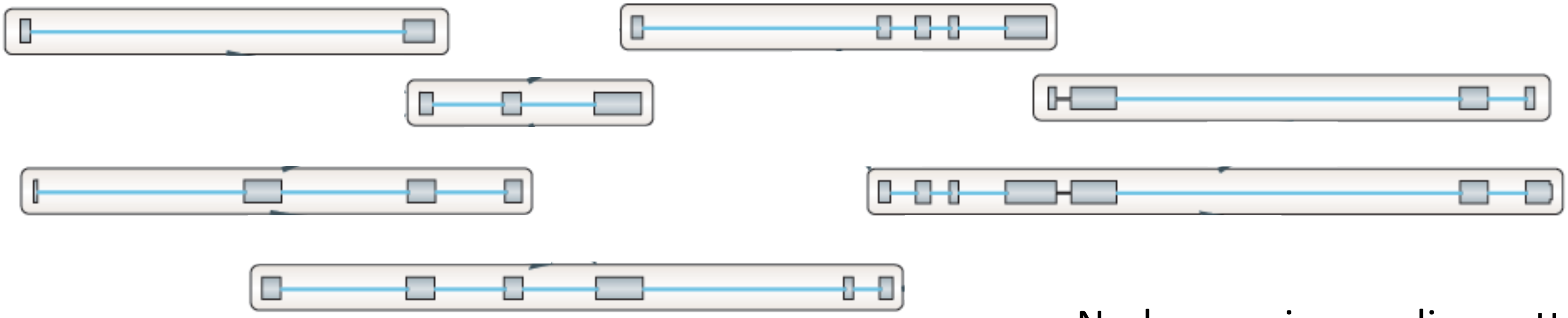
From Martin & Wang. Nature Reviews in Genetics. 2011

Genome-Guided Transcript Reconstruction

Splice-align reads to the genome



Construct graph from unique splice patterns of aligned reads.



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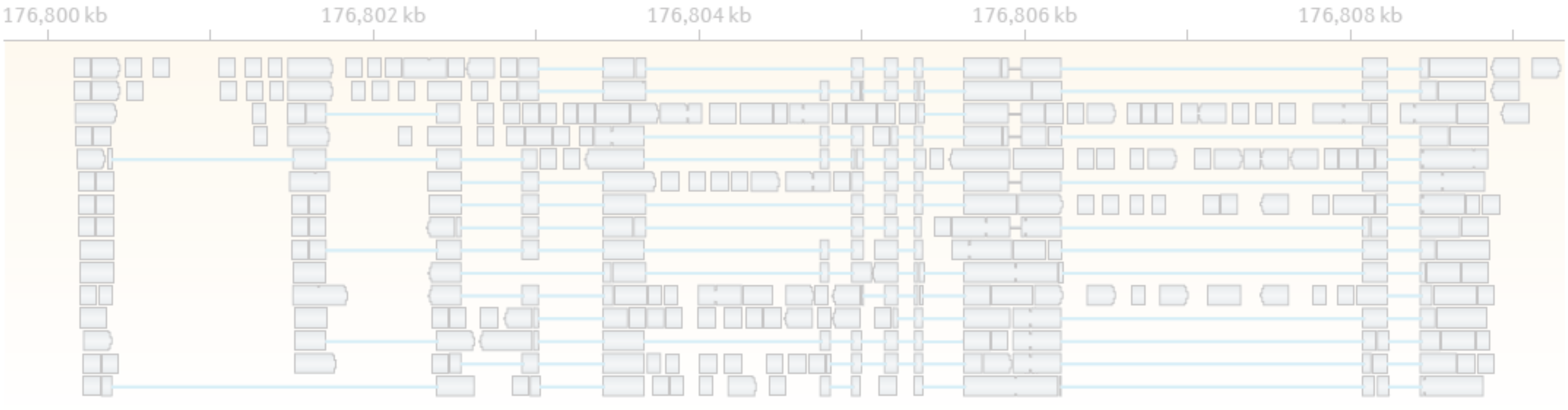

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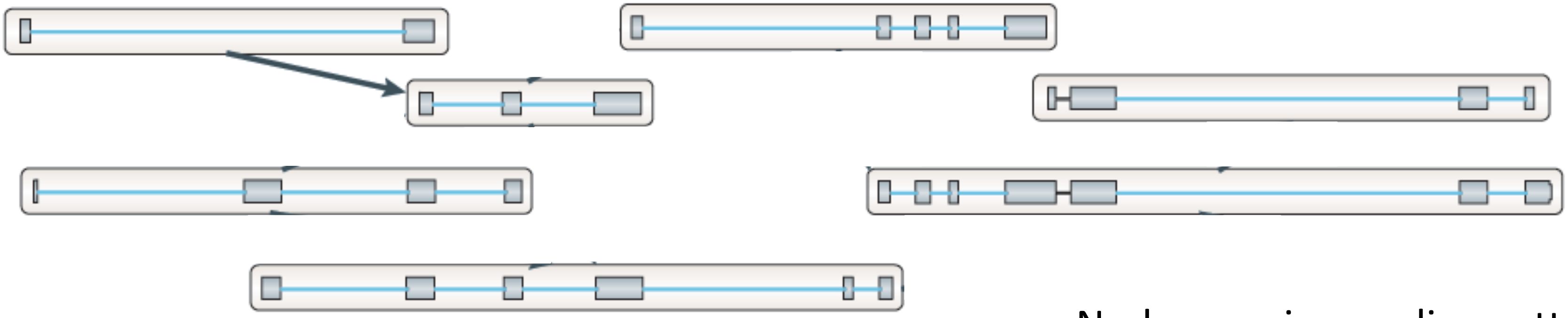
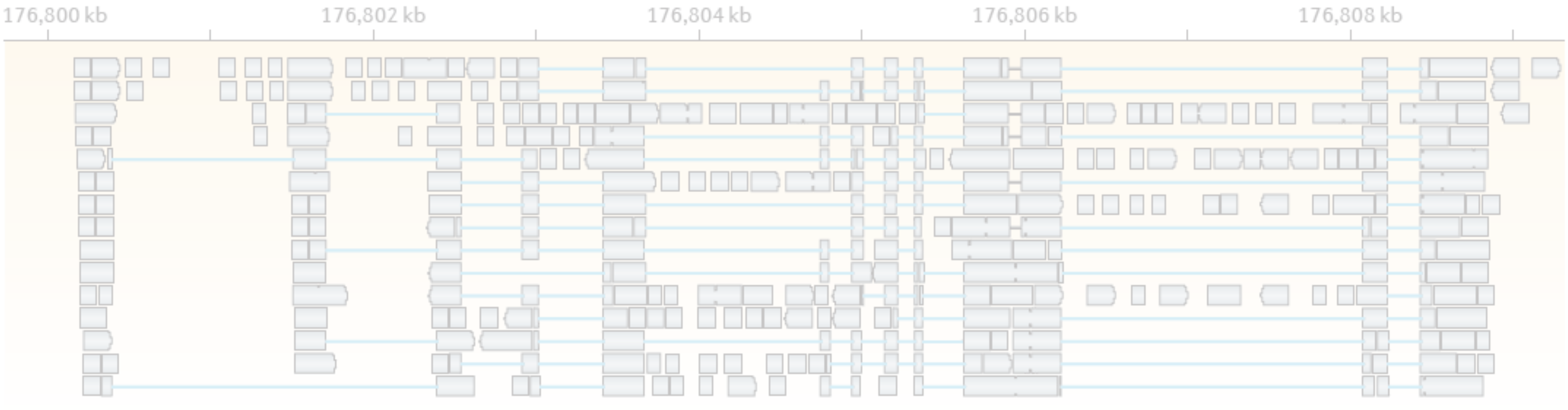


Nodes = unique splice patterns
Edges = compatible patterns

From Martin & Wang. Nature Reviews in Genetics. 2011

Genome-Guided Transcript Reconstruction

Splice-align reads to the genome



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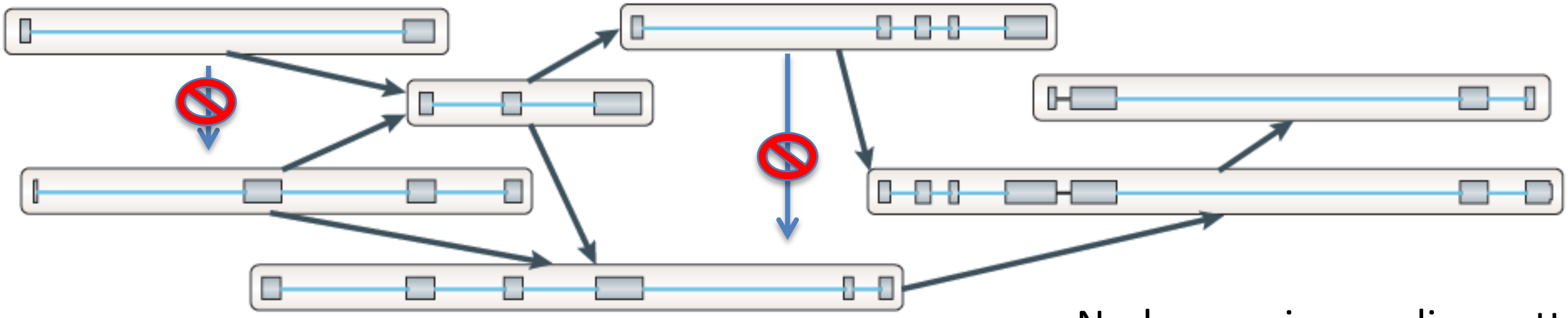

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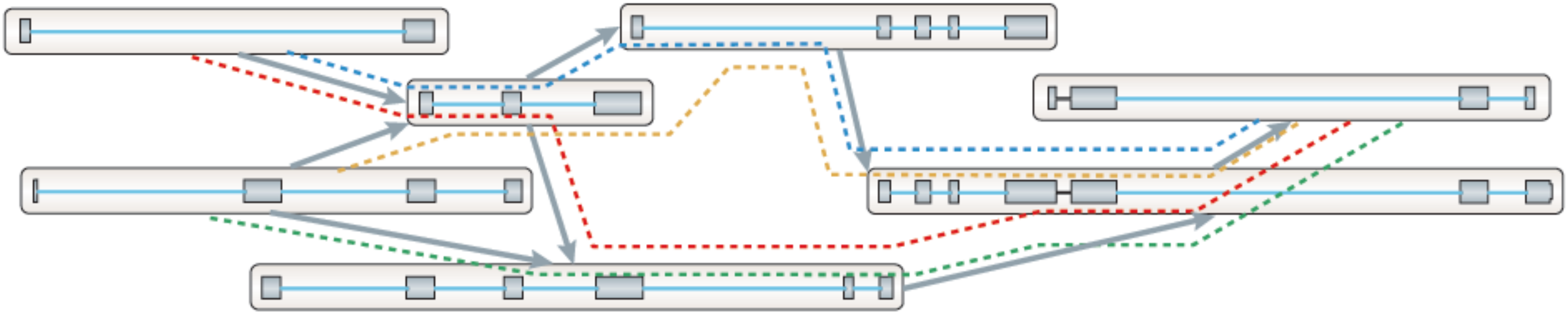
Genome-Guided Transcript Reconstruction

Traverse paths through the graph to assemble transcript isoforms



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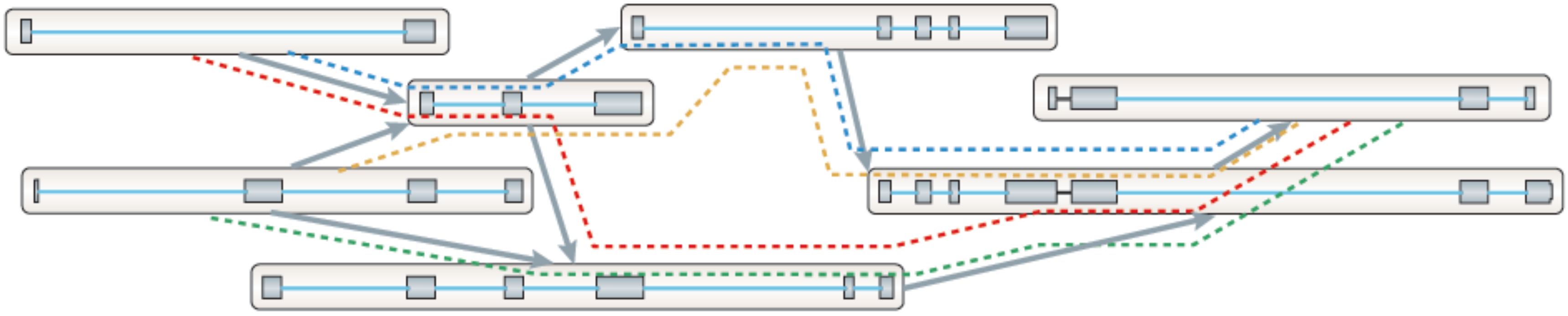
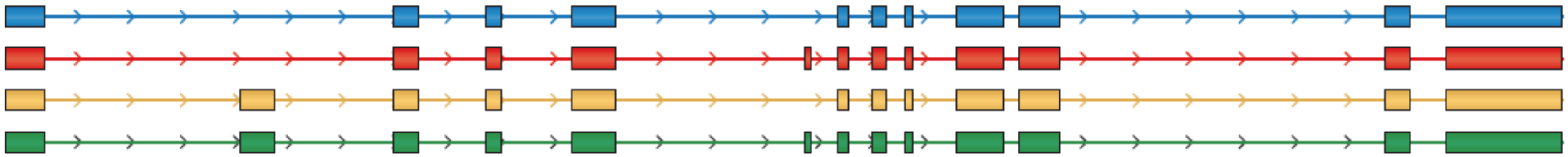
Genome-Guided Transcript Reconstruction

Traverse paths through the graph to assemble transcript isoforms



Reconstructed isoforms

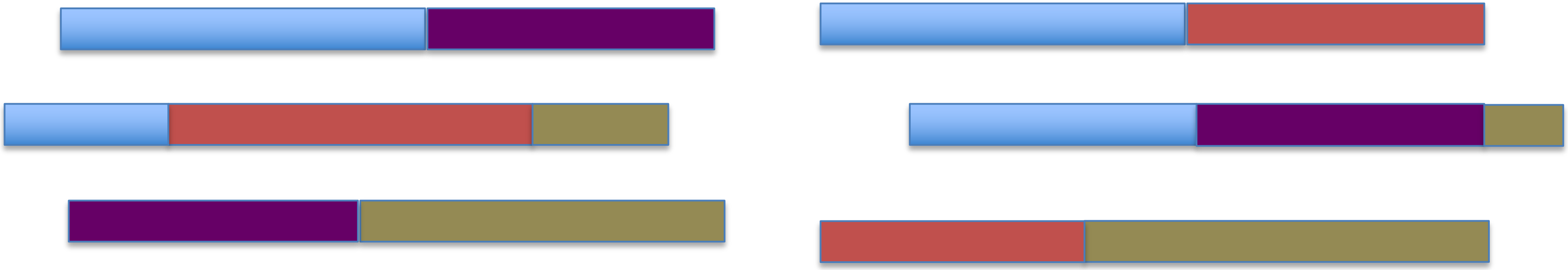


From Martin & Wang. Nature Reviews in Genetics. 2011

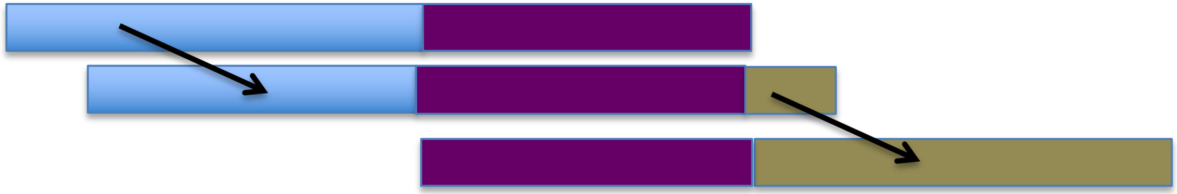
What if you don’t have a high quality reference genome sequence?

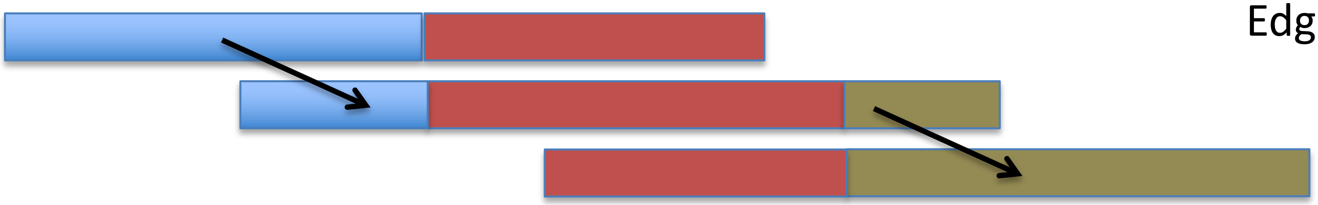
Genome-free de novo transcript reconstruction to the rescue.

Read Overlap Graph: Reads as nodes, overlaps as edges



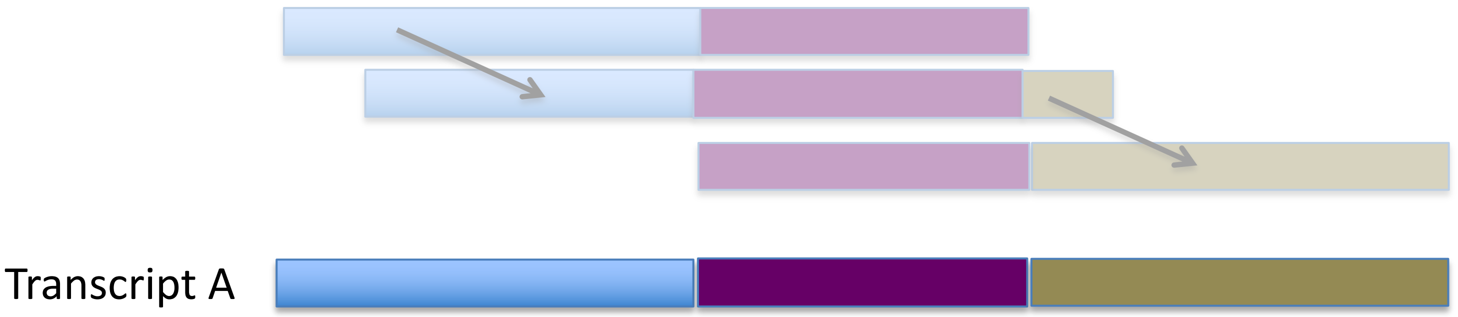
Read Overlap Graph: Reads as nodes, overlaps as edges





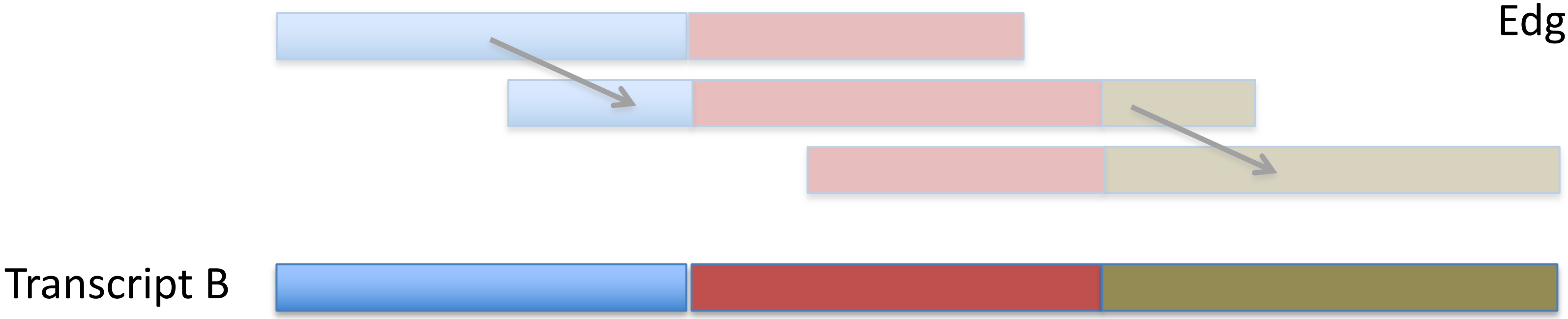
Node = read
Edge = overlap

Read Overlap Graph: Reads as nodes, overlaps as edges

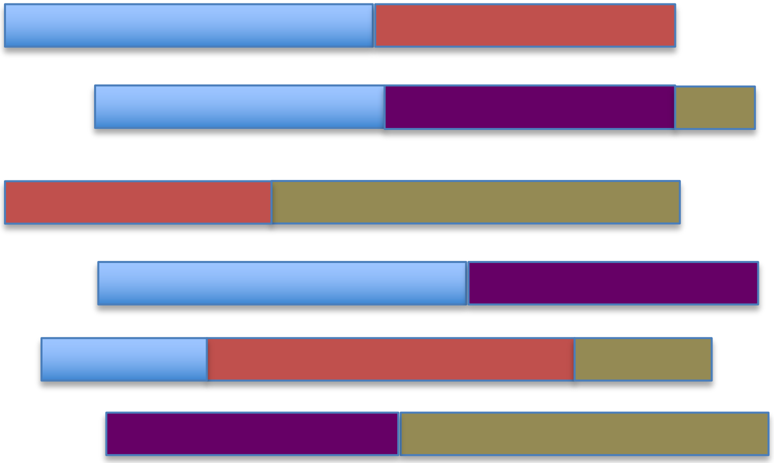


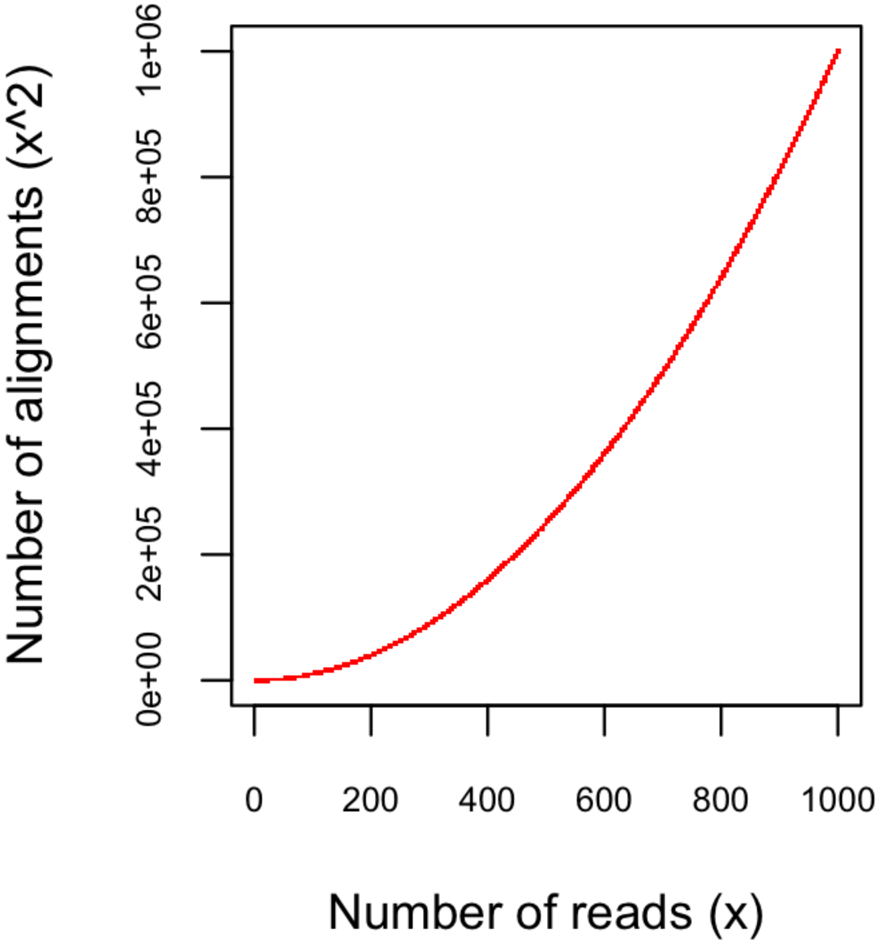
Generate consensus sequence where reads overlap

Node = read
Edge = overlap



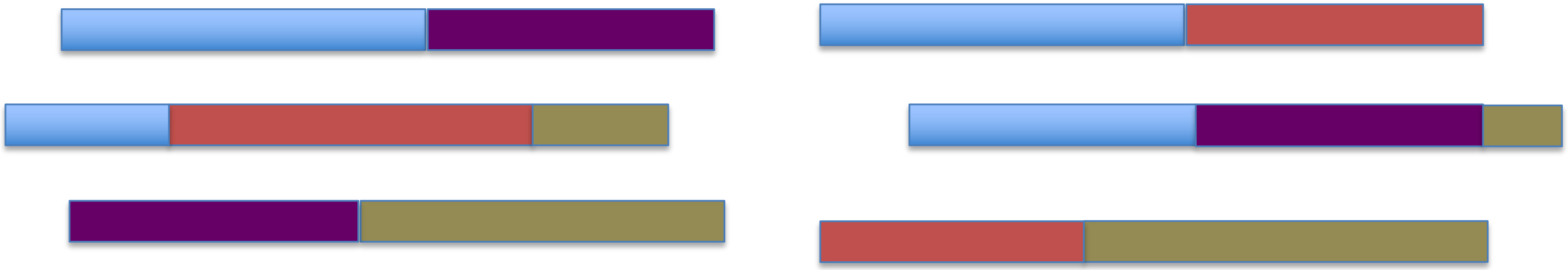
Finding pairwise overlaps between *n* reads involves ~ n^2 comparisons.





Impractical for typical RNA-Seq data (50M reads)

No genome to align to… De novo assembly required



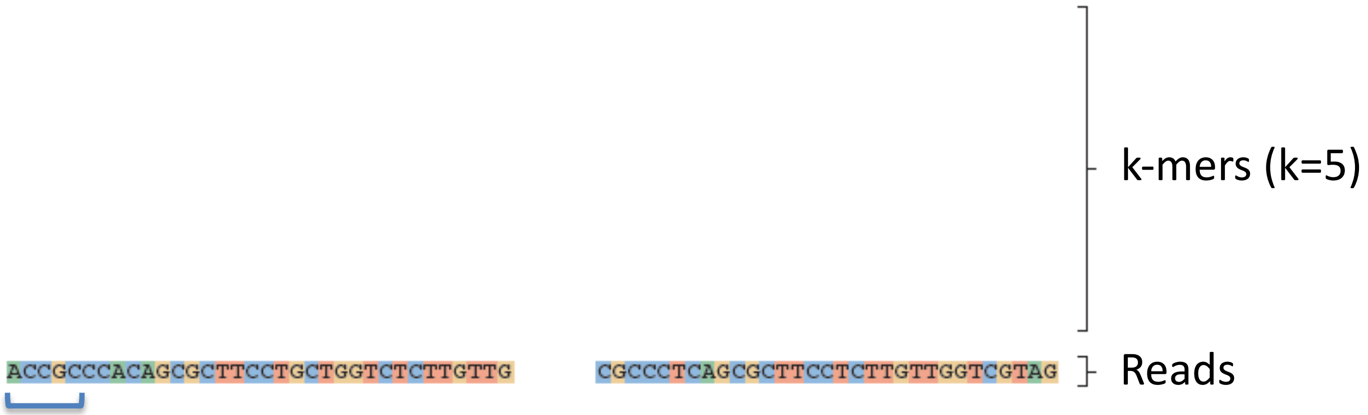
Want to avoid ***n***^{2} read alignments to define overlaps

Use a de Bruijn graph

Have you learned about the de Bruijn graph already?

Sequence Assembly via de Bruijn Graphs

Generate all substrings of length k from the reads


ACCGCCCACAGCGCTTCCTGCTGGTCTCTTGTTG
CGCCCTCAGCGCTTCCTCTTGTTGGTCGTAG

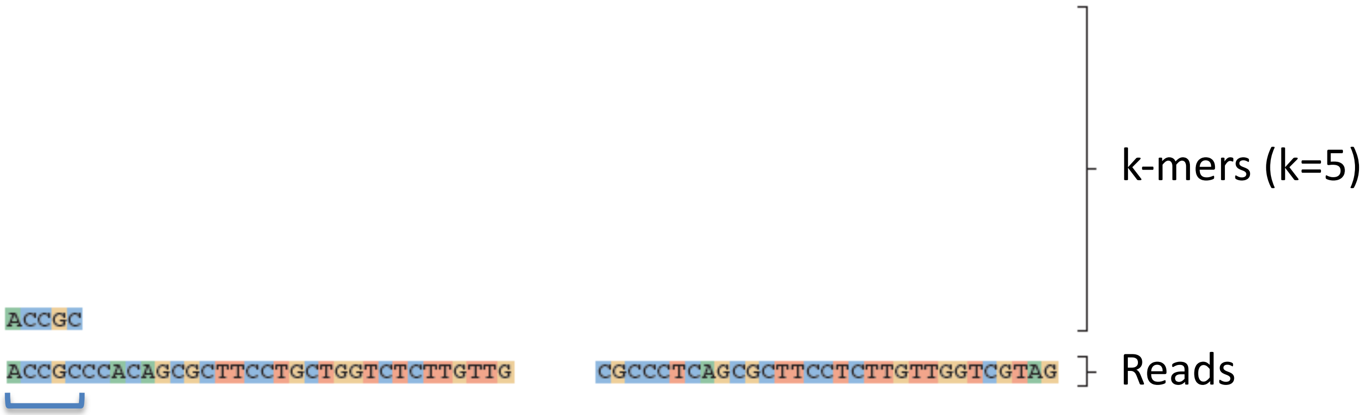
k-mers (k=5)

Reads

From Martin & Wang, Nat. Rev. Genet. 2011

Sequence Assembly via De Bruijn Graphs

Generate all substrings of length k from the reads



k-mers (k=5)
ACCGC
ACCGCCCACAGCGCTTCCTGCTGGTCTCTTGTTG
CGCCCTCAGCGCTTCCTCTTGTTGGTCGTAG
Reads

From Martin & Wang, Nat. Rev. Genet. 2011

Sequence Assembly via De Bruijn Graphs

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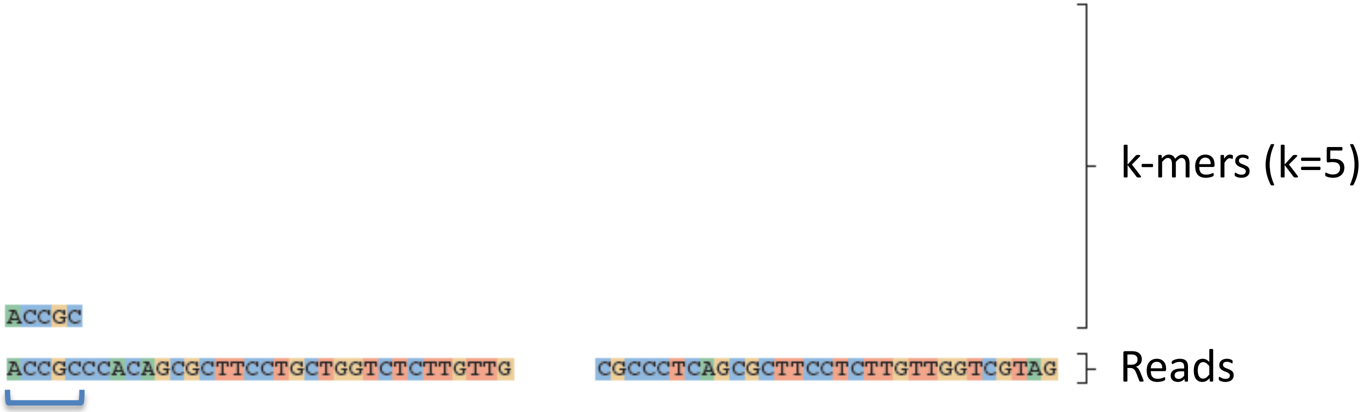


k-mers (k=5)

ACCGC

ACCGCCCACAGCGCTTCCTGCTGGTCTCTTGTTG   CGCCCTCAGCGCTTCCTCTTGTTGGTCGTAG

Reads

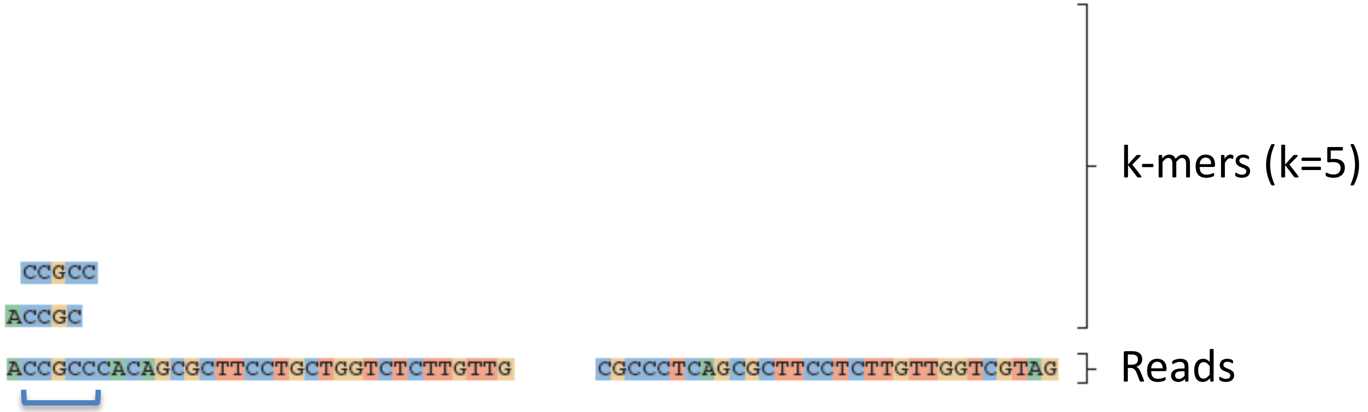
Construct the de Bruijn graph

Nodes = unique k-mers

From Martin & Wang, Nat. Rev. Genet. 2011

Sequence Assembly via De Bruijn Graphs

Generate all substrings of length k from the reads



CCGCC

ACCGC

ACCGCCCACAGCGCTTCCTGCTGGTCTCTTGTTG CGCCCTCAGCGCTTCCTCTTGTTGGTCGTAG

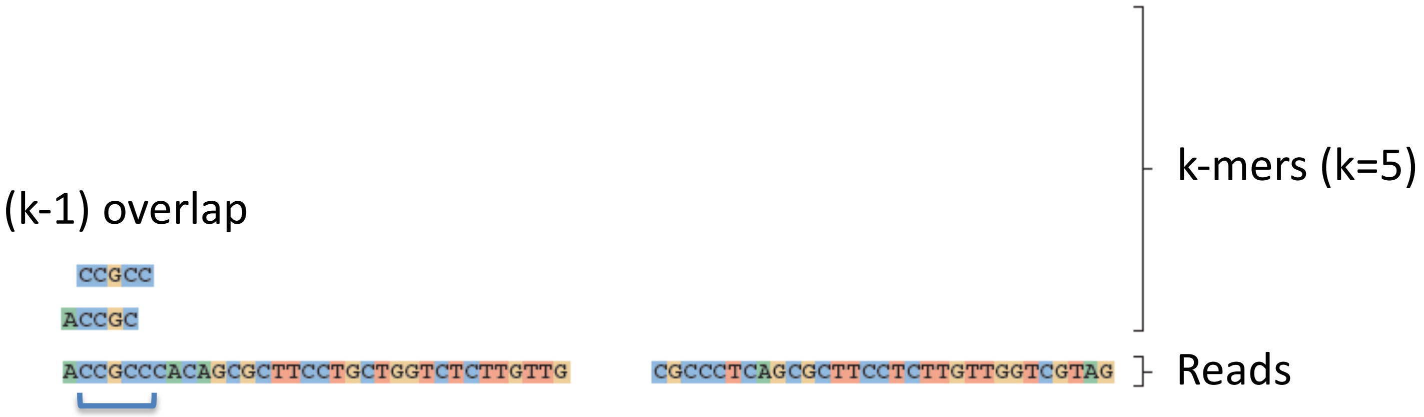
Construct the de Bruijn graph

From Martin & Wang, Nat. Rev. Genet. 2011

Nodes = unique k-mers
Edges = overlap by (k-1)

Sequence Assembly via De Bruijn Graphs

Generate all substrings of length k from the reads


k-mers (k=5)
(k-1) overlap
CCGCC
ACCGC
ACCGCCCACAGCGCTTCCTGCTGGTCTCTTGTTG
CGCCCTCAGCGCTTCCTCTTGTTGGTCGTAG
Reads

Construct the de Bruijn graph

ACCGC
CCGCC

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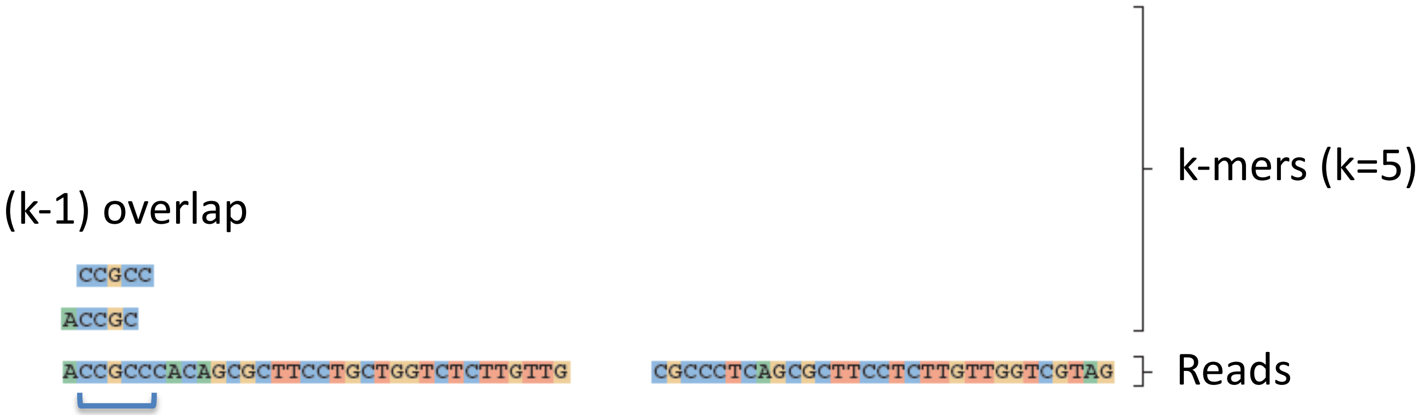
Edges = overlap by (k-1)

From Martin & Wang, Nat. Rev. Genet. 2011

Sequence Assembly via De Bruijn Graphs

Generate all substrings of length k from the reads

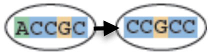

CCGCC
ACCGC
ACCGCCCACAGCGCTTCCTGCTGGTCTCTTGTTG
CGCCCTCAGCGCTTCCTCTTGTTGGTCGTAG

(k-1) overlap

k-mers (k=5)

Reads

Construct the de Bruijn graph



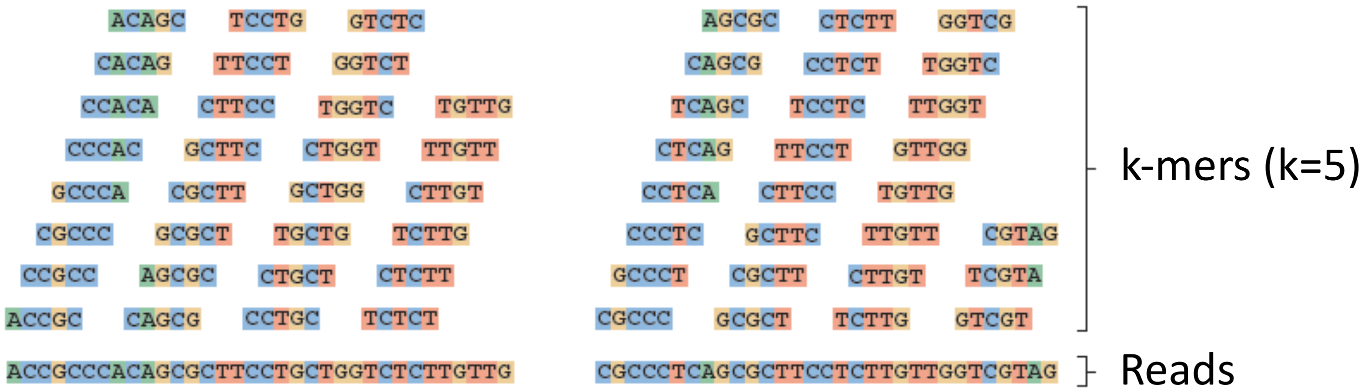
Nodes = unique k-mers

Edges = overlap by (k-1)

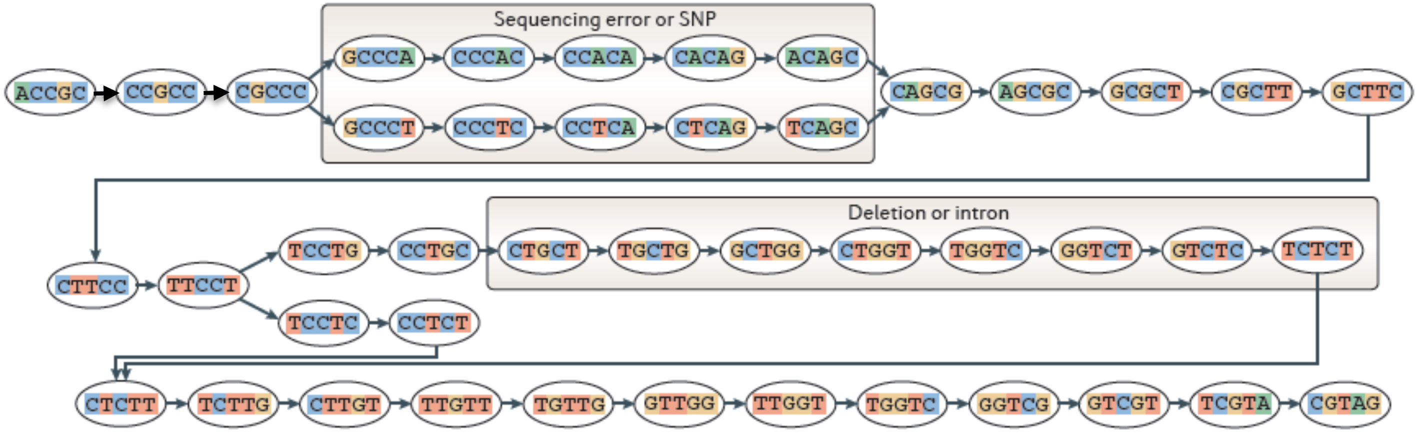
From Martin & Wang, Nat. Rev. Genet. 2011

Sequence Assembly via De Bruijn Graphs

Generate all substrings of length k from the reads



Construct the de Bruijn graph



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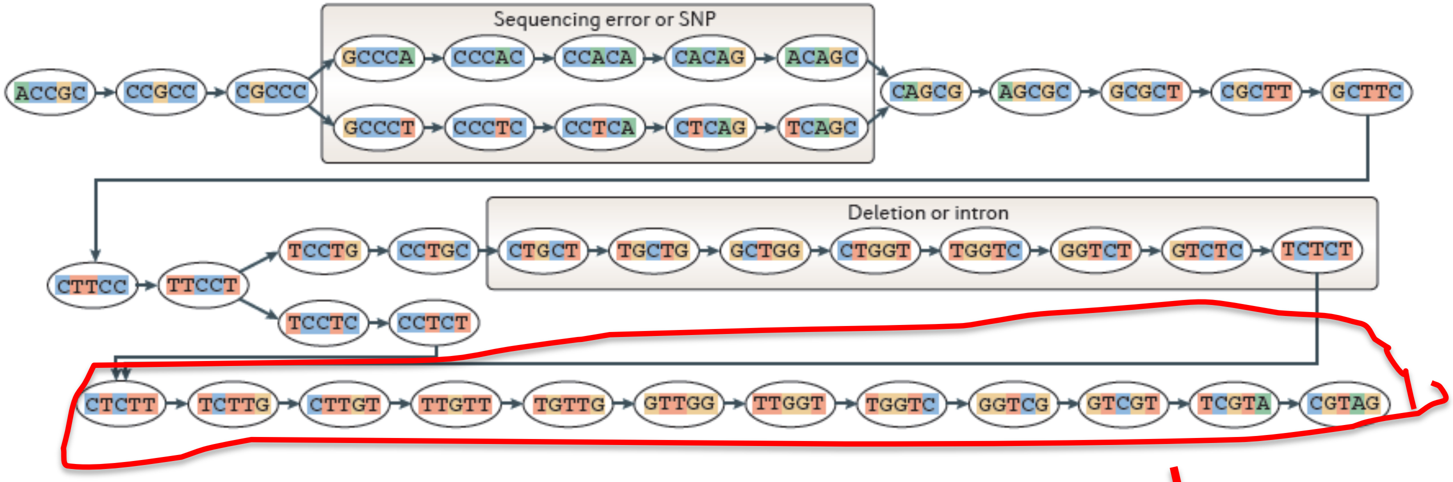
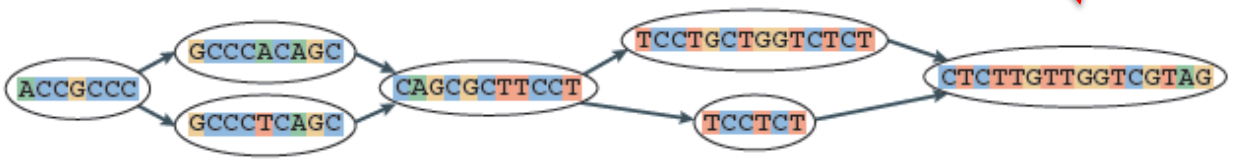
From Martin & Wang, Nat. Rev. Genet. 2011

Construct the de Bruijn graph

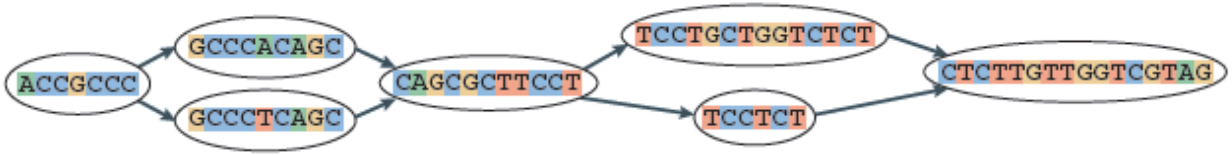


Collapse the de Bruijn graph

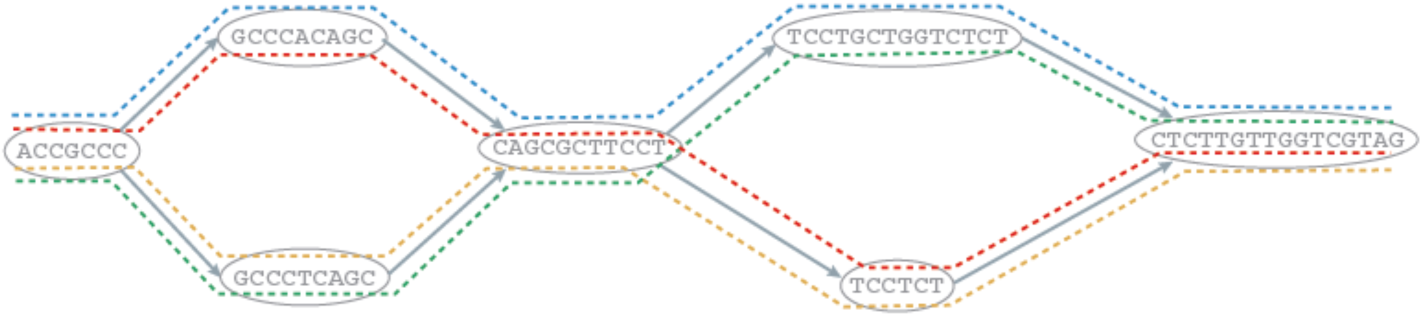


From Martin & Wang, Nat. Rev. Genet. 2011

Collapse the de Bruijn graph



Traverse the graph

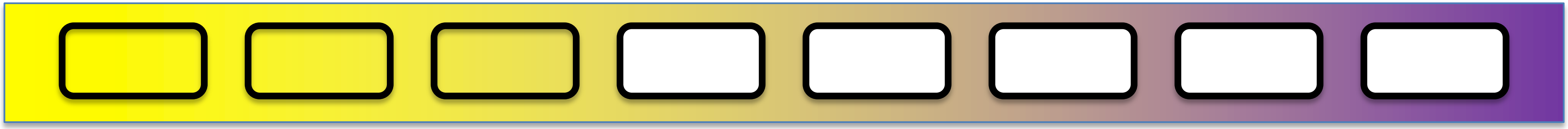


Assemble Transcript Isoforms

```
------ ACCGCCCACAGCGCTTCCTGCTGGTCTCTTGTTGGTCGTAG
------ ACCGCCCACAGCGCTTCCT--------CTTGTTGGTCGTAG
------ ACCGCCCTCAGCGCTTCCT--------CTTGTTGGTCGTAG
------ ACCGCCCTCAGCGCTTCCTGCTGGTCTCTTGTTGGTCGTAG
```

From Martin & Wang, Nat. Rev. Genet. 2011

Part 3. Trinity De novo Assembly



Contrasting Genome and Transcriptome Assembly

Genome Assembly

- Uniform coverage
- Single contig per locus
- Double-stranded

Transcriptome Assembly

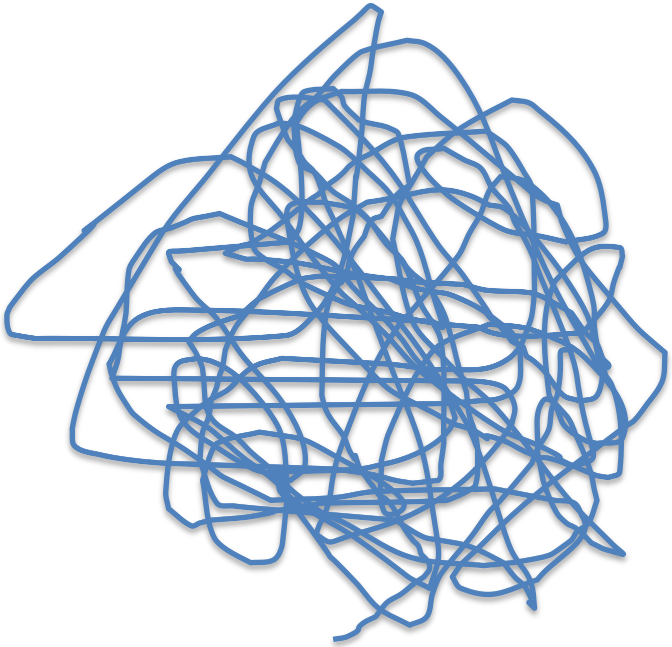
- Exponentially distributed coverage levels
- Multiple contigs per locus (alt splicing)
- Strand-specific

Trinity

Trinity Aggregates Isolated Transcript Graphs

Genome Assembly

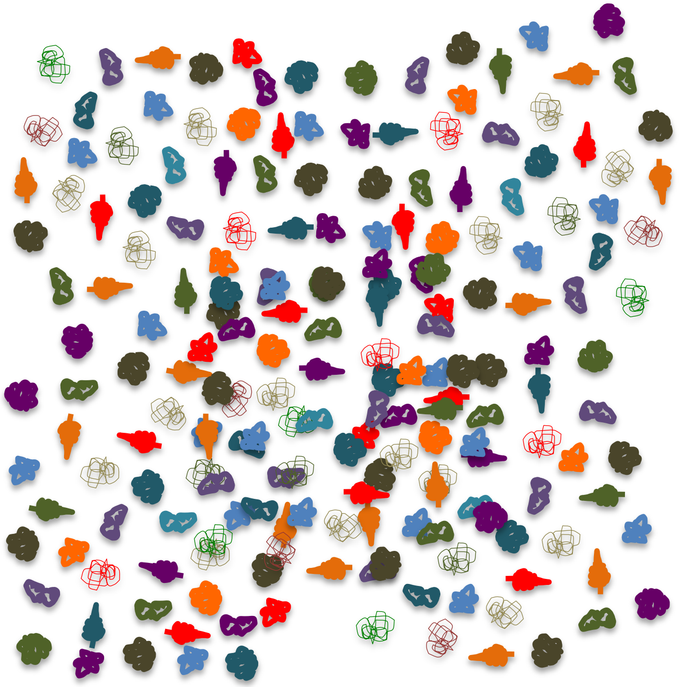
Single Massive Graph



Entire chromosomes represented.

Trinity Transcriptome Assembly

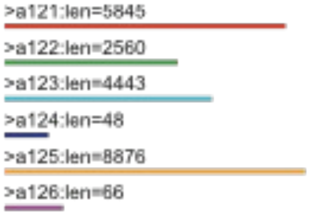
Many Thousands of Small Graphs



Ideally, one graph per expressed gene.

Trinity – How it works:

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



>a121:len=5845

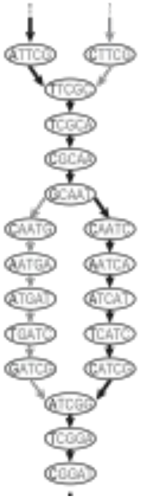
>a122:len=2560

>a123:len=4443

>a124:len=48

>a125:len=8876

>a126:len=66



Thousands of disjoint graphs

...CTTCGCAA...TGATCGGAT...

...ATTCGCAA...TCATCGGAT...

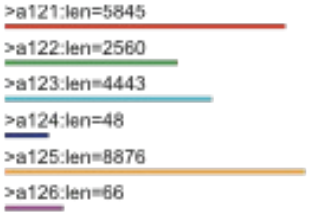
Trinity – How it works:

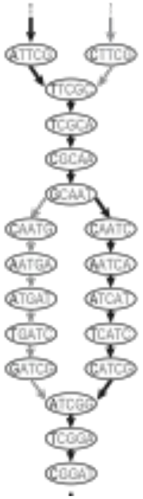
Younger
me

Manfred
Grabherr

Moran
Yassour

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





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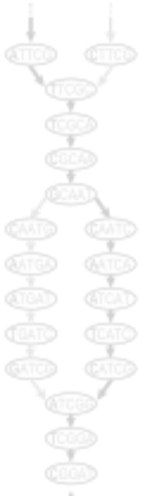
>a126:len=66



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

Thousands of disjoint graphs

Inchworm Algorithm

- Decompose all reads into overlapping Kmers => hashtable(kmer, count)

Read: **AATGTGAAAACTGGATTACATGCTGGTATGTC…**

```
AATGTGA
  ATGTGAA        Overlapping kmers of length (k)
    TGTGAAA
       …
```

Kmer Catalog (hashtable)

Kmer	Count among all reads
AATGTGA	4
ATGTGAA	2
TGTGAAA	1
GATTACA	9

Inchworm Algorithm

- Decompose all reads into overlapping Kmers => hashtable(kmer, count)
- Identify seed kmer as most abundant Kmer, ignoring low-complexity kmers.

ETHAN HAWKE UMA THURMAN JUDE LAW
FROM ANDREW NICCOL, WRITER OF "THE TRUMAN SHOW"
GATTACA
THERE IS NO GENE FOR THE HUMAN SPIRIT
DVD

https://en.wikipedia.org/wiki/Gattaca

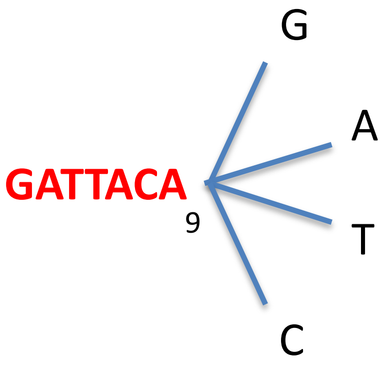
GATTACA$_{9}$

Kmer Catalog (hashtable)

Kmer	Count among all reads
AATGTGA	4
ATGTGAA	2
TGTGAAA	1
GATTACA	9

Inchworm Algorithm

- Decompose all reads into overlapping Kmers => hashtable(kmer, count)
- Identify seed kmer as most abundant Kmer, ignoring low-complexity kmers.
- Extend kmer at 3’ end, guided by coverage.



G

A

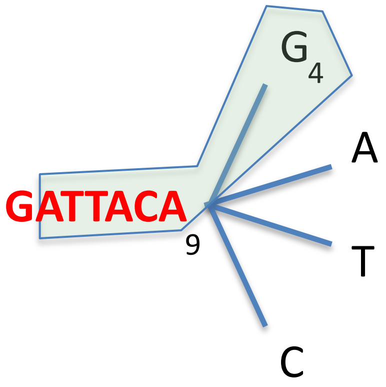
GATTACA

9

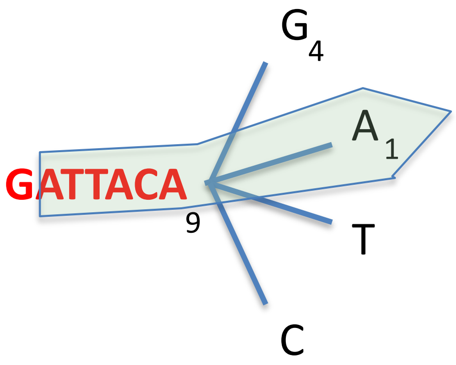
T

C

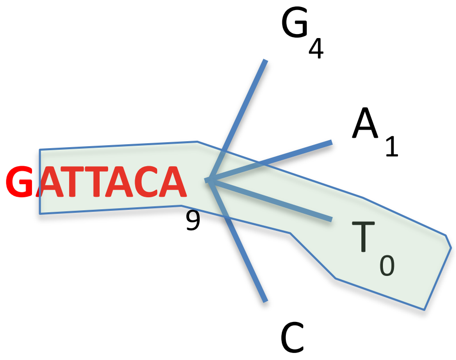
Inchworm Algorithm



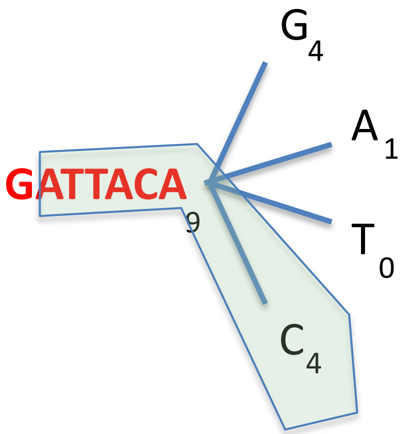
Inchworm Algorithm



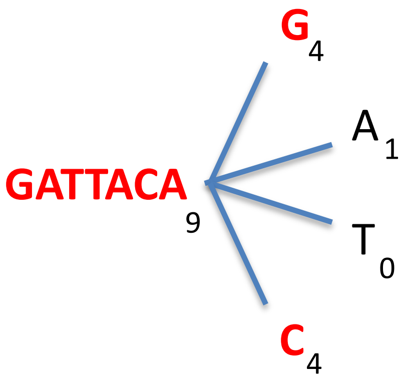
Inchworm Algorithm



Inchworm Algorithm



Inchworm Algorithm



G_4

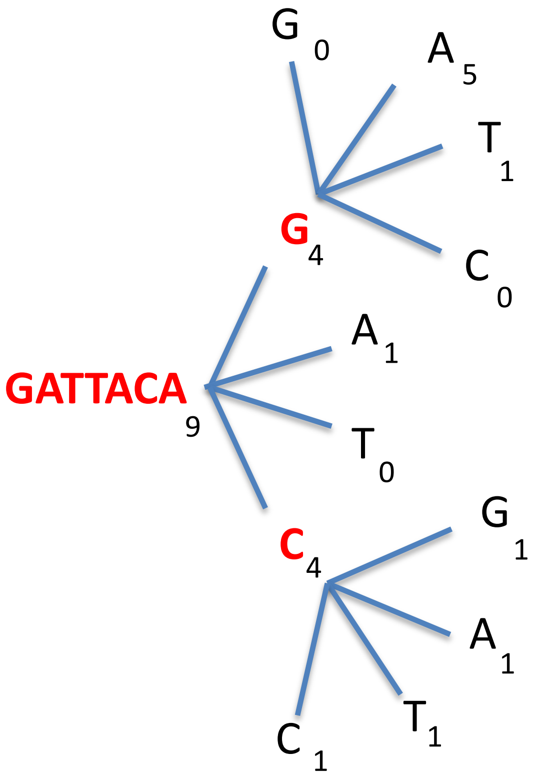
A_1

GATTACA_9

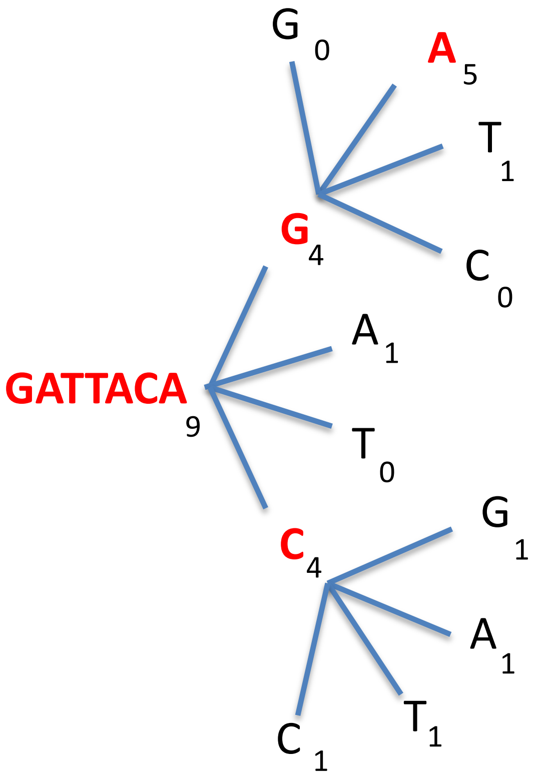
T_0

C_4

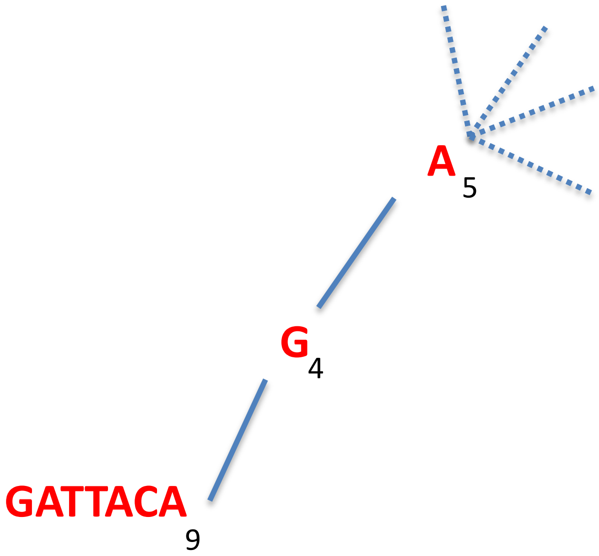
Inchworm Algorithm



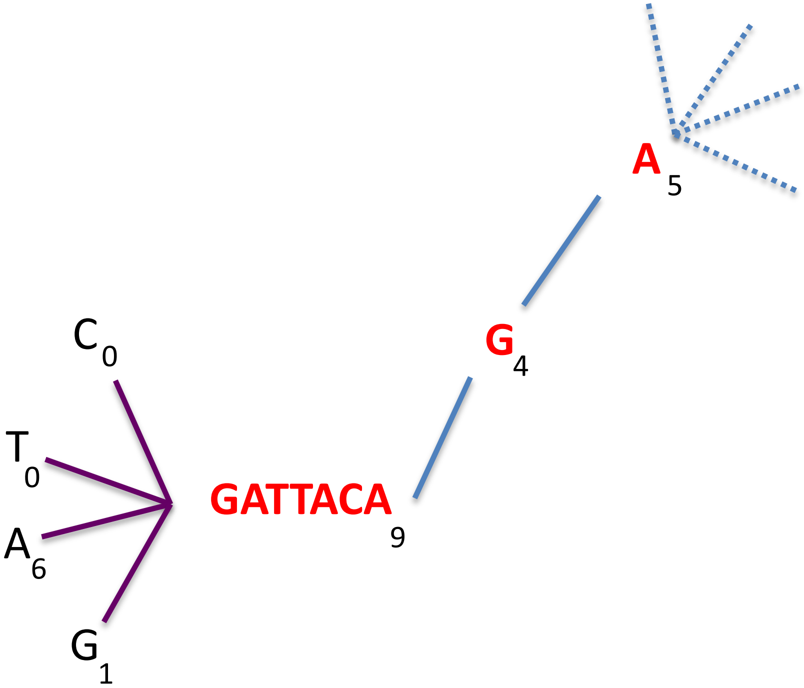
Inchworm Algorithm



Inchworm Algorithm



Inchworm Algorithm



C_0

T_0

A_6

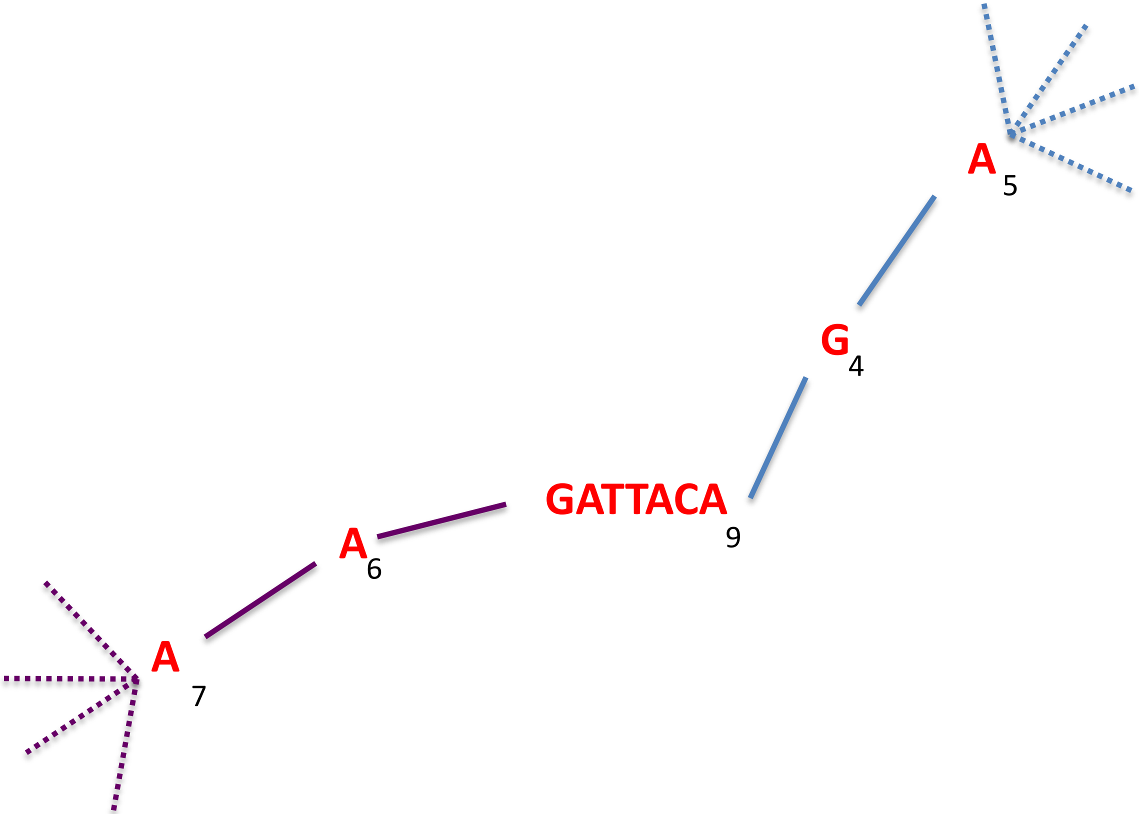
G_1

GATTACA$_9$

G$_4$

A$_5$

Inchworm Algorithm



Report contig: ....AAGATTACAGA....

Remove assembled kmers from catalog, then repeat the entire process.

Trinity – How it works:

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

>a121:len=5845

>a122:len=2560

>a123:len=4443

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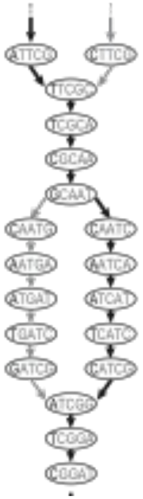
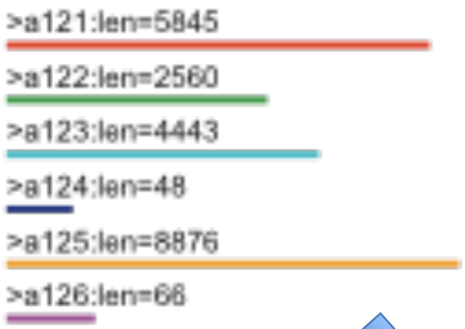


Thousands of disjoint graphs

...CTTCGCAA...TGATCGGAT...

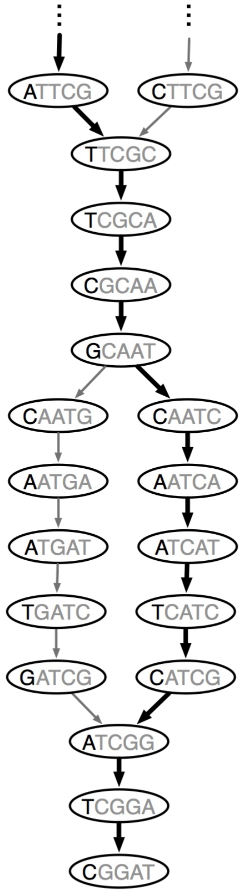
...ATTCGCAA...TCATCGGAT...

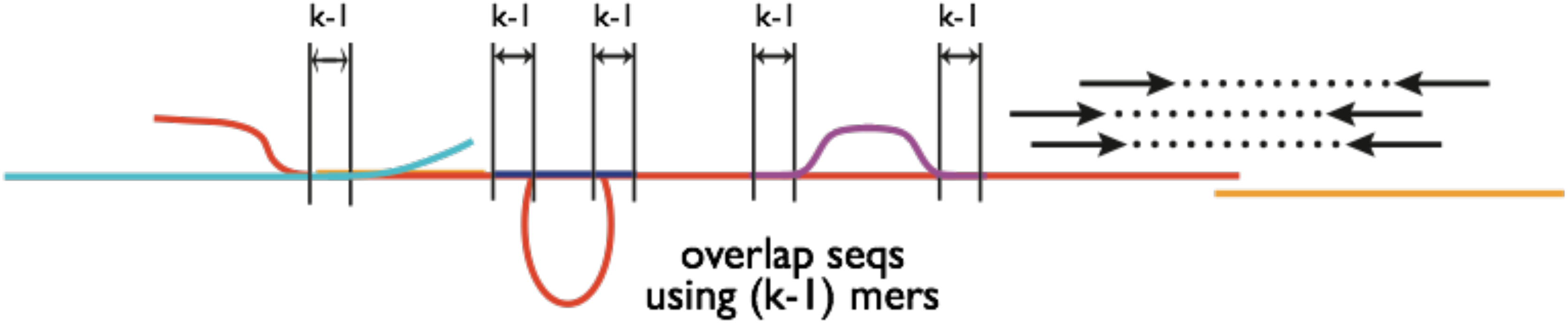
Chrysalis



Integrate isoforms via k-1 overlaps

Build de Bruijn Graphs (ideally, one per gene)





Thousands of Chrysalis Clusters

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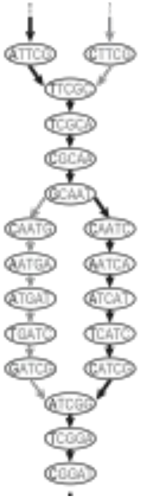
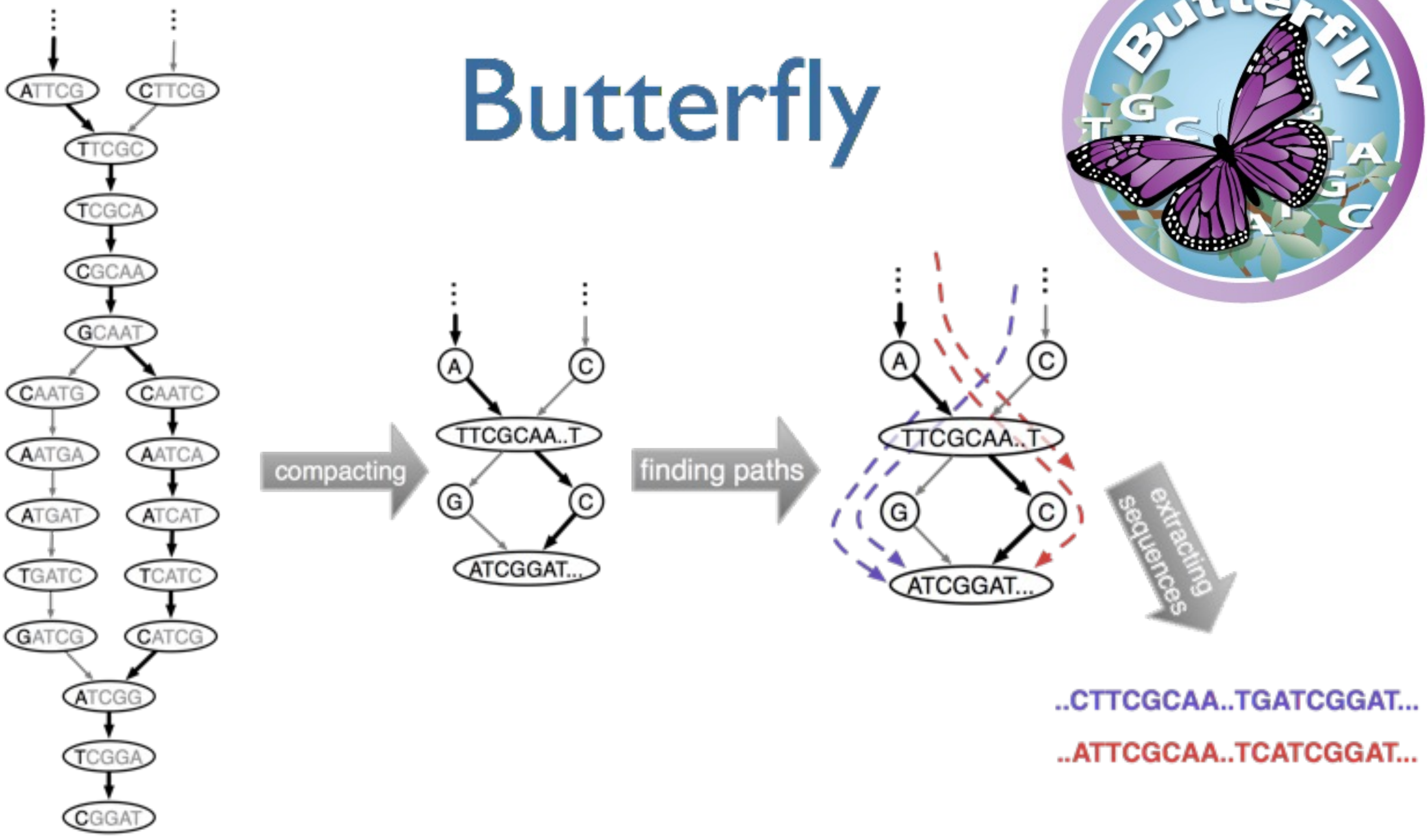


...CTTCGCAA...TGATCGGAT...

...ATTCGCAA...TCATCGGAT...

Thousands of disjoint graphs

Butterfly



de Bruijn graph

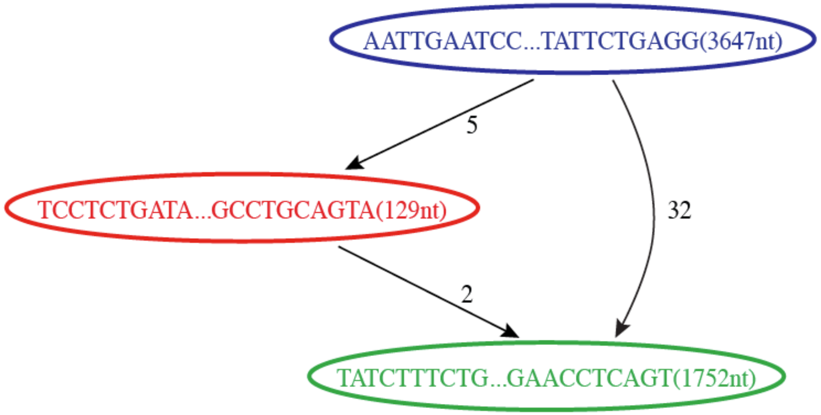
compact graph

compact graph with reads

sequences
(isoforms and paralogs)

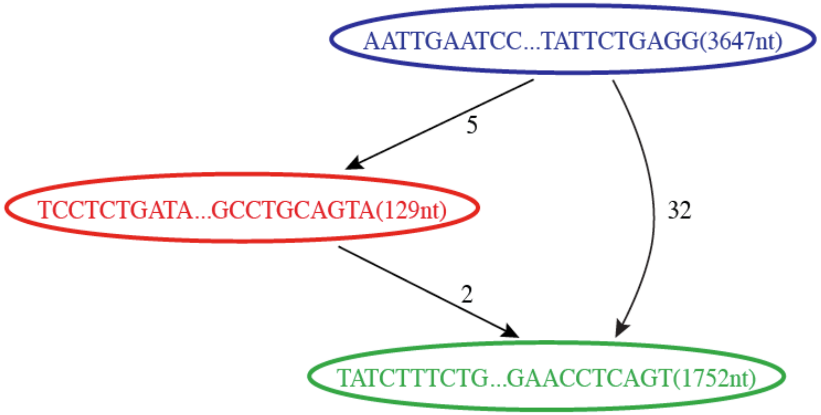
Butterfly Example 1:
Reconstruction of Alternatively Spliced Transcripts

Butterfly's Compacted Sequence Graph


AATTGAATCC...TATTCTGAGG(3647nt)
5
32
TCCTCTGATA...GCCTGCAGTA(129nt)
2
TATCTTTCTG...GAACCTCAGT(1752nt)

Reconstruction of Alternatively Spliced Transcripts

Butterfly's Compacted Sequence Graph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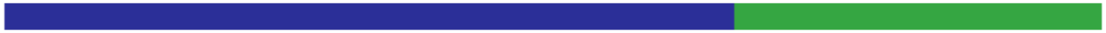
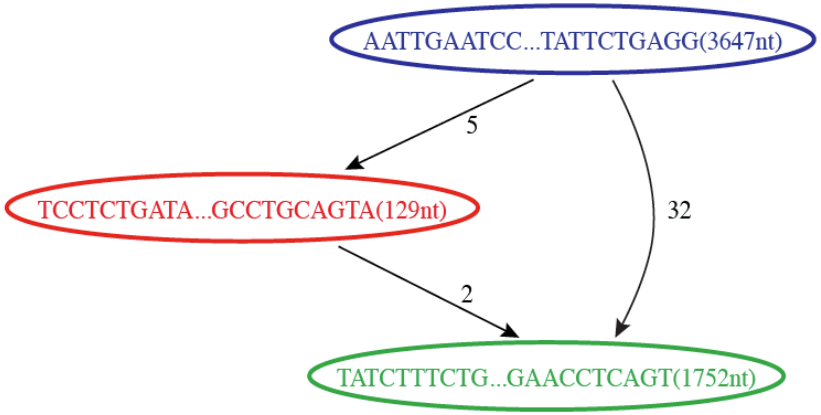
32
TCCTCTGATA...GCCTGCAGTA(129nt)
2
TATCTTTCTG...GAACCTCAGT(1752nt)

Reconstructed Transcripts



Reconstruction of Alternatively Spliced Transcripts

Butterfly's Compacted Sequence Graph


AATTGAATCC...TATTCTGAGG(3647nt)
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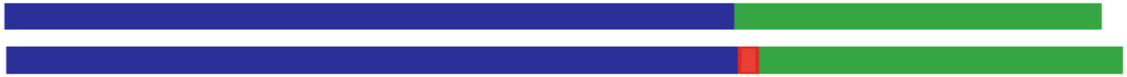
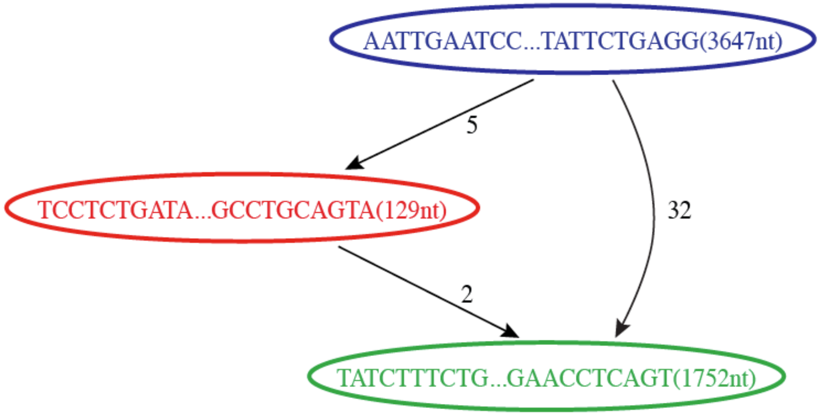
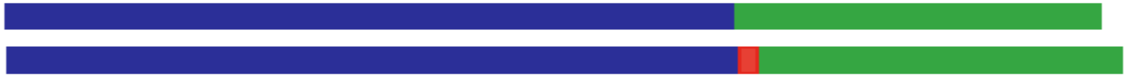


Reconstruction of Alternatively Spliced Transcripts

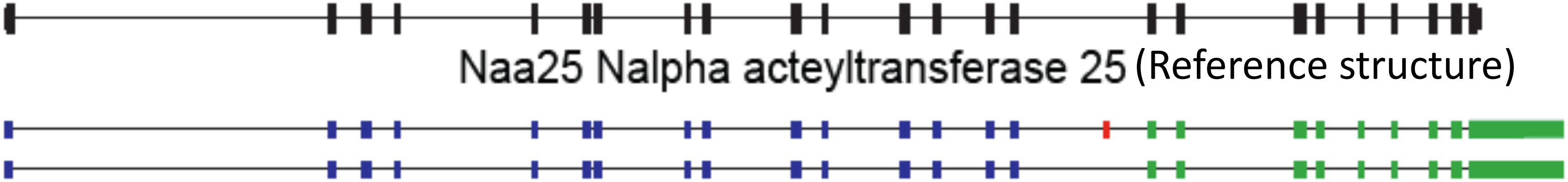
Butterfly's Compacted Sequence Graph


AATTGAATCC...TATTCTGAGG(3647nt)
5
32
TCCTCTGATA...GCCTGCAGTA(129nt)
2
TATCTTTCTG...GAACCTCAGT(1752nt)

Reconstructed Transcripts



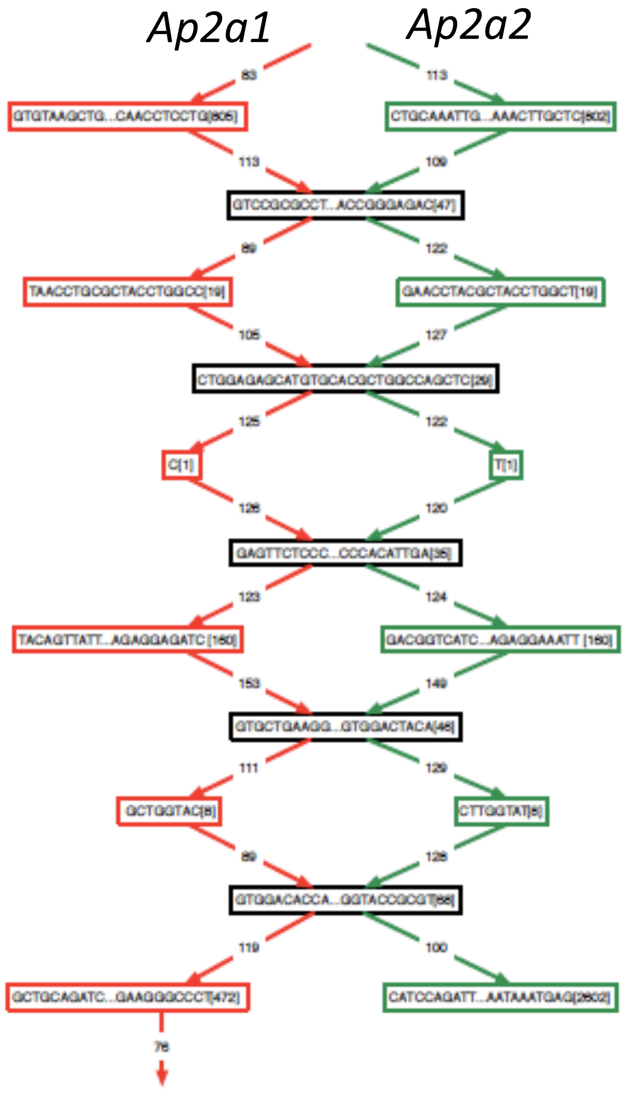
Aligned to Mouse Genome



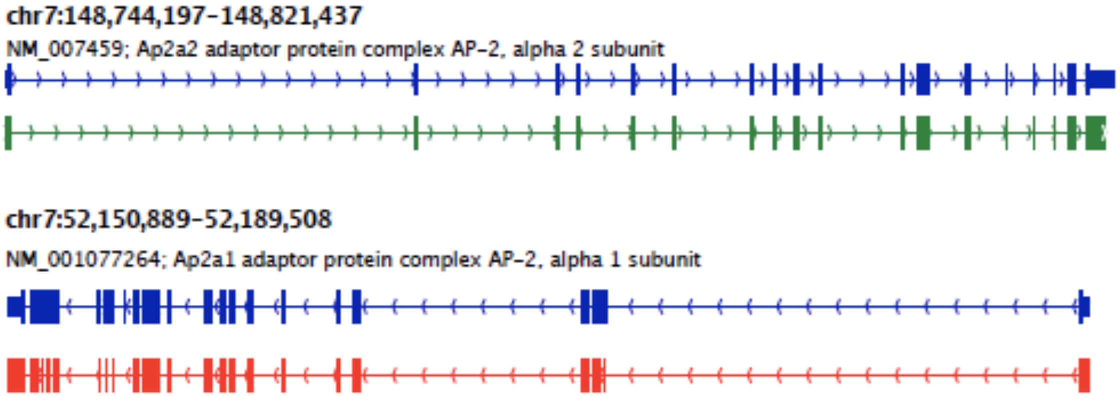
(Reference structure)

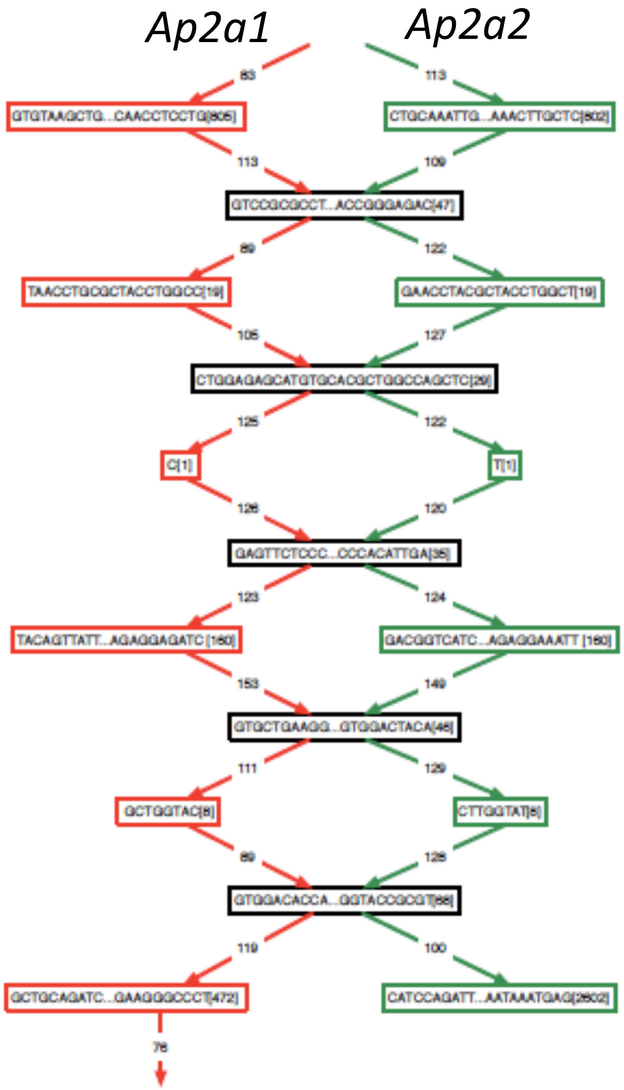
Butterfly Example 2:

Teasing Apart Transcripts of Paralogous Genes


Ap2a1
Ap2a2
83
113
GTGTAAGCTG...CAACCTCCTG[806]
CTGCAAATTG...AAACTTGCTC[802]
113
109
GTCCGCGCCT...ACCGGGAGAC[47]
89
122
TAACCTGCGCTACCTGGCC[19]
GAACCTACGCTACCTGGCT[19]
105
127
CTGGAGAGCATGTGCACGCTGGCCAGCTC[29]
125
122
C[1]
T[1]
128
120
GAGTTCTCCC...CCCACATTGA[35]
123
124
TACAGTTATT...AGAGGAGATC [160]
GACGGTCATC...AGAGGAAATT [160]
153
149
GTGCTGAAGG...GTGGACTACA[46]
111
129
GCTGGTAC[8]
CTTGGTAT[8]
89
128
GTGGACACCA...GGTACCGCGT[68]
119
100
GCTGCAGATC...GAAGGGCCCT[472]
CATCCAGATT...AATAAATGAG[2802]
78

Teasing Apart Transcripts of Paralogous Genes


chr7:148,744,197-148,821,437
NM_007459; Ap2a2 adaptor protein complex AP-2, alpha 2 subunit
chr7:52,150,889-52,189,508
NM_001077264; Ap2a1 adaptor protein complex AP-2, alpha 1 subunit


Ap2a1
Ap2a2
83
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GTGTAAGCTG...CAACCTCCTG[806]
CTGCAAATTG...AAACTTGCTC[802]
113
109
GTCCGCGCCT...ACCGGGAGAC[47]
89
122
TAACCTGCGCTACCTGGCC[19]
GAACCTACGCTACCTGGCT[19]
105
127
CTGGAGAGCATGTGCACGCTGGCCAGCTC[29]
125
122
C[1]
T[1]
126
120
GAGTTCTCCC...CCCACATTGA[35]
123
124
TACAGTTATT...AGAGGAGATC [160]
GACGGTCATC...AGAGGAAATT [160]
153
149
GTGCTGAAGG...GTGGACTACA[46]
111
129
GCTGGTAC[8]
CTTGGTAT[8]
89
128
GTGGACACCA...GGTACCGCGT[68]
119
100
GCTGCAGATC...GAAGGGCCCT[472]
CATCCAGATT...AATAAATGAG[2602]
76

Strand-specific RNA-Seq is Preferred

Computationally: fewer confounding graph structures in de novo assembly:

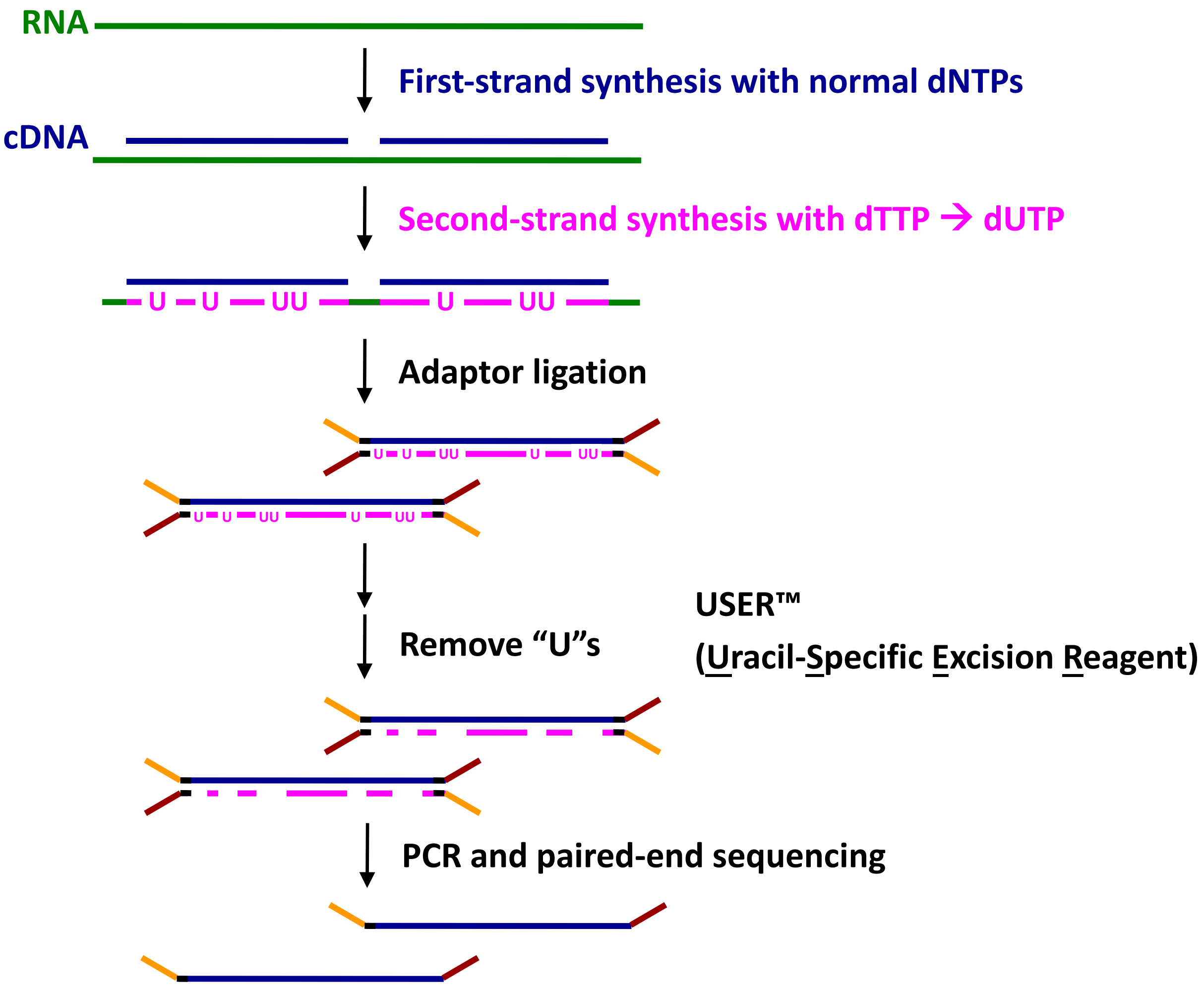
ex. Forward != reverse complement

(GGAA != TTCC)

Biologically: separate sense vs. antisense transcription

Illumina TruSeq Stranded mRNA Kit:

dUTP 2nd Strand Method: Our Favorite



RNA

First-strand synthesis with normal dNTPs

cDNA

Second-strand synthesis with dTTP → dUTP

Adaptor ligation

Remove "U"s

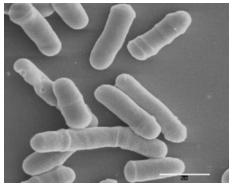
USER™
(Uracil-Specific Excision Reagent)

PCR and paired-end sequencing

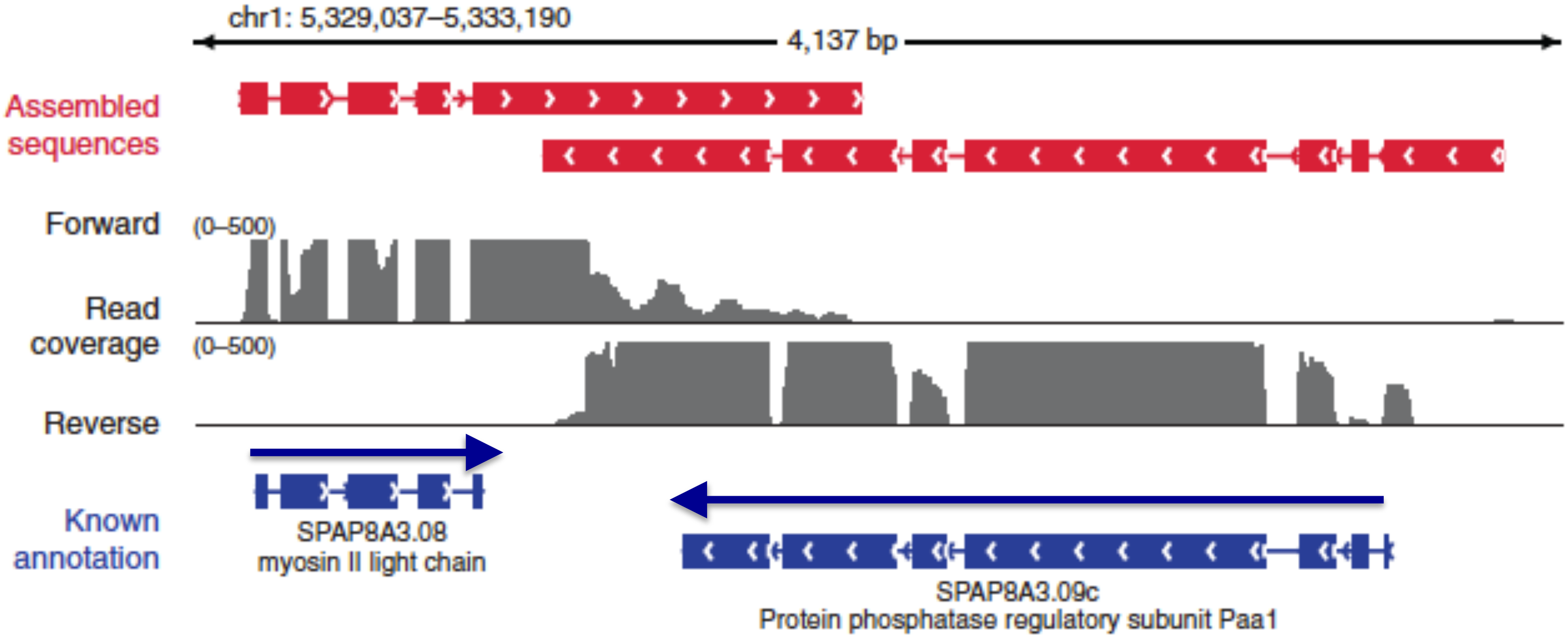
Modified from Parkhomchuk *et al.* (2009) *Nucleic Acids Res.* 37:e123

Slide courtesy of Joshua Levin, Broad Institute.

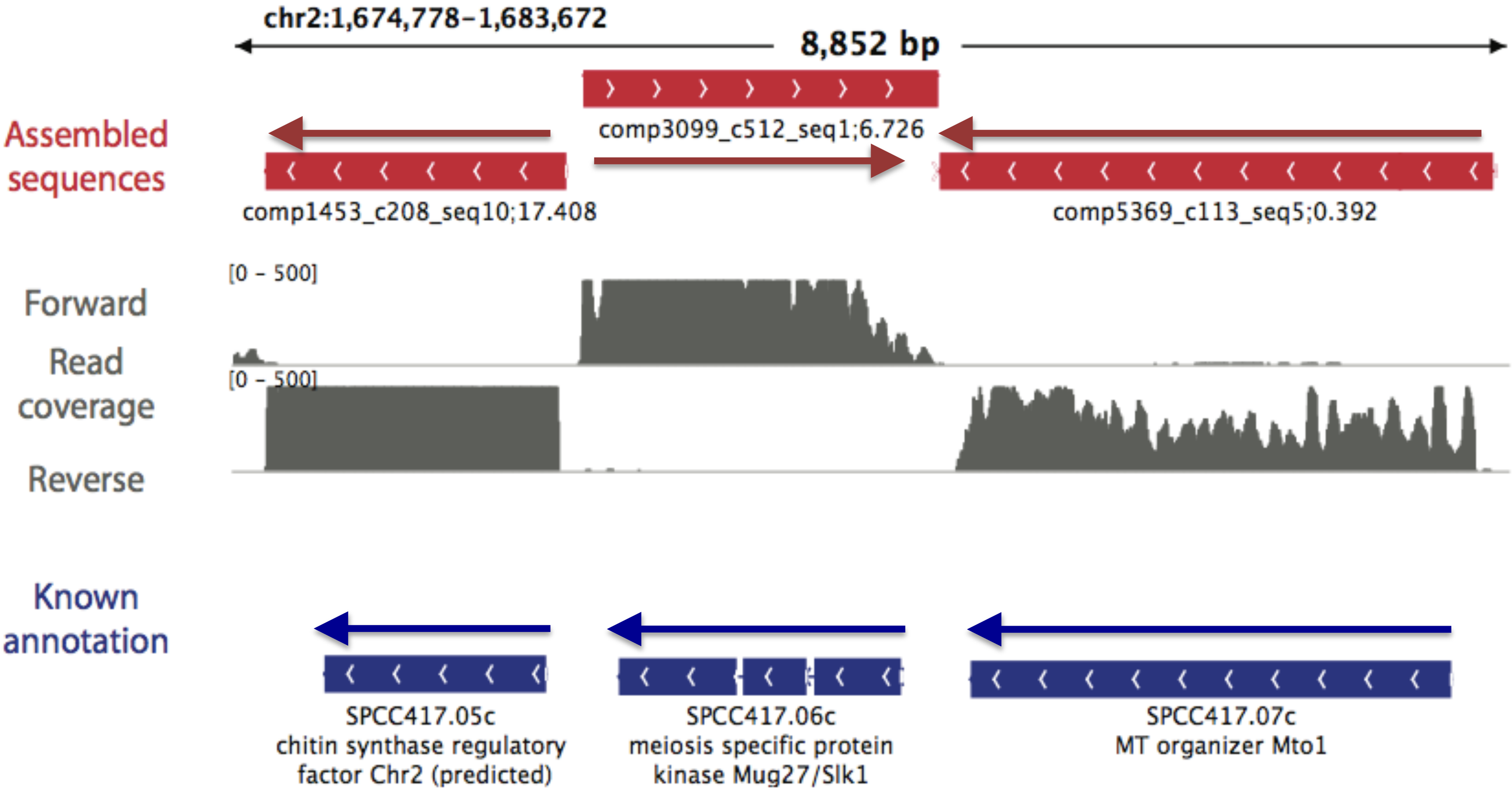
Overlapping UTRs from Opposite Strands



Schizosacharomyces pombe
(fission yeast)



Antisense-dominated Transcription



chr2:1,674,778–1,683,672

8,852 bp

Assembled sequences

comp3099_c512_seq1;6.726

comp1453_c208_seq10;17.408

comp5369_c113_seq5;0.392

Forward Read coverage Reverse

[0 - 500]

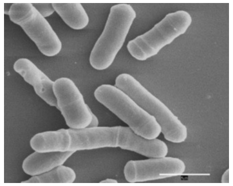
[0 - 500]

Known annotation

SPCC417.05c
chitin synthase regulatory factor Chr2 (predicted)

SPCC417.06c
meiosis specific protein kinase Mug27/Slk1

SPCC417.07c
MT organizer Mto1



Trinity is a Highly Effective and Popular RNA-Seq Assembler

Nature Biotechnology, 2011

Thousands of routine users.

>15k literature citations

Freely Available, Well-supported,
Open Source Software

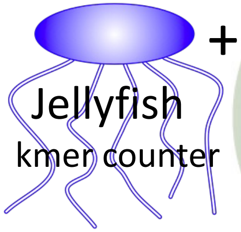
GitHub

http://trinityrnaseq.github.io

Trinity – Today, Many More Components

(off-the-shelf and into the Trinity ecosystem)

Rob Patro

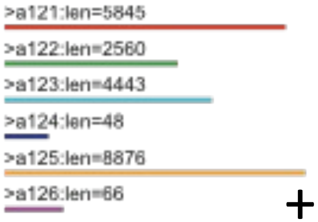


+

Jellyfish

kmer counter

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

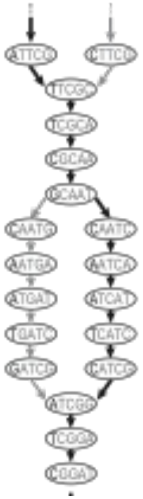


```
>a121:len=5845
>a122:len=2560
>a123:len=4443
>a124:len=48
>a125:len=8876
>a126:len=66
```

+

(Capture paired-end links between inchworm contigs)

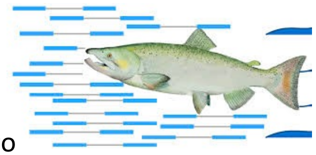
Ben Langmead



...CTTCGCAA...TGATCGGAT...

...ATTCGCAA...TCATCGGAT...

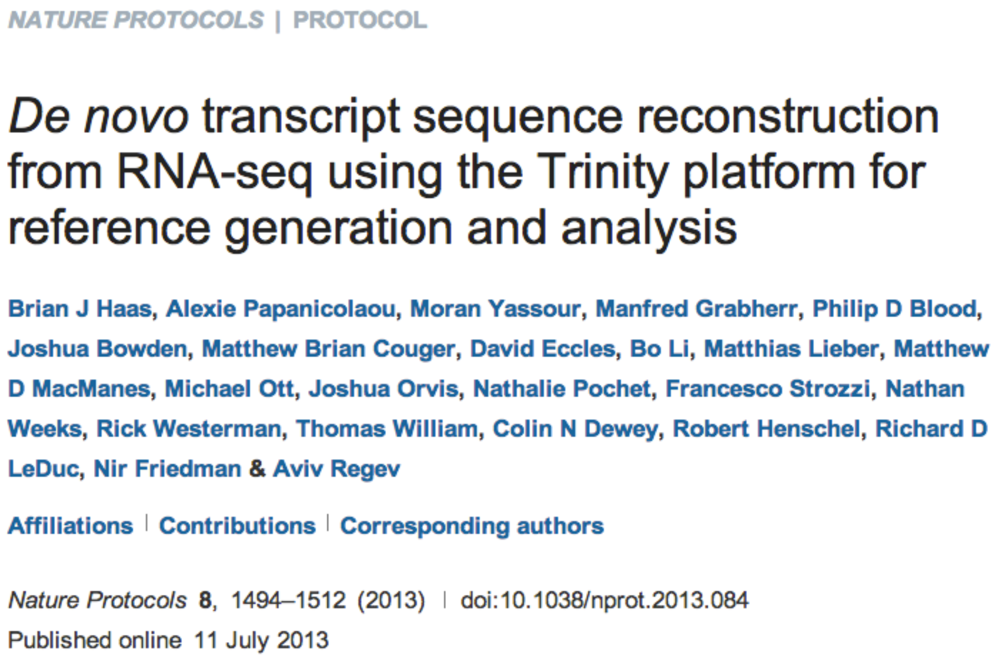
+



Rob Patro

Salmon expression quantification (eliminate assembly artifacts) nature protocols

Transcriptome Assembly is Just the End of the Beginning…



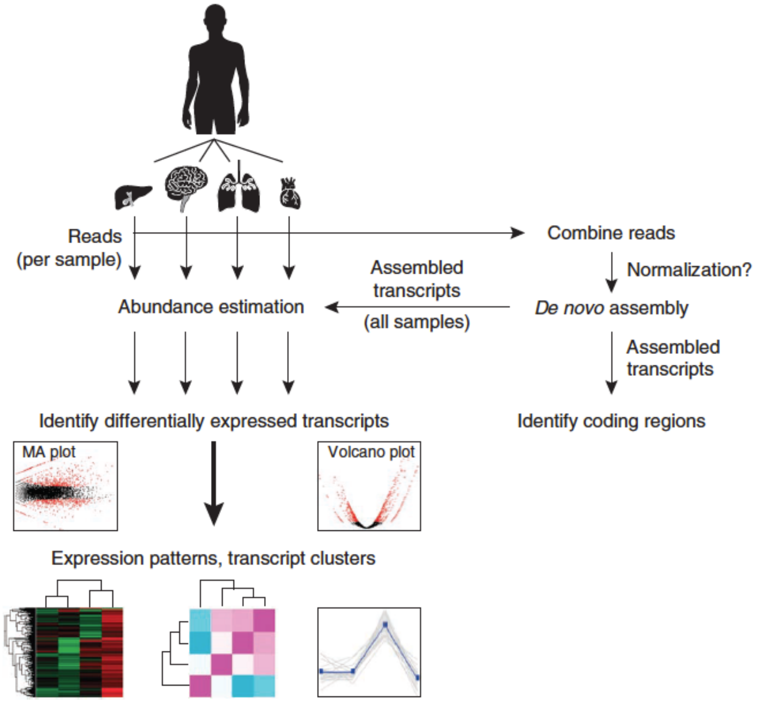
NATURE PROTOCOLS | PROTOCOL

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

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

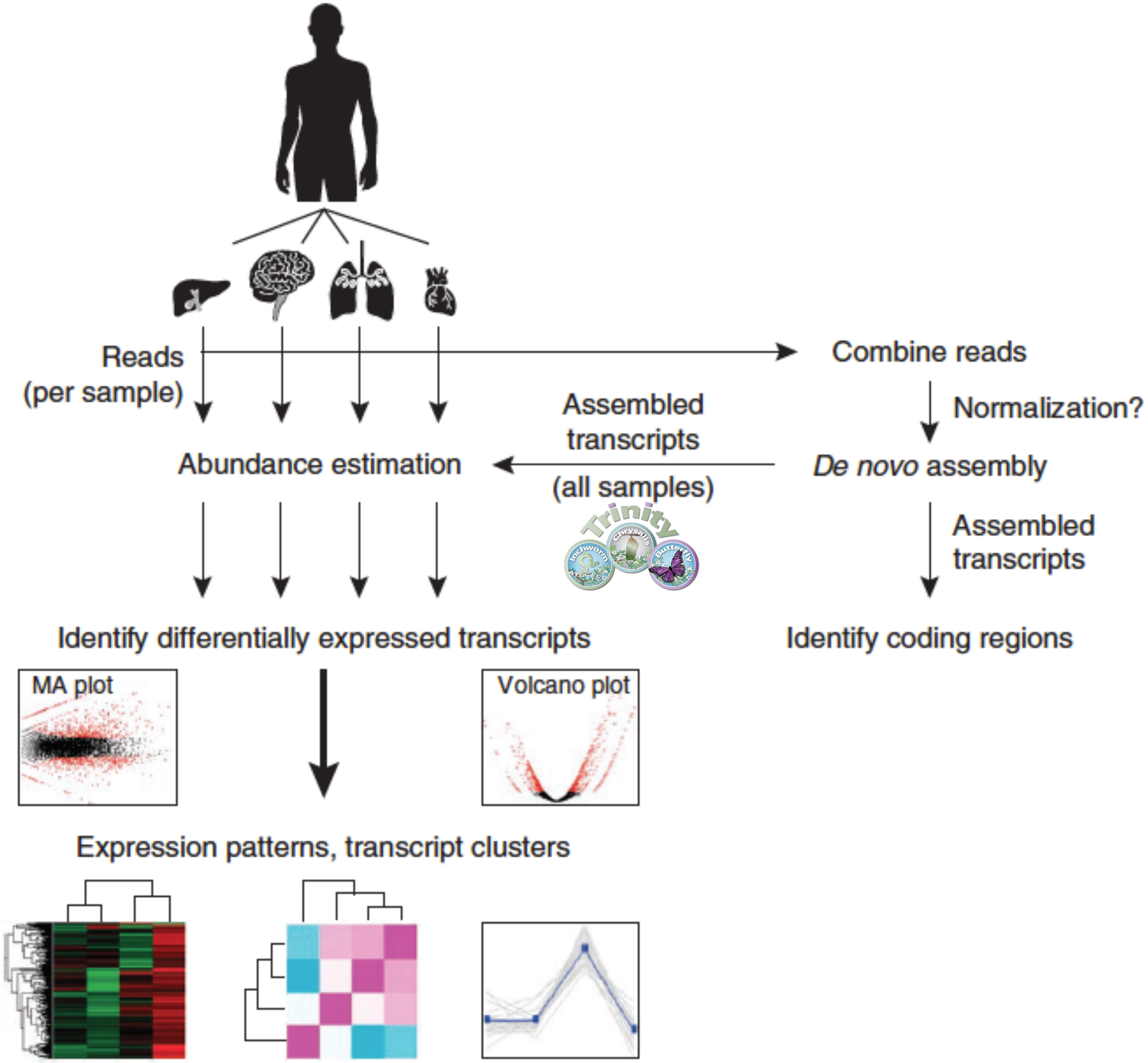
Affiliations | Contributions | Corresponding authors

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



Trinity Framework for De novo Transcriptome Assembly and Analysis

(focus of the transcriptomics lab)



Reads (per sample)

Combine reads

Normalization?

De novo assembly

Assembled transcripts (all samples)

Abundance estimation

Assembled transcripts

Identify differentially expressed transcripts

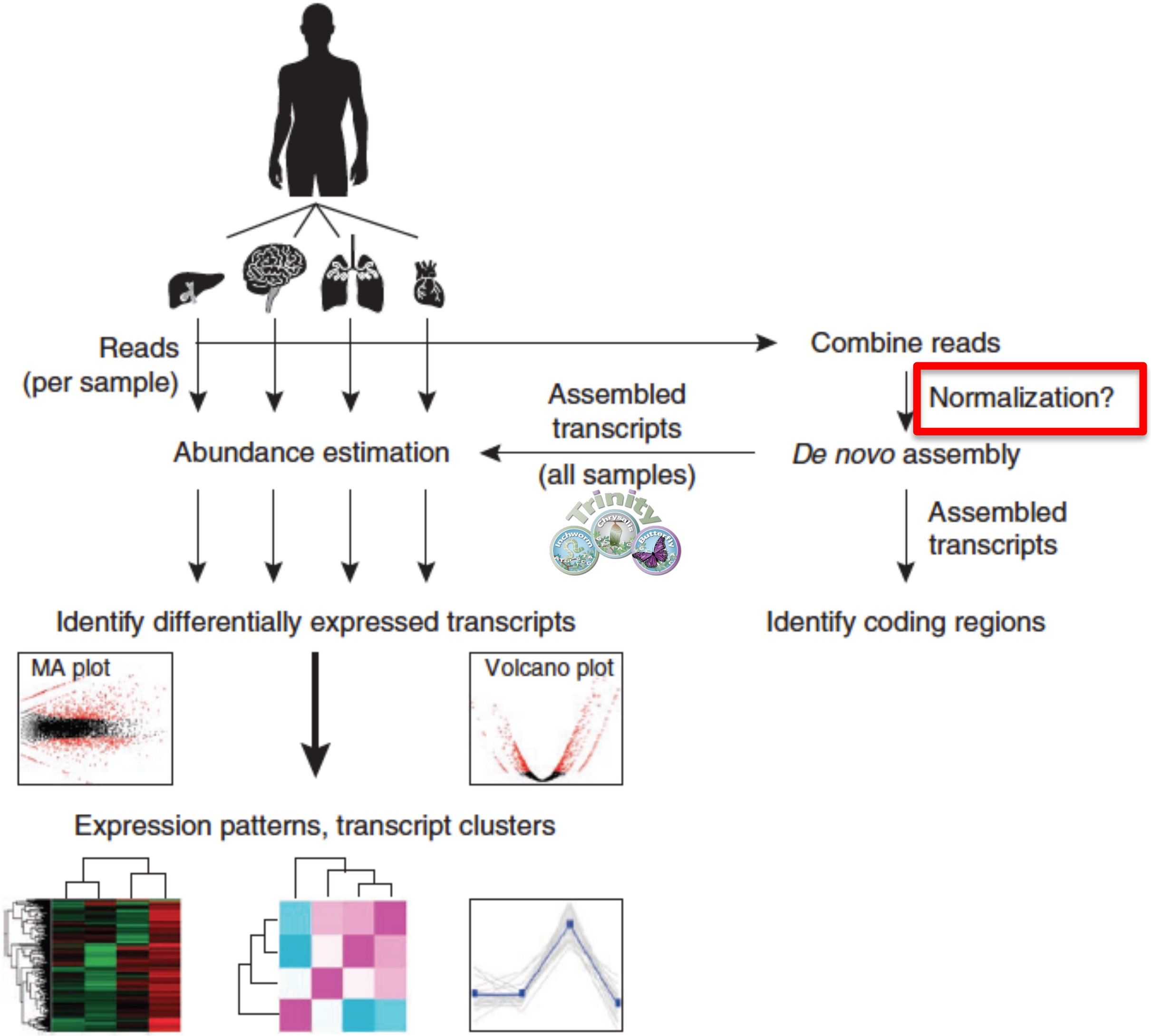
Identify coding regions

Expression patterns, transcript clusters

Bioconductor,
& Trinity

Trinity Framework for De novo Transcriptome Assembly and Analysis

(focus of the transcriptomics lab)



Reads (per sample)

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Assembled transcripts (all samples)

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Assembled transcripts

Identify differentially expressed transcripts

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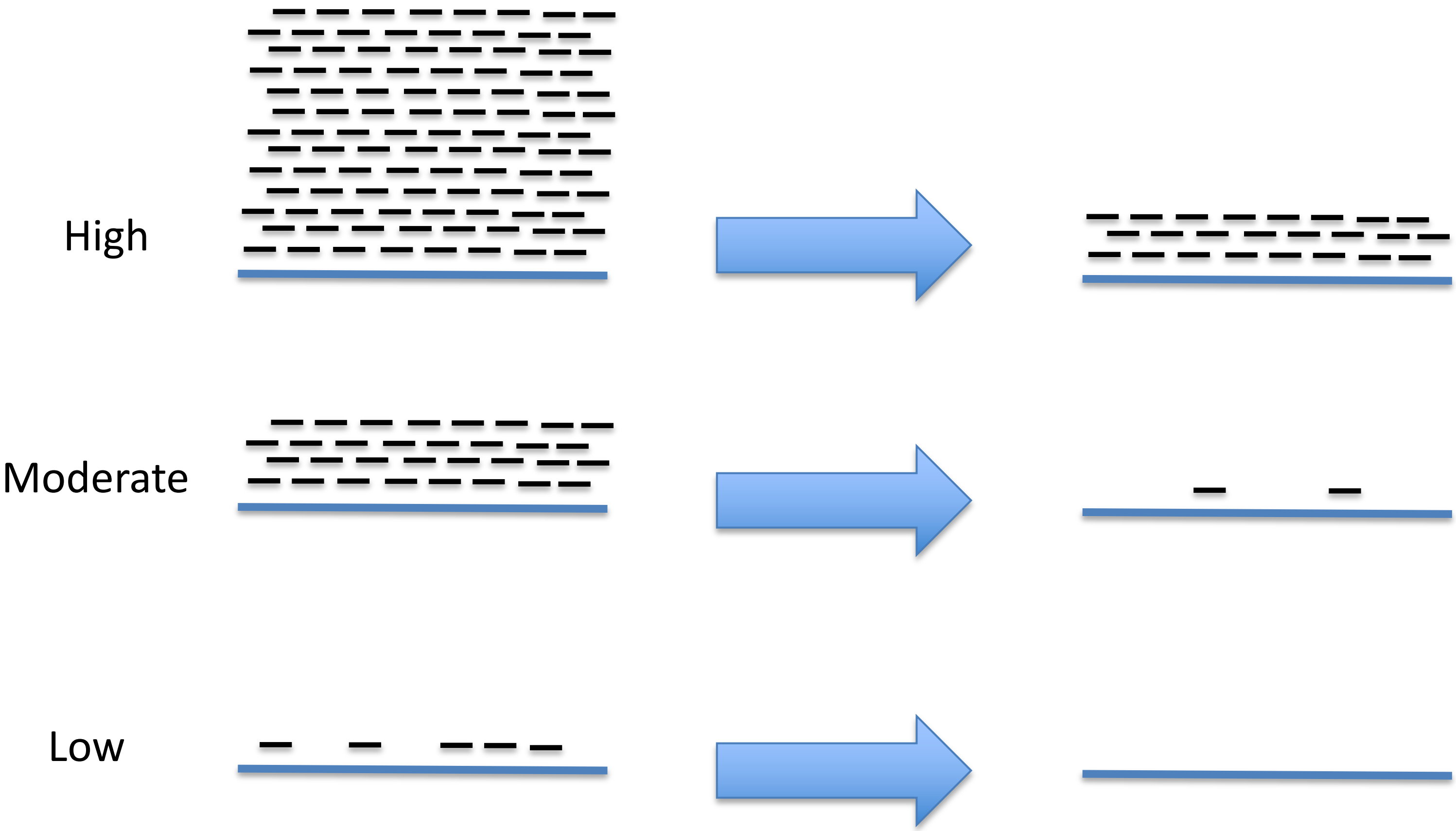
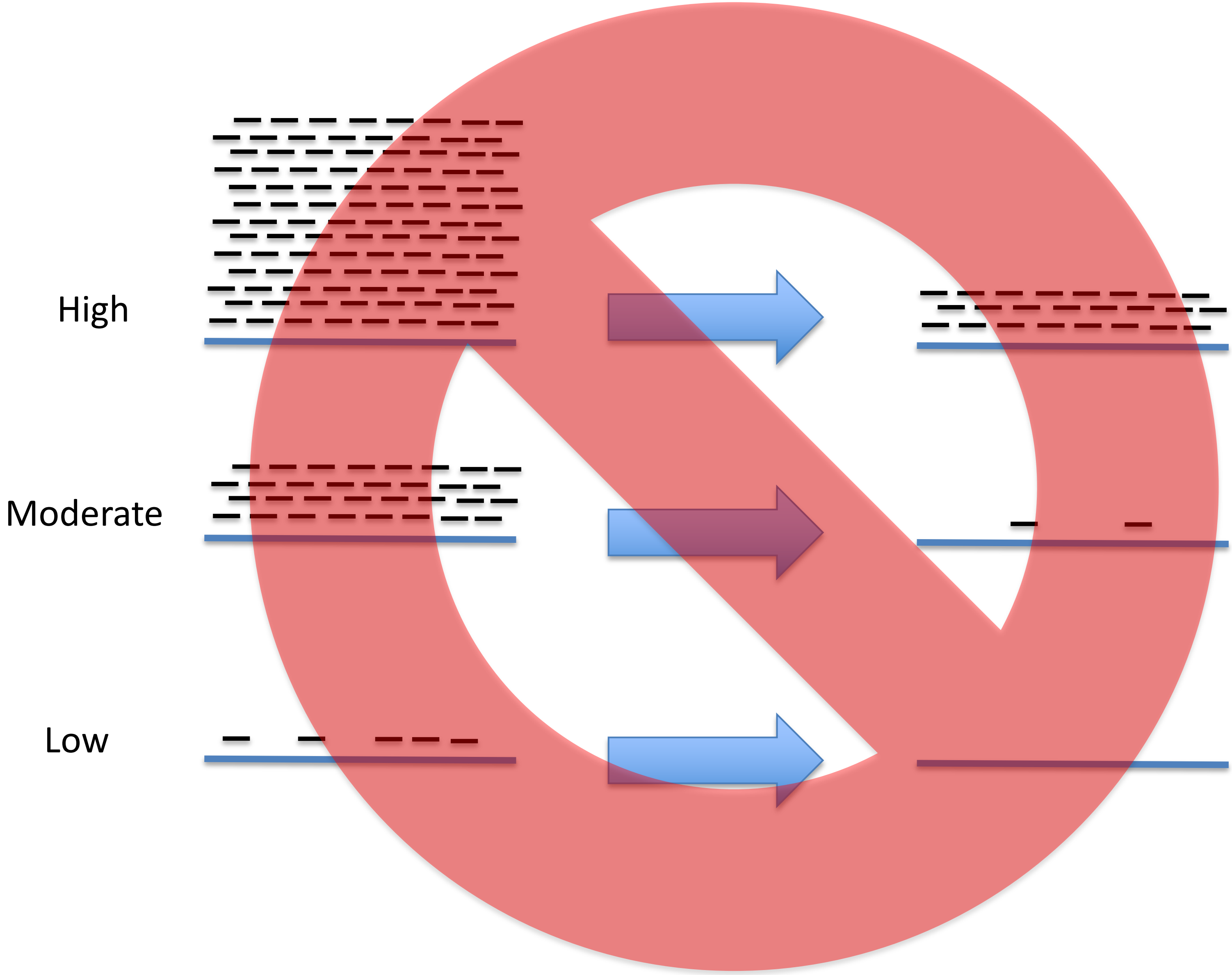
Expression patterns, transcript clusters

Bioconductor,
& Trinity

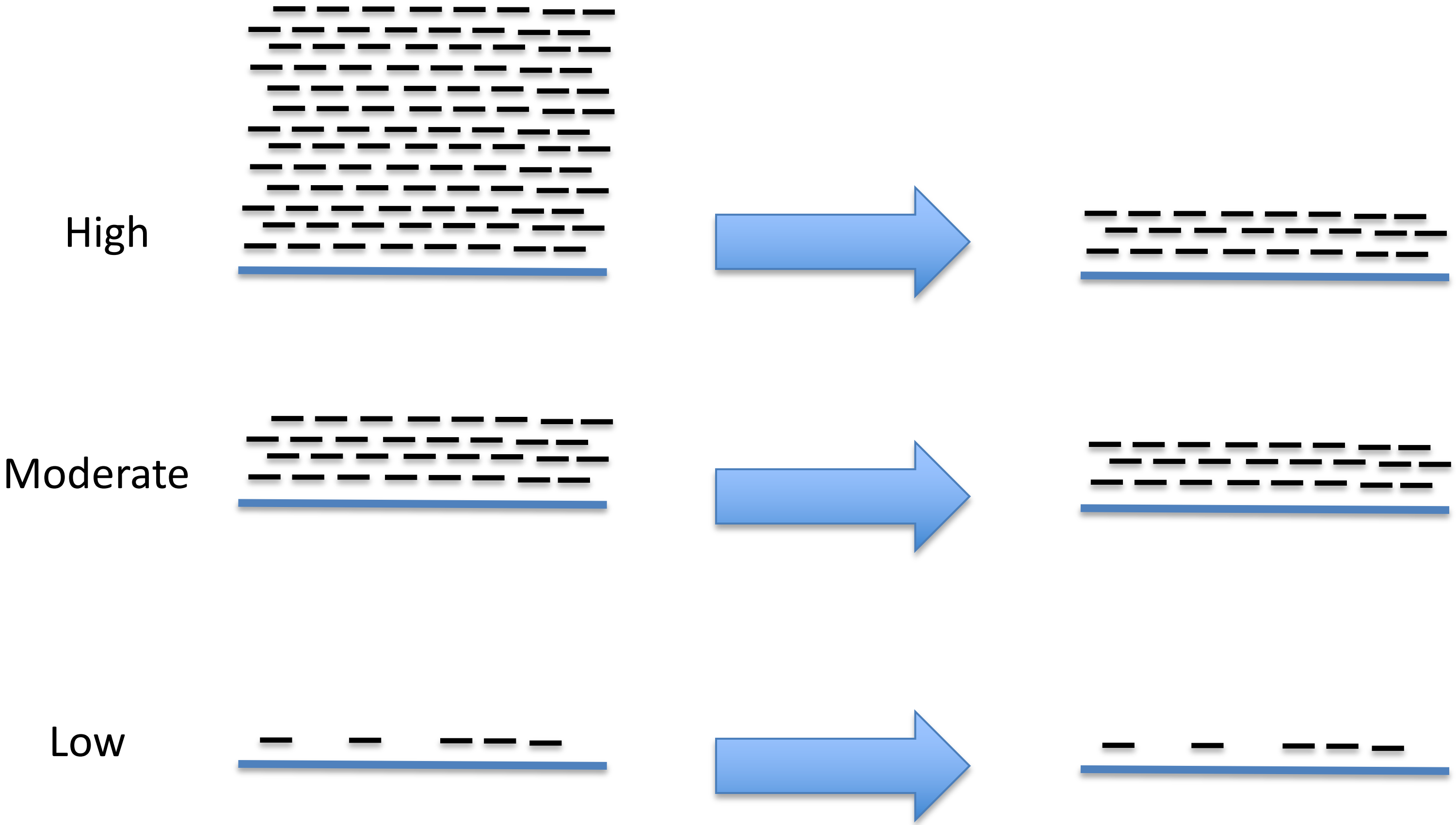
Could sub-sample the reads



Could sub-sample the reads



In silico normalization of reads

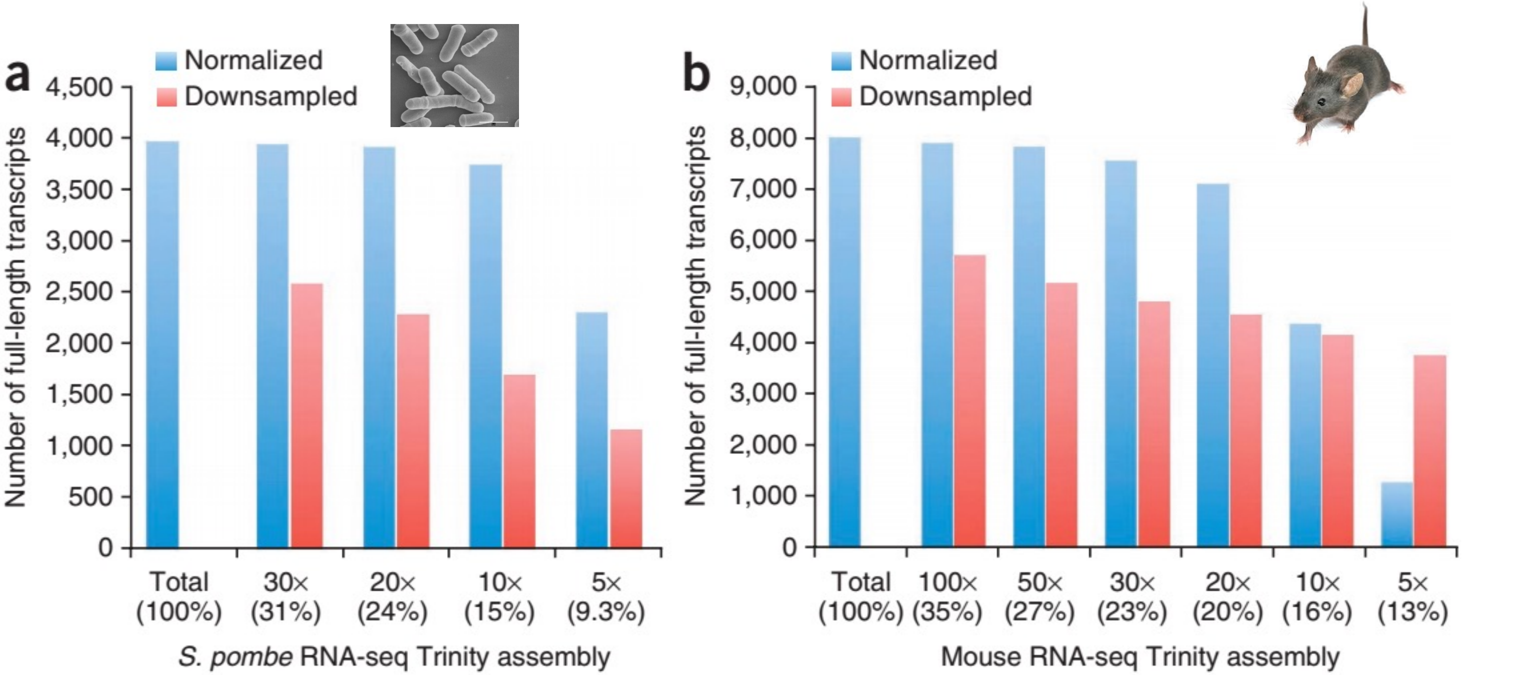


Select reads according to the probability:

$$P(\text{select read}) = \text{Min}\left(\frac{\text{target\_coverage(read)}}{\text{observed\_coverage(read)}}, 1\right)$$

Inspired by C. Titus Brown's Diginorm

Impact of Normalization on *De novo* Full-length Transcript Reconstruction

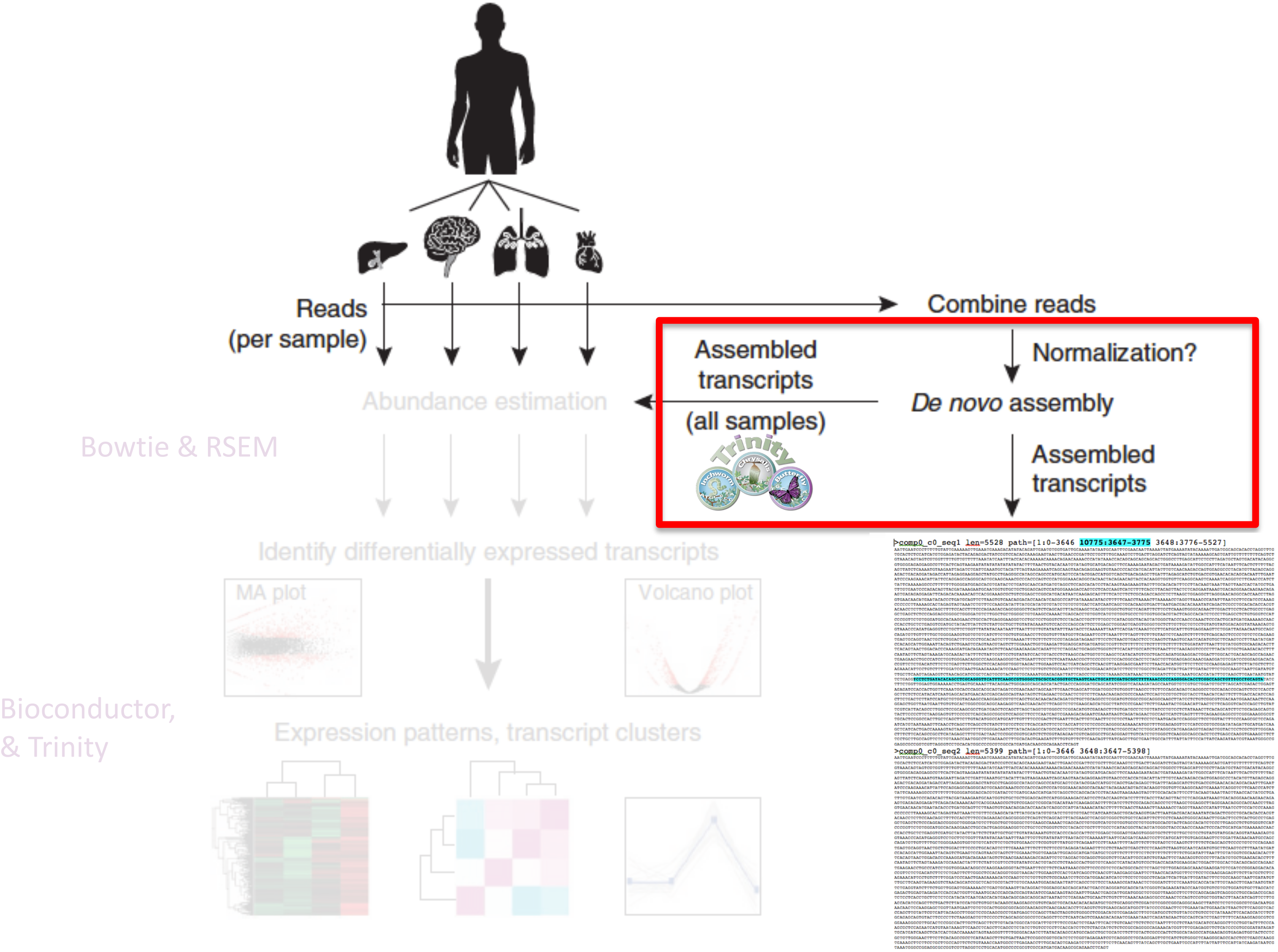


Largely retain full-length reconstruction, but use less RAM and assemble much faster.

Can go from >1 billion reads down to < 100 M reads used in assembly.

Haas et al., 2013

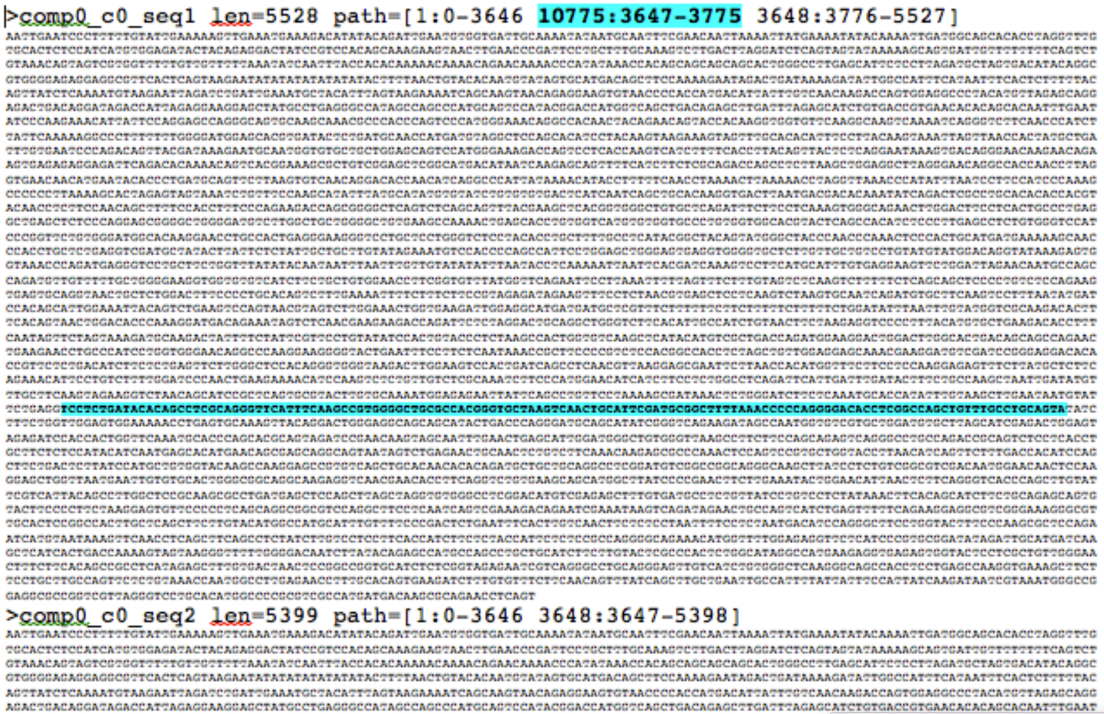
The product of Trinity: a Fasta file of assembled transcripts



```
>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]
```

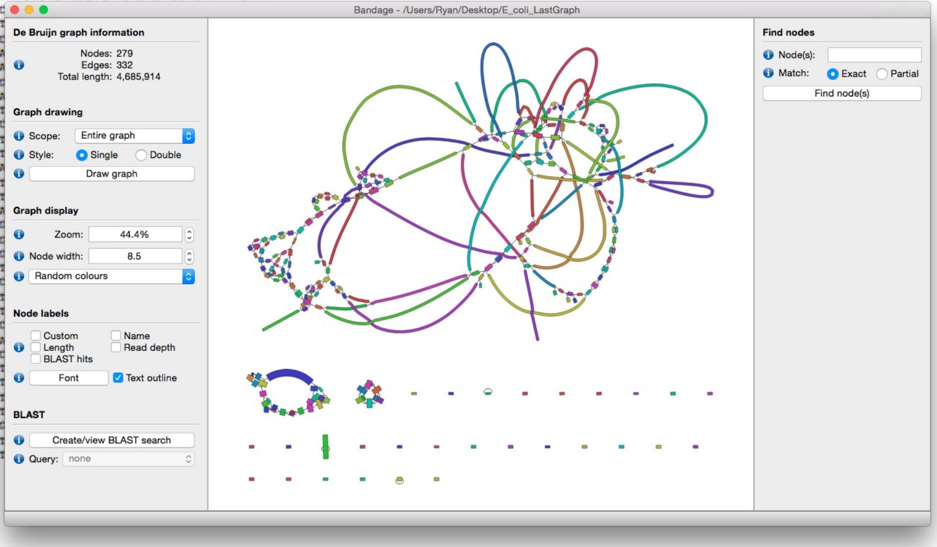
Trinity output: A multi-fasta file



```
>comp0_c0_seq1 len=5528 path=[1:0-3646 10775:3647-3775 3648:3776-5527]
[illegible]
>comp0_c0_seq2 len=5399 path=[1:0-3646 3648:3647-5398]
[illegible]
```

Can visualize using Bandage

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



Bandage - /Users/Ryan/Desktop/E_coli_LastGraph

De Bruijn graph information

Nodes: 279
Edges: 332
Total length: 4,685,914

Graph drawing

Scope: Entire graph
Style: ● Single ○ Double

Graph display

Zoom: 44.4%
Node width: 8.5
Random colours

Node labels

☐ Custom ☐ Name
☐ Length ☐ Read depth
☐ BLAST hits
☑ Text outline

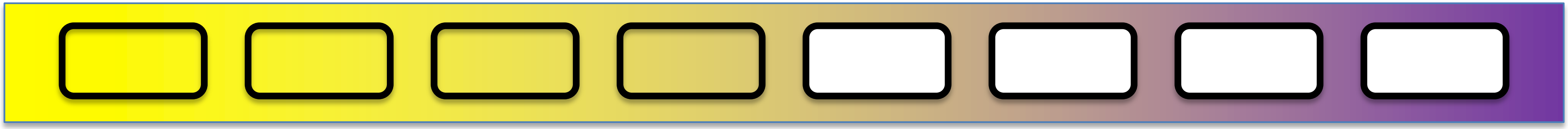
BLAST

Query: none

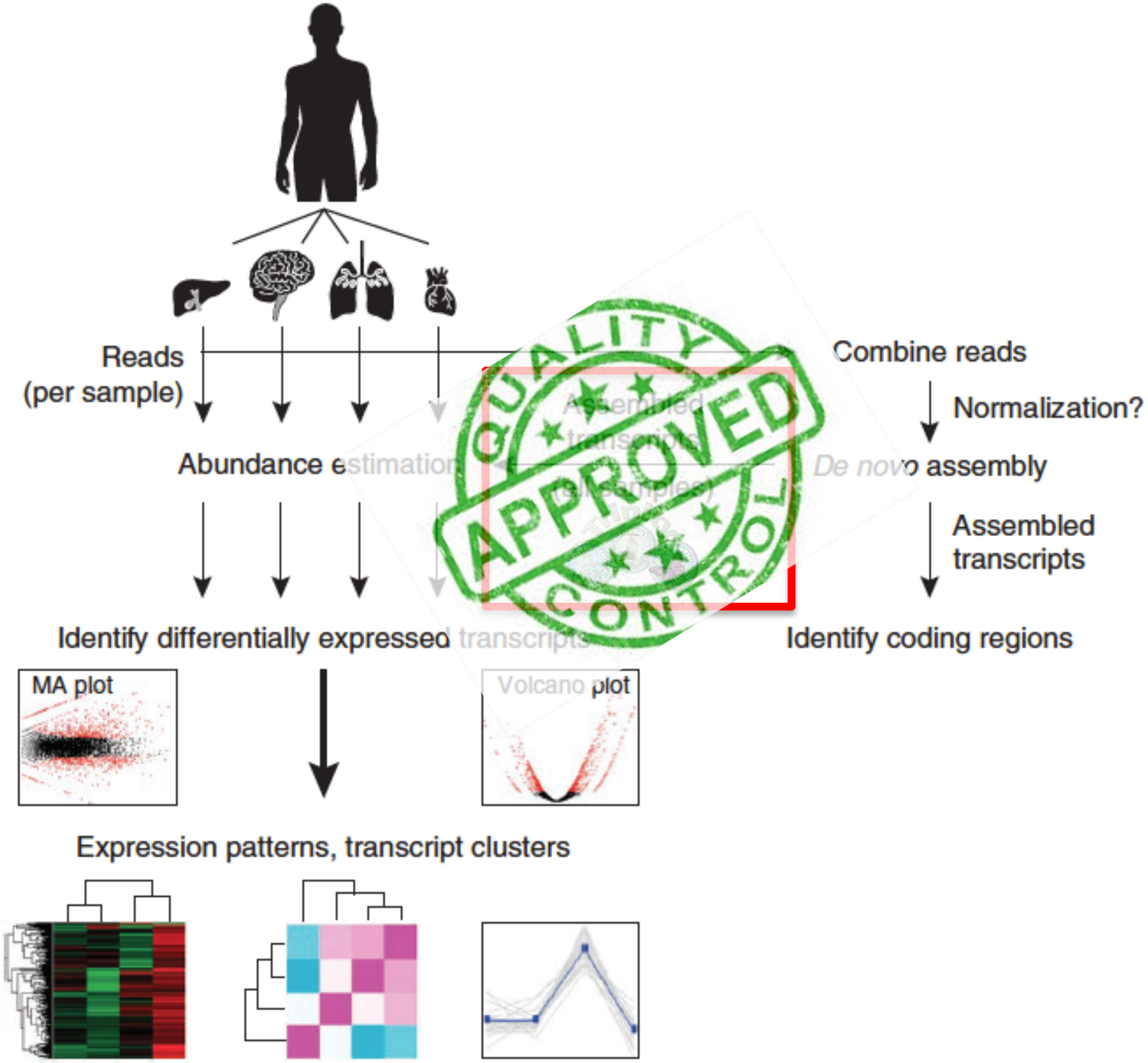
Find nodes

Node(s):
Match: ● Exact ○ Partial

Part 4. Transcriptome Quality Assessment



Evaluating the quality of your transcriptome assembly



Reads
(per sample)

Combine reads

Normalization?

Abundance estimation

Assembled transcripts (all samples)

De novo assembly

Assembled transcripts

Identify differentially expressed transcripts

Identify coding regions

MA plot

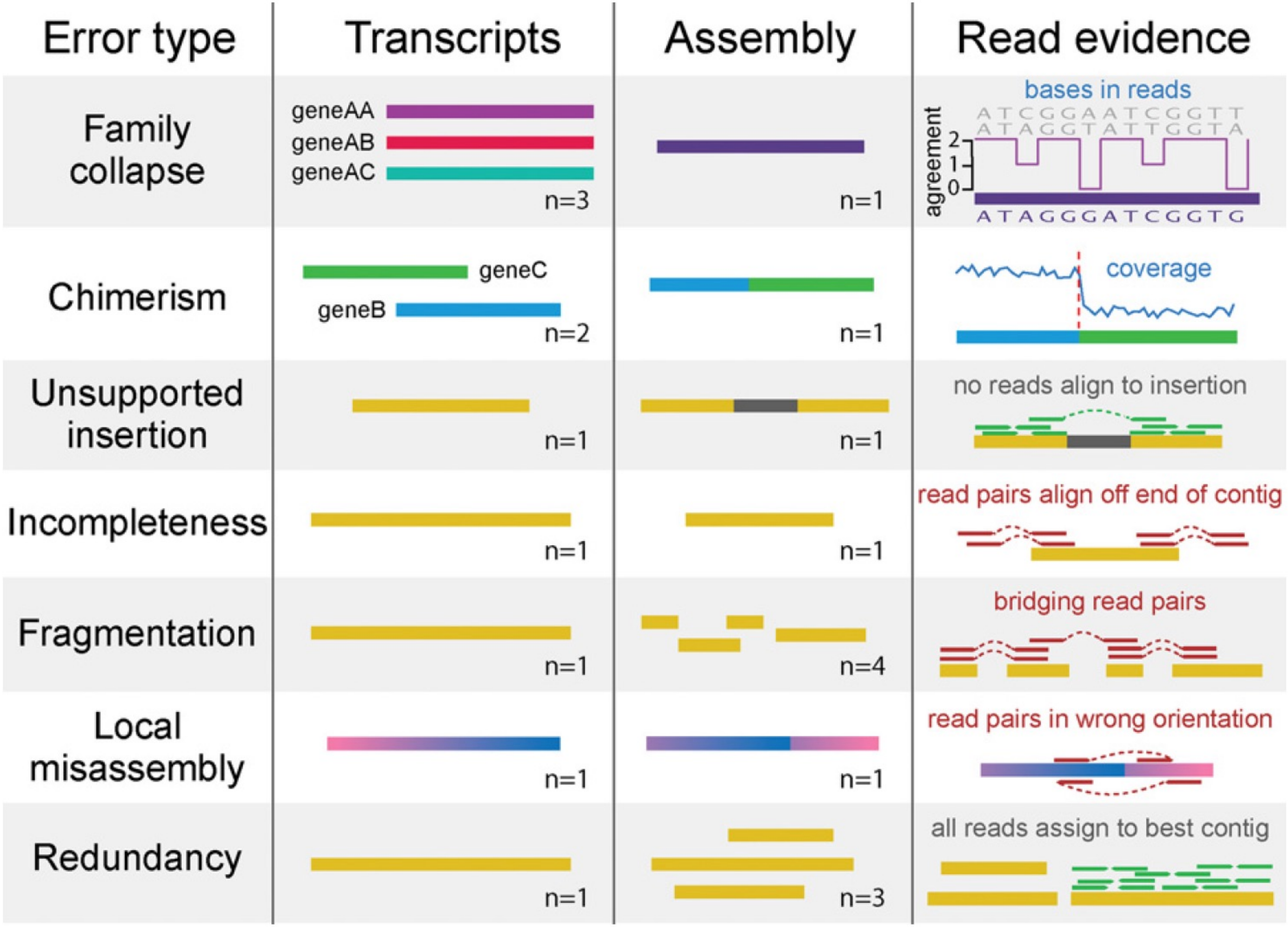
Volcano plot

Expression patterns, transcript clusters

QUALITY
APPROVED
CONTROL

Bioconductor,
& Trinity

De novo Transcriptome Assembly is Prone to Certain Types of Errors

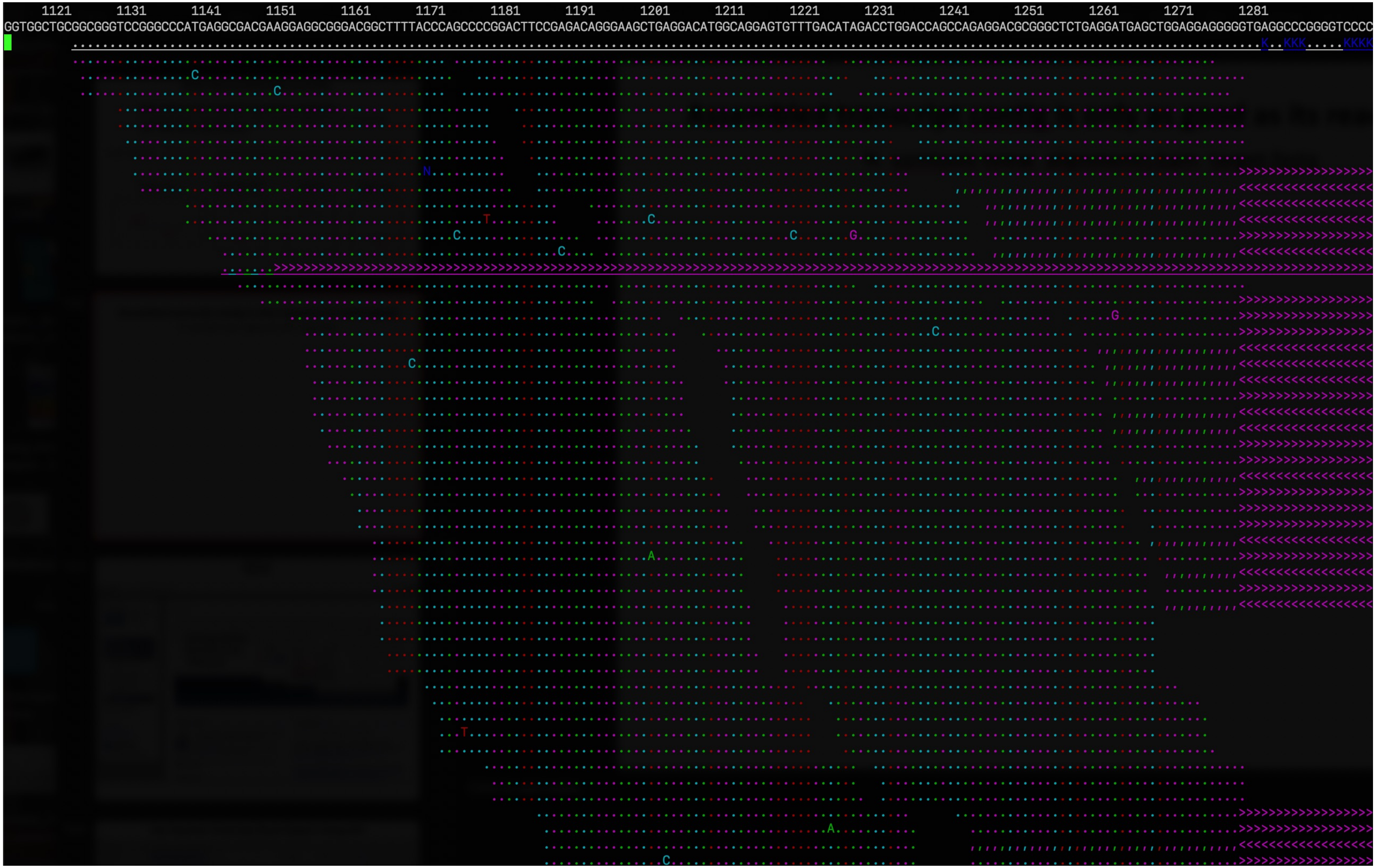


Error type	Transcripts	Assembly	Read evidence
Family collapse	geneAA geneAB geneAC n=3	n=1	bases in reads
Chimerism	geneC geneB n=2	n=1	coverage
Unsupported insertion	n=1	n=1	no reads align to insertion
Incompleteness	n=1	n=1	read pairs align off end of contig
Fragmentation	n=1	n=4	bridging read pairs
Local misassembly	n=1	n=1	read pairs in wrong orientation
Redundancy	n=1	n=3	all reads assign to best contig

Smith-Unna et al. Genome Research, 2016

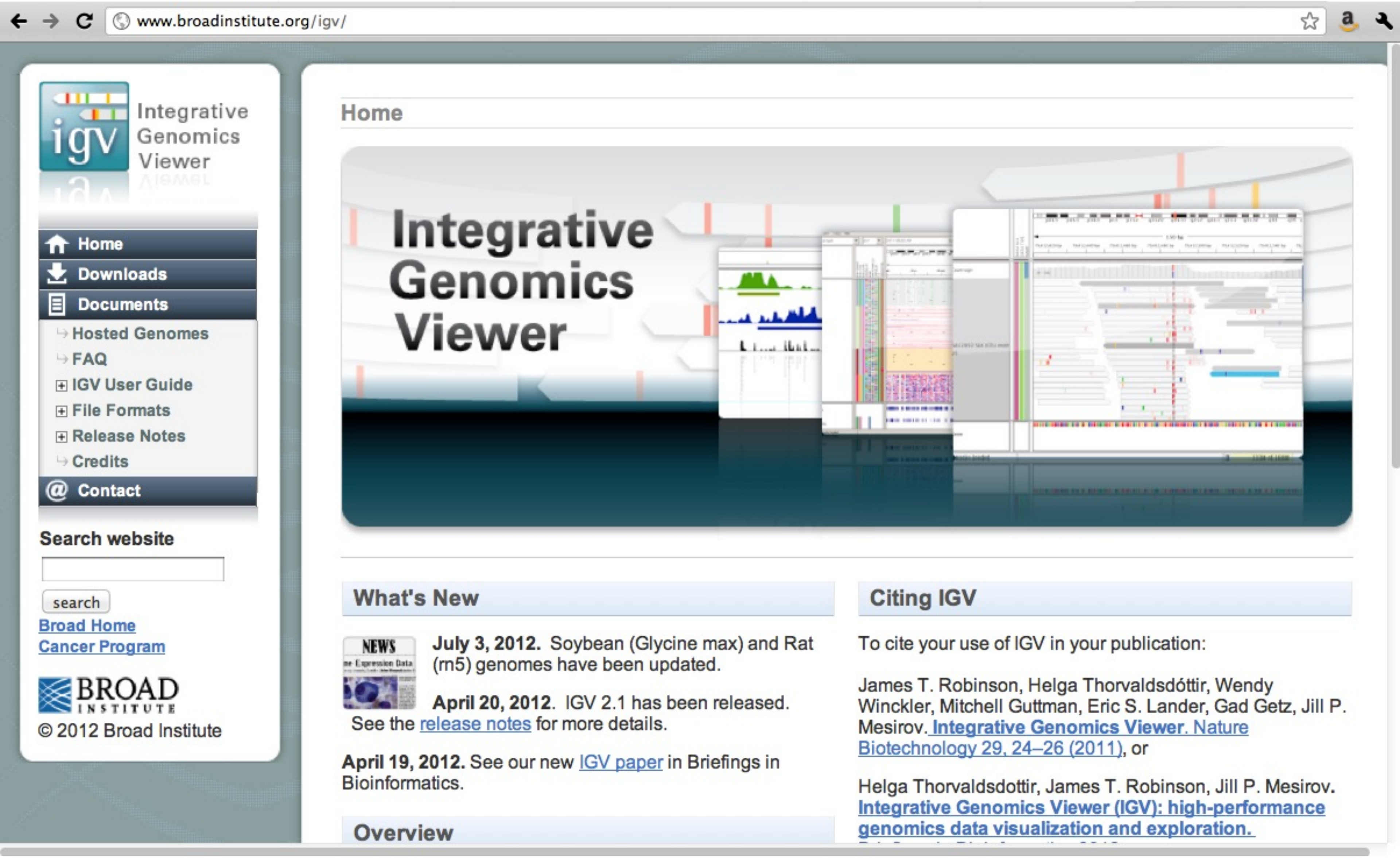
Assembled transcript contig is only as good as its read support.

```
%  samtools tview  alignments.bam  target.fasta
```



```
     1121      1131      1141      1151      1161      1171      1181      1191      1201      1211      1221      1231      1241      1251      1261      1271      1281
GGTGGCTGCGGCGGGTCCGGGCCCATGAGGCGACGAAGGAGGCGGGACGGCTTTTACCCAGCCCCGGACTTCCGAGACAGGGAAGCTGAGGACATGGCAGGAGTGTTTGACATAGACCTGGACCAGCCAGAGGACGCGGGCTCTGAGGATGAGCTGGAGGAGGGGGTGAGGCCCGGGGTCCCC
```

IGV



Home

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

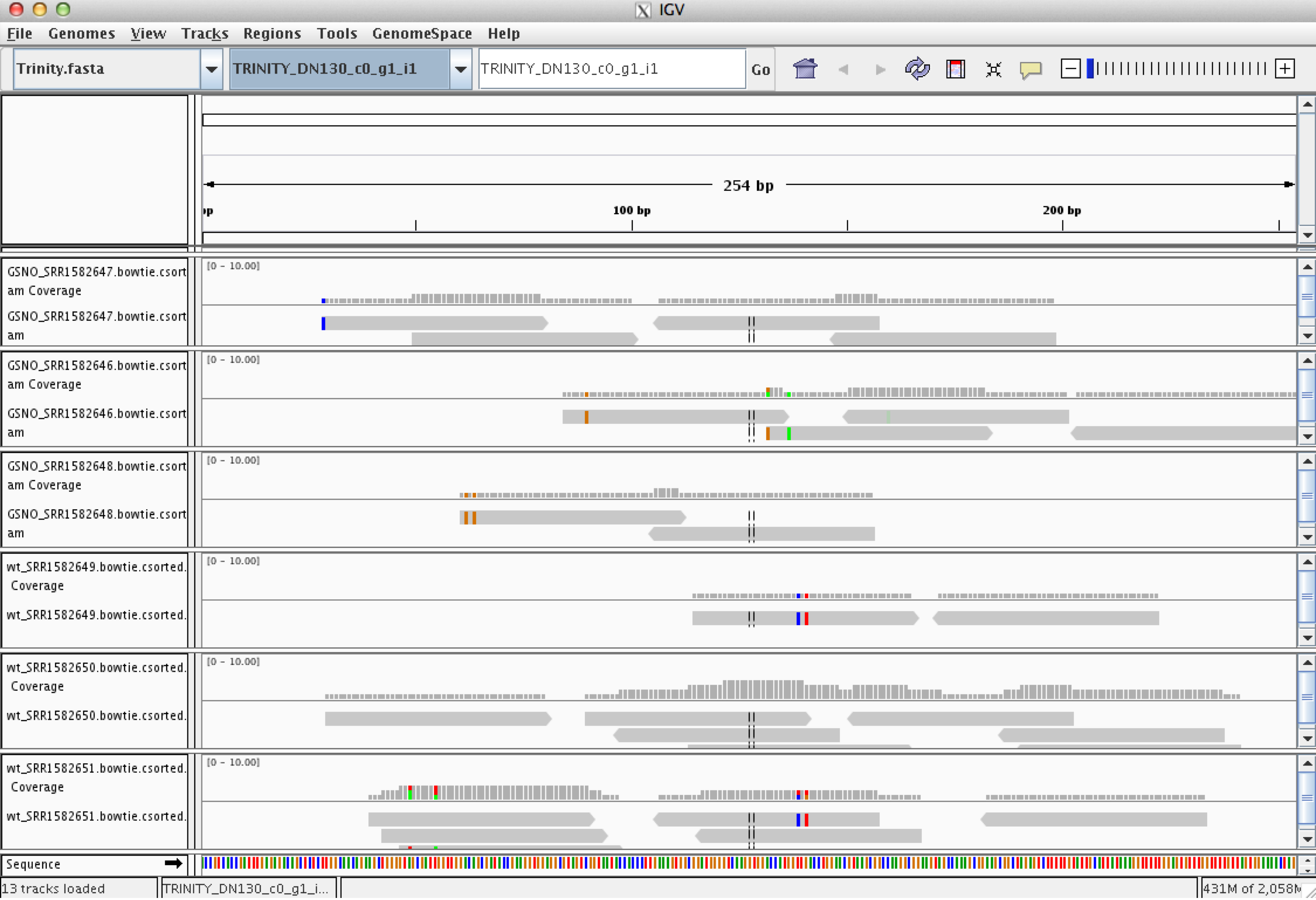
Citing IGV

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

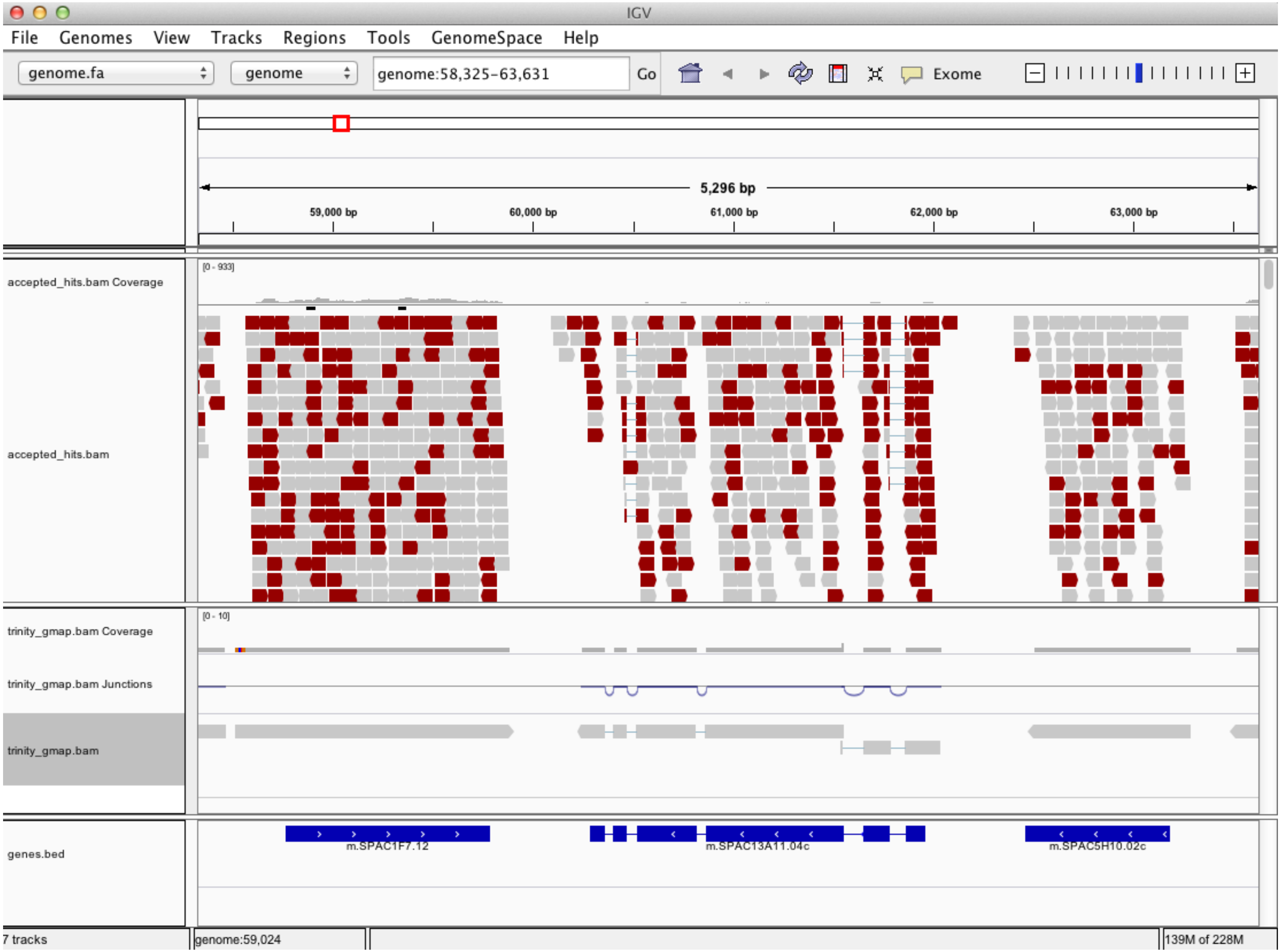
Helga Thorvaldsdottir, James T. Robinson, Jill P. Mesirov. **Integrative Genomics Viewer (IGV): high-performance genomics data visualization and exploration.**

Can Examine Transcript Read Support Using IGV


IGV
File Genomes View Tracks Regions Tools GenomeSpace Help
Trinity.fasta
TRINITY_DN130_c0_g1_i1
TRINITY_DN130_c0_g1_i1
Go
254 bp
100 bp
200 bp
GSNO_SRR1582647.bowtie.csortam Coverage
GSNO_SRR1582647.bowtie.csortam
GSNO_SRR1582646.bowtie.csortam Coverage
GSNO_SRR1582646.bowtie.csortam
GSNO_SRR1582648.bowtie.csortam Coverage
GSNO_SRR1582648.bowtie.csortam
wt_SRR1582649.bowtie.csorted. Coverage
wt_SRR1582649.bowtie.csorted.
wt_SRR1582650.bowtie.csorted. Coverage
wt_SRR1582650.bowtie.csorted.
wt_SRR1582651.bowtie.csorted. Coverage
wt_SRR1582651.bowtie.csorted.
[0 - 10.00]
Sequence
13 tracks loaded
TRINITY_DN130_c0_g1_i...
431M of 2,058M

Can align Trinity transcripts to genome scaffolds to examine intron/exon structures

(Trinity transcripts aligned to the genome using GMAP)

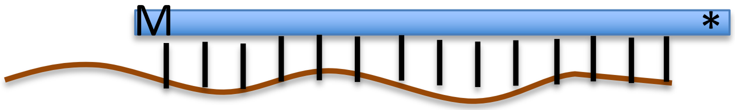


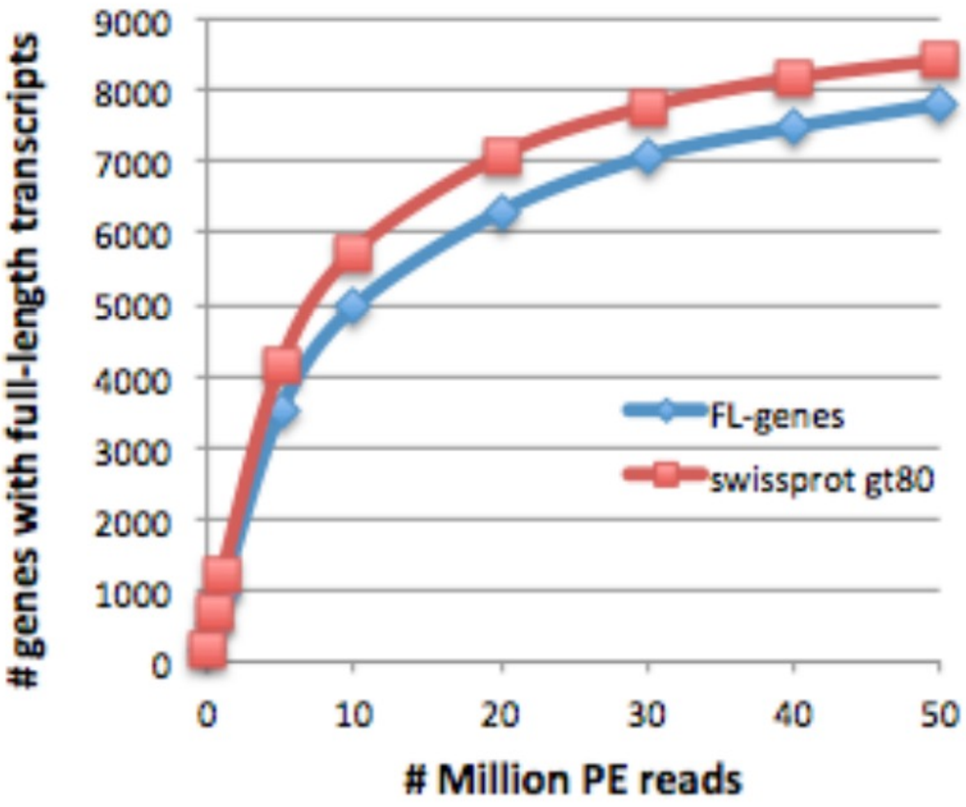
Evaluating the quality of your transcriptome assembly

Full-length Transcript Detection via BLASTX

Known protein (SWISSPROT)

Trinity transcript

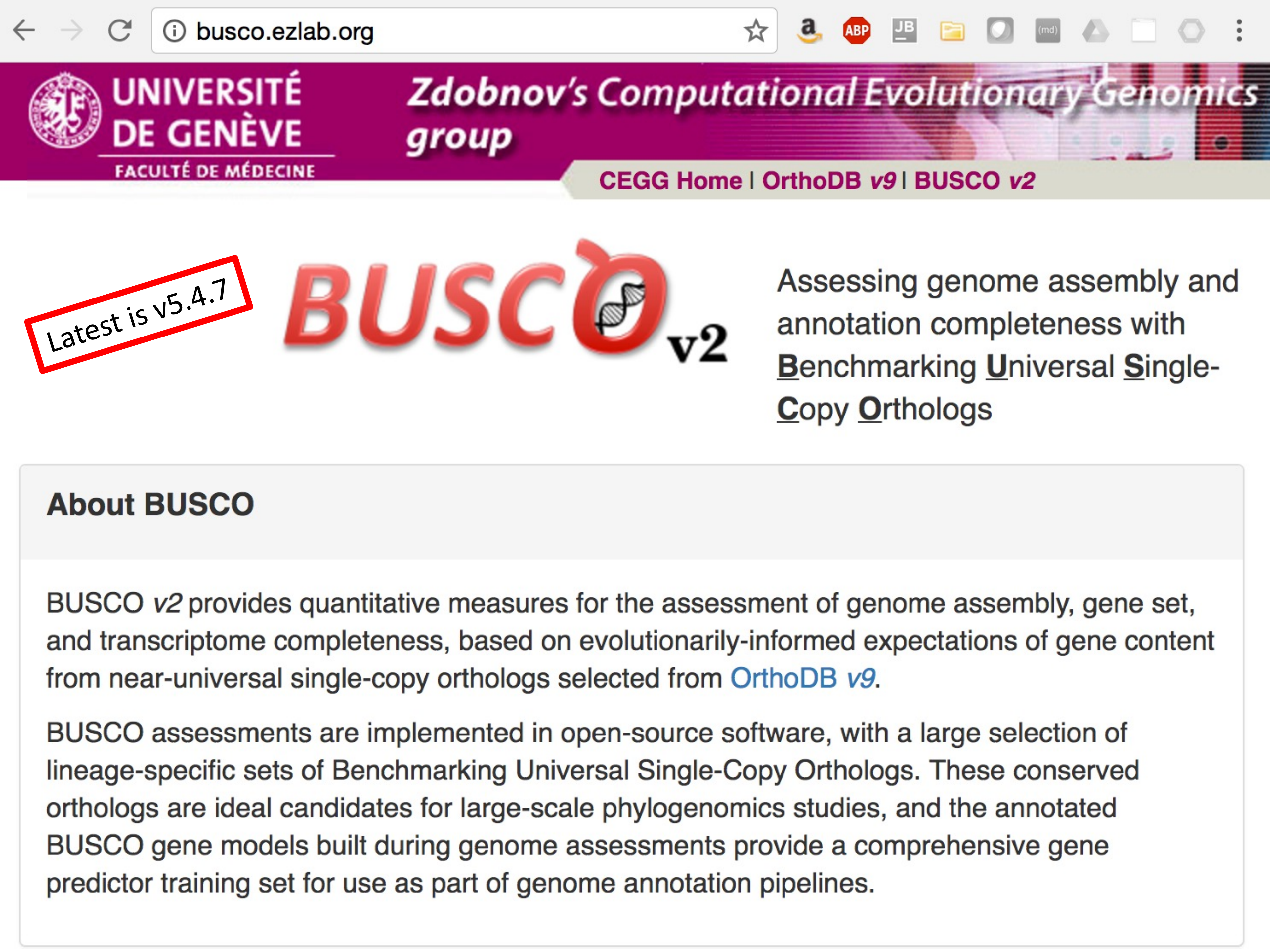




Have you sequenced deeply enough?

* Mouse transcriptome

Haas et al. Nat. Protoc. 2013


busco.ezlab.org
UNIVERSITÉ DE GENÈVE
FACULTÉ DE MÉDECINE
Zdobnov's Computational Evolutionary Genomics group
CEGG Home | OrthoDB *v9* | BUSCO *v2*
BUSCO v2

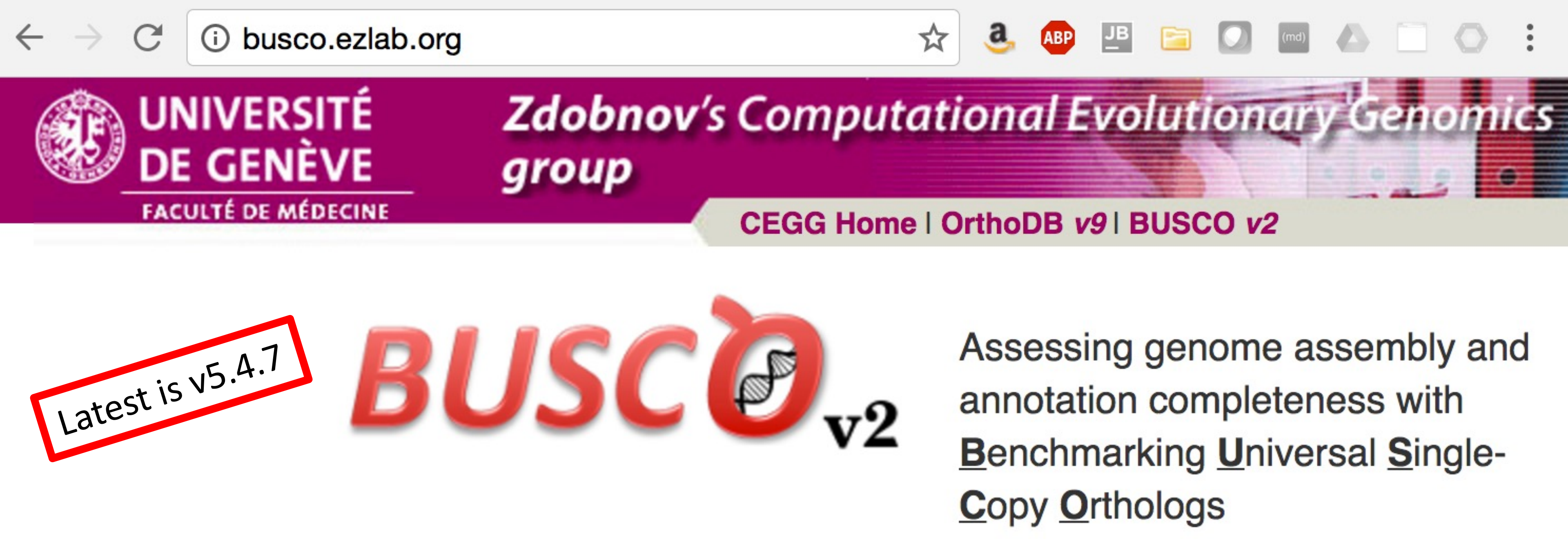
Latest is v5.4.7

Assessing genome assembly and annotation completeness with Benchmarking Universal Single-Copy Orthologs

About BUSCO

BUSCO *v2* provides quantitative measures for the assessment of genome assembly, gene set, and transcriptome completeness, based on evolutionarily-informed expectations of gene content from near-universal single-copy orthologs selected from OrthoDB *v9*.

BUSCO assessments are implemented in open-source software, with a large selection of lineage-specific sets of Benchmarking Universal Single-Copy Orthologs. These conserved orthologs are ideal candidates for large-scale phylogenomics studies, and the annotated BUSCO gene models built during genome assessments provide a comprehensive gene predictor training set for use as part of genome annotation pipelines.

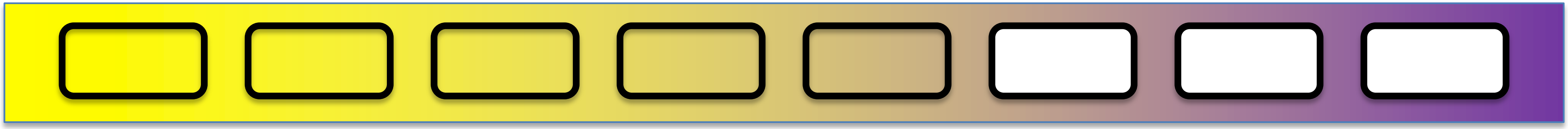

busco.ezlab.org
UNIVERSITÉ DE GENÈVE
FACULTÉ DE MÉDECINE
Zdobnov's Computational Evolutionary Genomics group
CEGG Home | OrthoDB v9 | BUSCO v2
BUSCO v2

Latest is v5.4.7

Assessing genome assembly and annotation completeness with Benchmarking Universal Single-Copy Orthologs

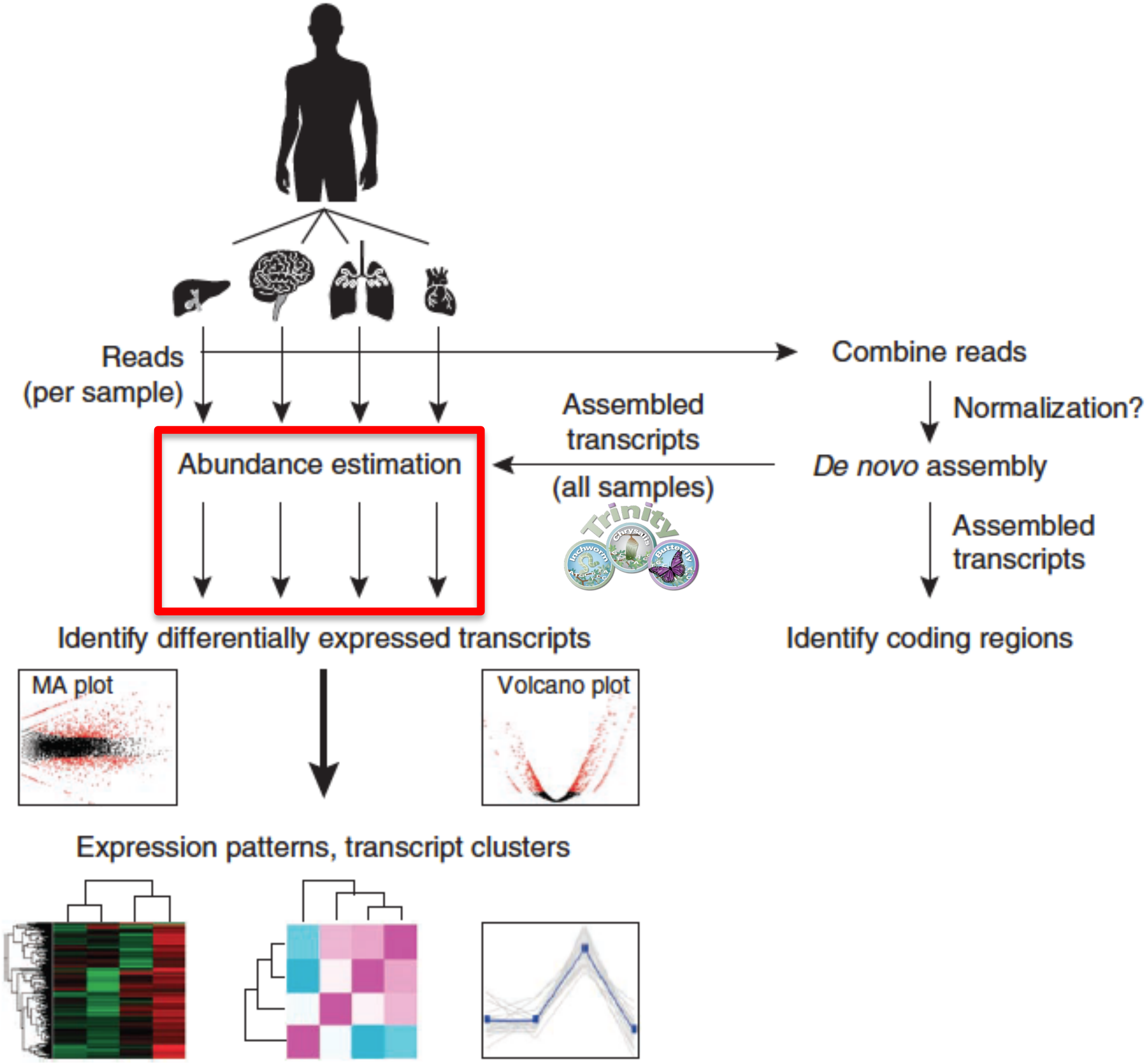
```
#Summarized BUSCO benchmarking for file: Trinity.fasta
#BUSCO was run in mode: trans

Summarized benchmarks in BUSCO notation:
        C:88%[D:53%],F:4.5%,M:7.3%,n:3023

Representing:
        1045    Complete Single-copy BUSCOs
        1617    Complete Duplicated BUSCOs
        139     Fragmented BUSCOs
        222     Missing BUSCOs
        3023    Total BUSCO groups searched
```

Part 5. Expression Quantification



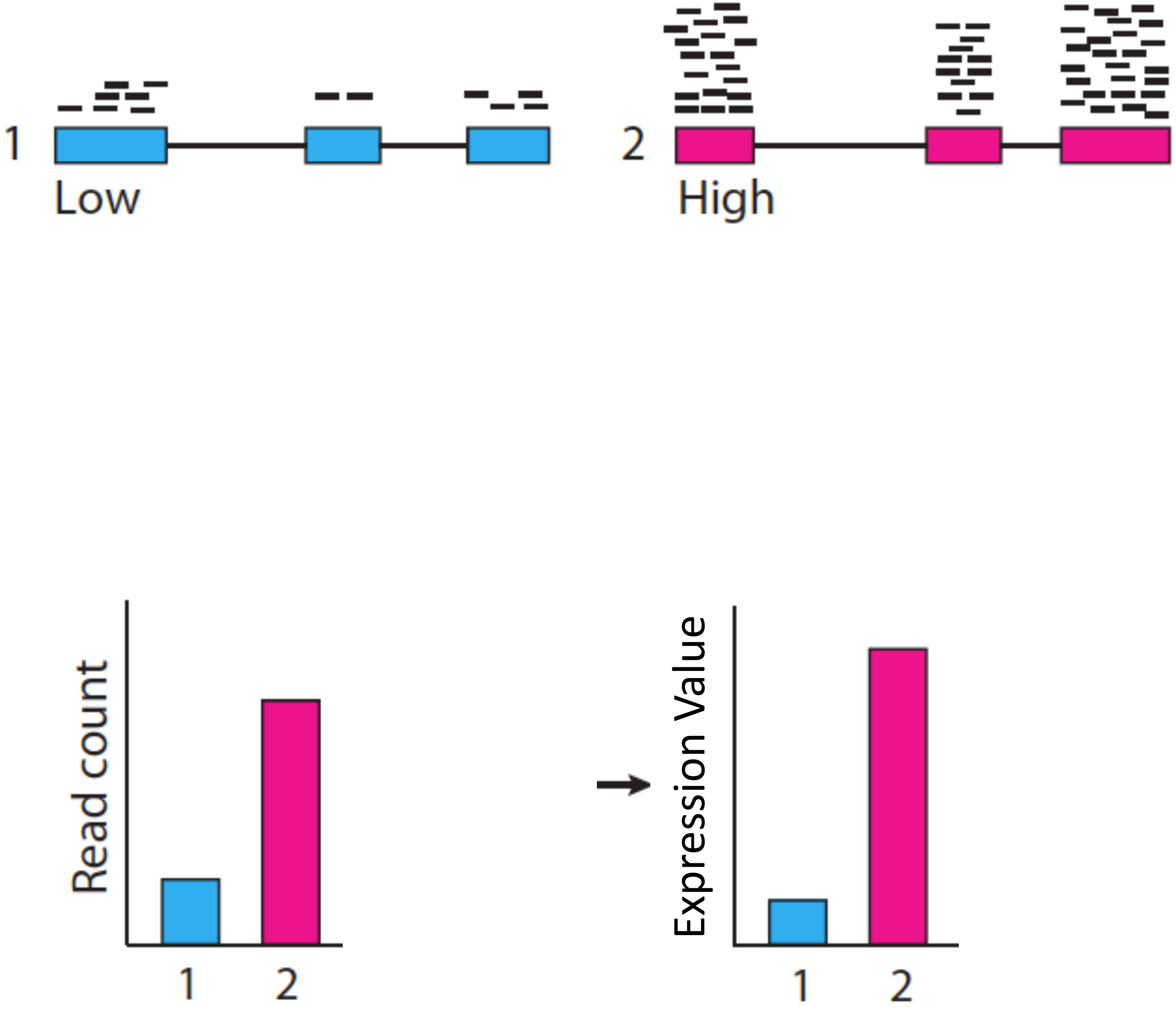
Abundance Estimation

(Aka. Computing Expression Values)



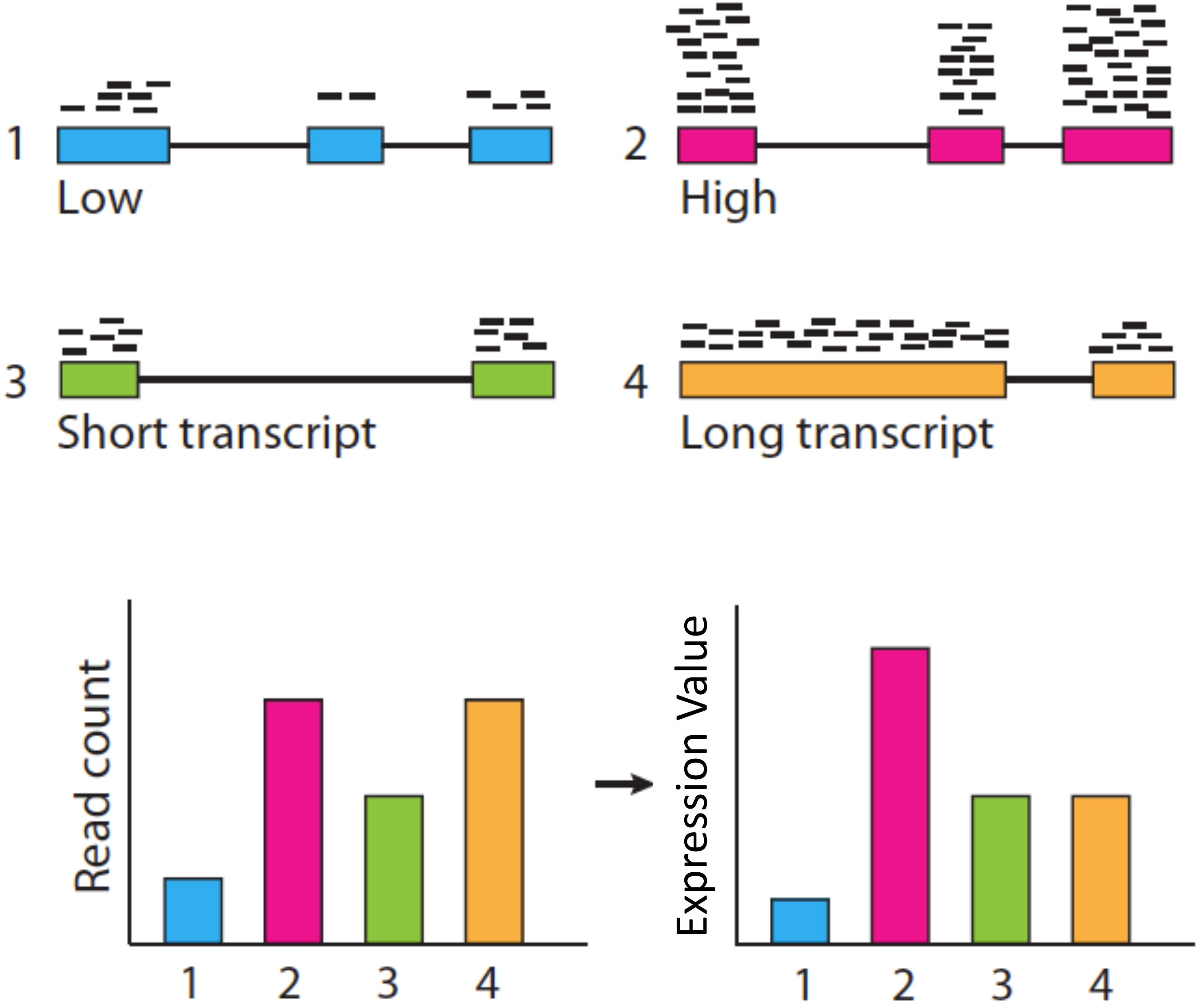
Bioconductor,
& Trinity

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)

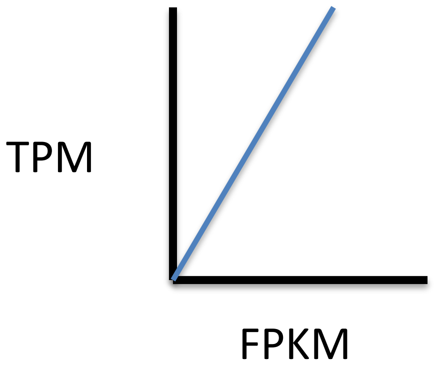
$$TPM_i = \frac{FPKM_i}{\sum_j FPKM} * 1e6$$

Preferred metric for measuring expression

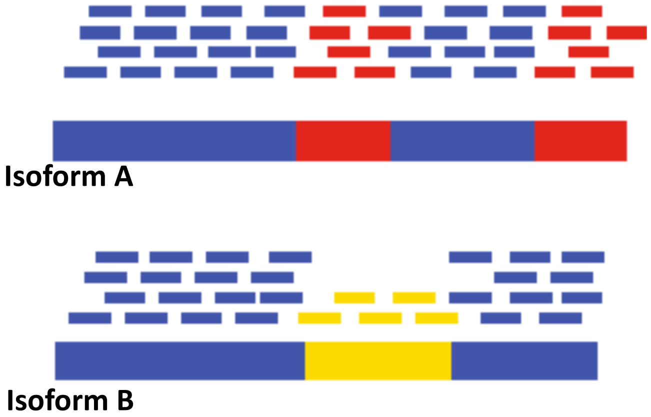
- Better reflects transcript concentration in the sample.
- Nicely sums to 1 million

Linear relationship between TPM and FPKM values.

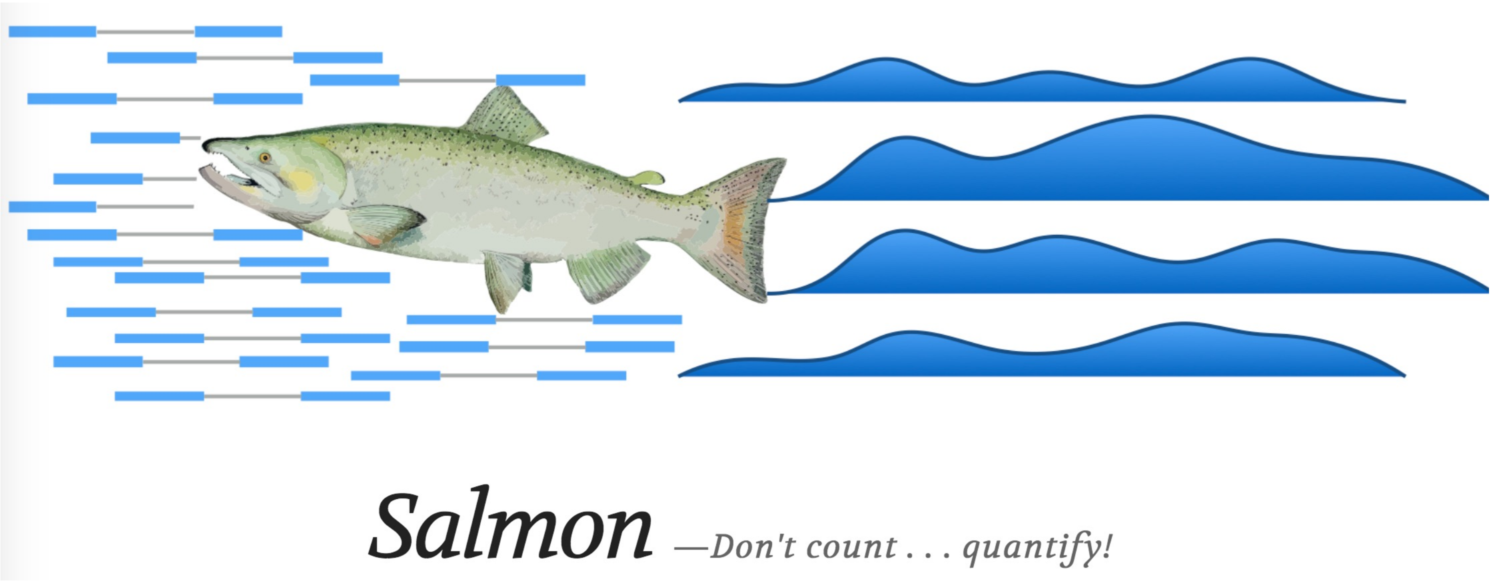
Both are valid metrics, but best to be consistent.



Multiply-mapped Reads Confound Abundance Estimation

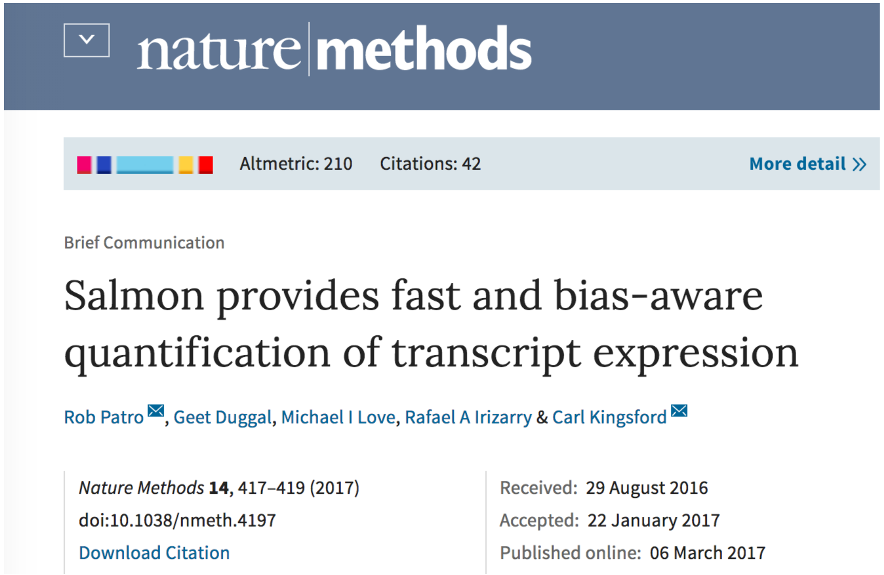


Blue = multiply-mapped reads
Red, Yellow = uniquely-mapped reads



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

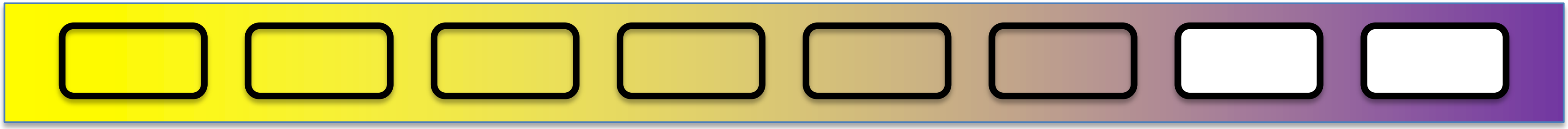
Rob Patro ✉, Geet Duggal, Michael I Love, Rafael A Irizarry & Carl Kingsford ✉

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 6. Differential Expression

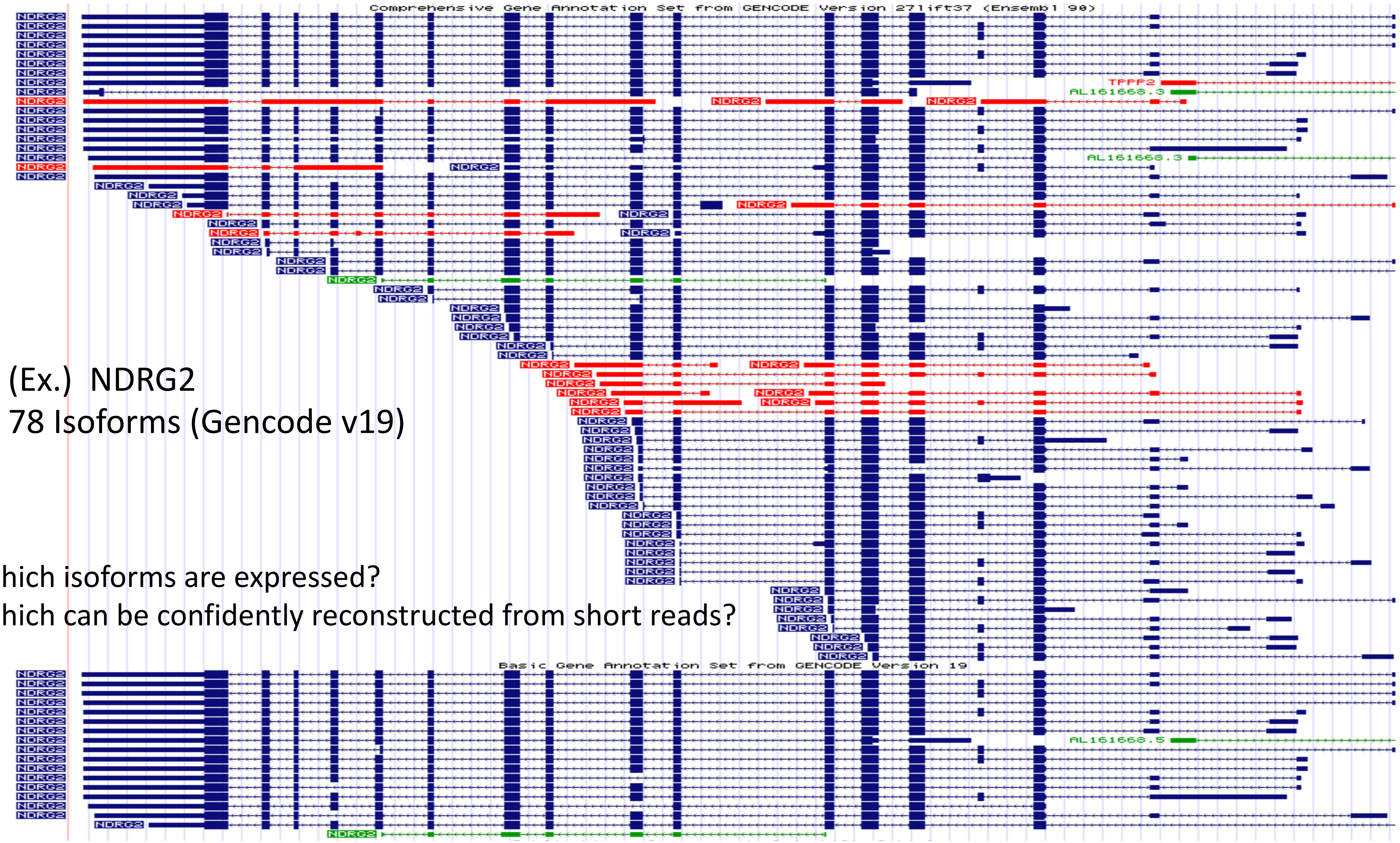


Differential Expression Analysis

After Dinner!! -- Thanks, Rachel !!

Thx, Charlotte Soneson! ☺

Transcript Reconstruction or Expression Analysis can be Quite Difficult at Complex Loci

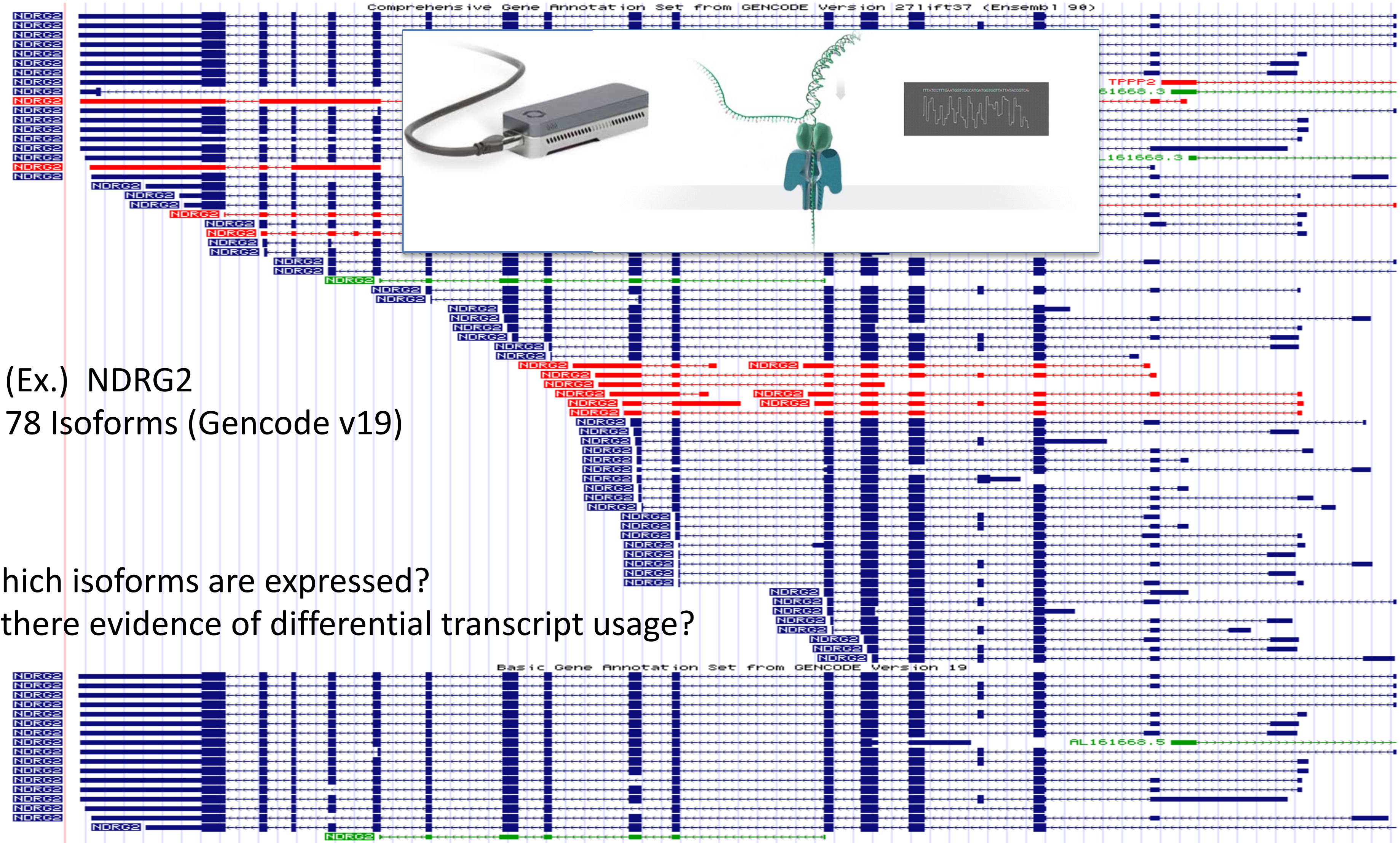


Comprehensive Gene Annotation Set from GENCODE Version 27lift37 (Ensembl 90)
NDRG2
TPPP2
AL161668.3
Basic Gene Annotation Set from GENCODE Version 19
AL161668.5

(Ex.) NDRG2
78 Isoforms (Gencode v19)

Which isoforms are expressed?
Which can be confidently reconstructed from short reads?

Too complex… don’t guess from short reads, use long reads.


Comprehensive Gene Annotation Set from GENCODE Version 27lift37 (Ensembl 90)
NDRG2
TPPP2
AL161668.3
Basic Gene Annotation Set from GENCODE Version 19
AL161668.5

(Ex.) NDRG2
78 Isoforms (Gencode v19)

Which isoforms are expressed?
Is there evidence of differential transcript usage?

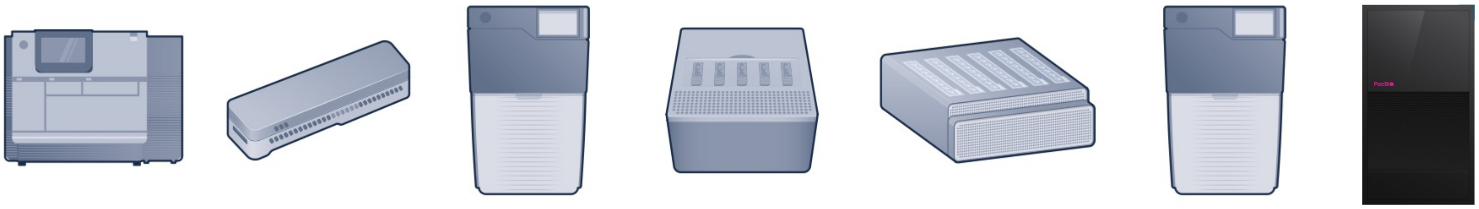
Editorial | Published: 12 January 2023

Method of the Year 2022: long-read sequencing

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

Inflection point for LR transcriptomics

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	
~5 million	1–10 million	400,000	10–30 million	30–150 million	4 million	8 million	Throughput
>1%	5–10%	>1%	5–10%	5–10%	>1%	>1%	Error rate



MAS-seq → **40-120 million cDNA reads**

Info on error rates for long reads – impressive!!

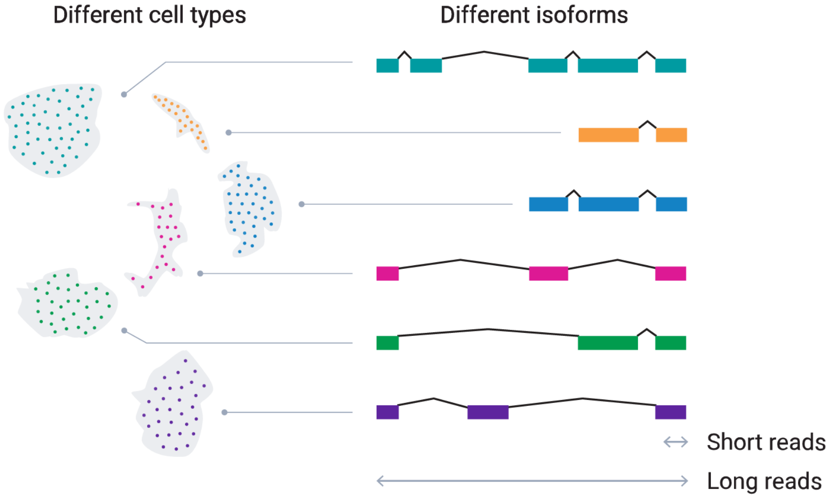
https://nanoporetech.com/accuracy

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

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

Q30 Q40

Long reads for Single Cell Transcriptomes!!



Key Points

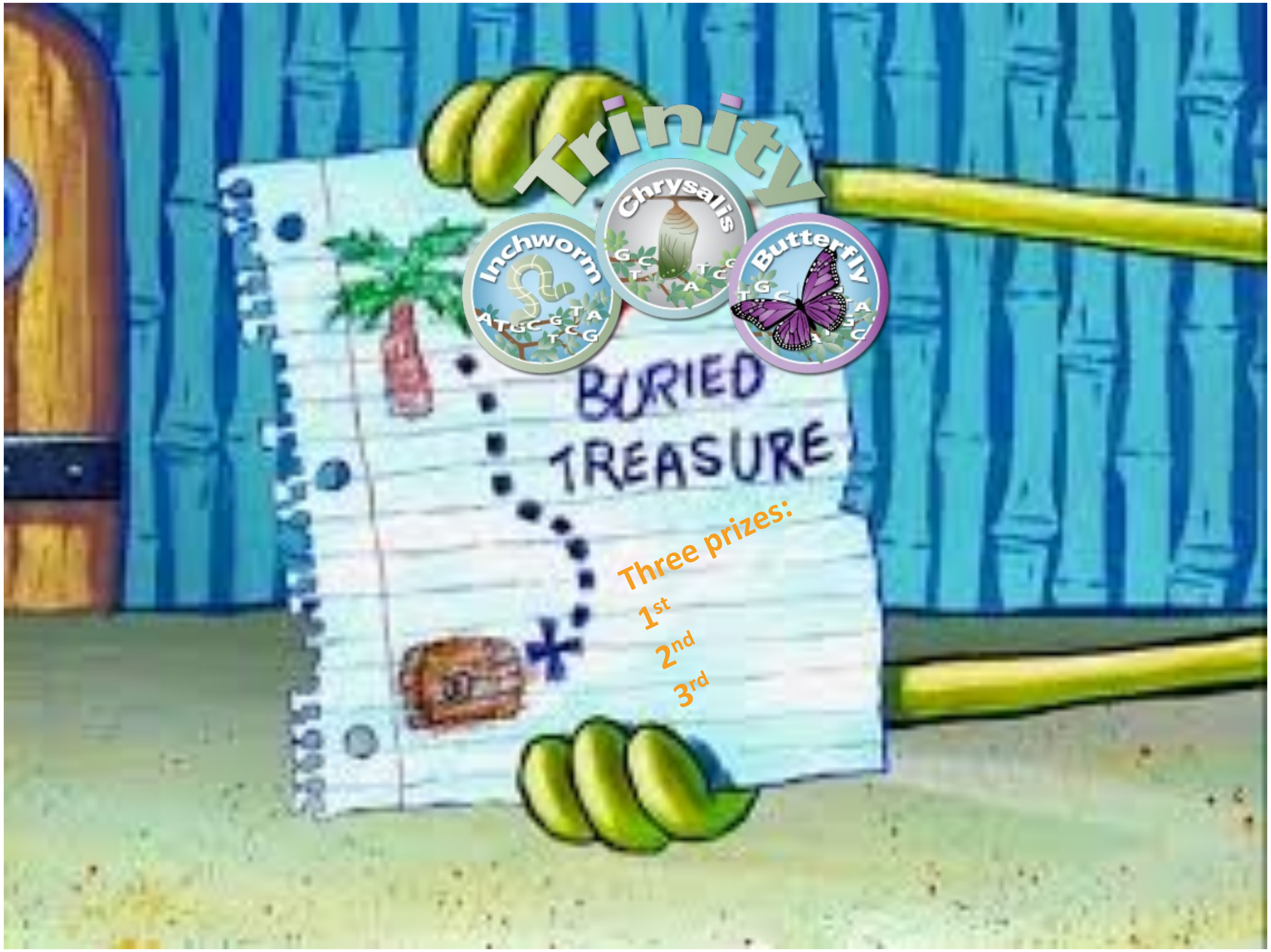
- RNA-seq enables many aspects of biology to be studied at single base & single cell resolution plus spatial context.
- Different isolation/capture methods available
- Reconstruction typically involves graph reconstruction from reads or alignments and path traversal.
- Do strand-specific sequencing whenever possible (eg. TruSeq)
- For QC – examine read support and full-length reconstruction stats.
- Latest advancements: long read transcriptome sequencing yields isoform structure info at single cell resolution (eg. MAS-seq).

Running Trinity

(on small sets of reads)

```
Trinity --left reads.left.fa \
        --right reads.right.fa \
        --seqType fa \
        --max_memory 1G \
        --CPU 1 \
        --output trinity_outdir \
        --no_normalize_reads
```

Trinity Treasure Hunt!!! ☺



Will provide link to the challenge via slack – stay tuned, will start ~ 8pm

Slack channel: #transcriptomicslab