RNA-seq bioinfo analysis

Bilille training
16-17 septembre 2021
Camille Marchet - Pierre Pericard

General Introduction

Goals

This course main goals:

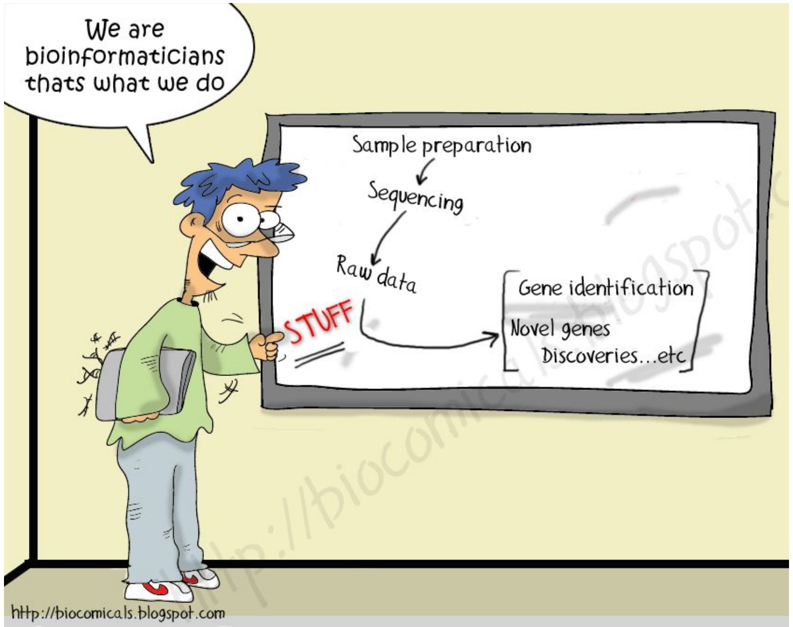
- An overview of RNA-seq data analysis

- Identify the (key issues/points) (critical steps/parameters)

Warning !

This is NOT a course to train you as a bioinformatician, and this course will NOT allow you to design an analysis pipeline set-up for your specific needs

This course WILL give you the basis information to understand and run a generic RNA-seq analysis, its key steps and problematics, and how to interact with bioinformaticians/bioanalysts that can analyze your RNA-seq datasets



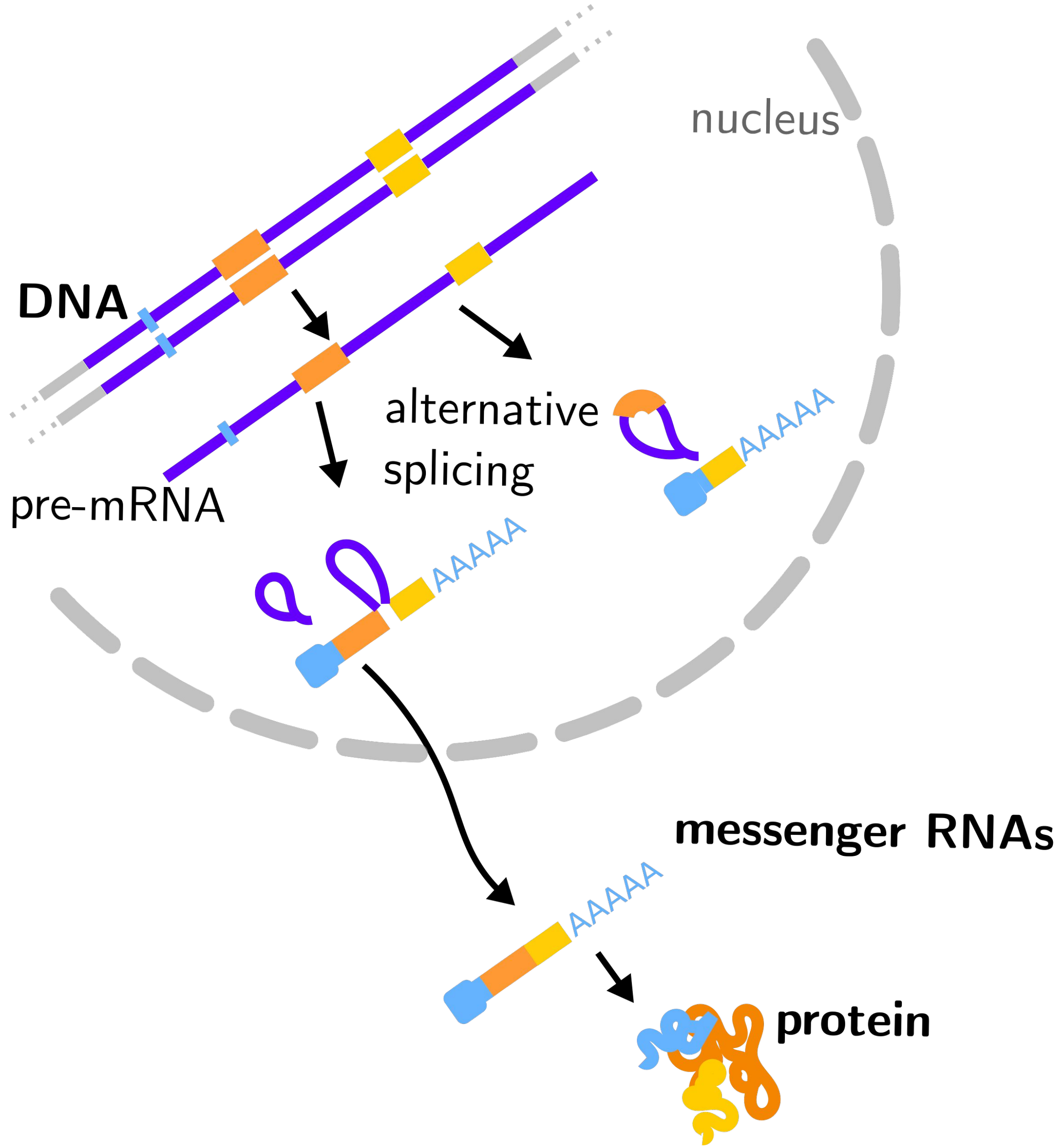
Preliminary

Transcriptome/transcript

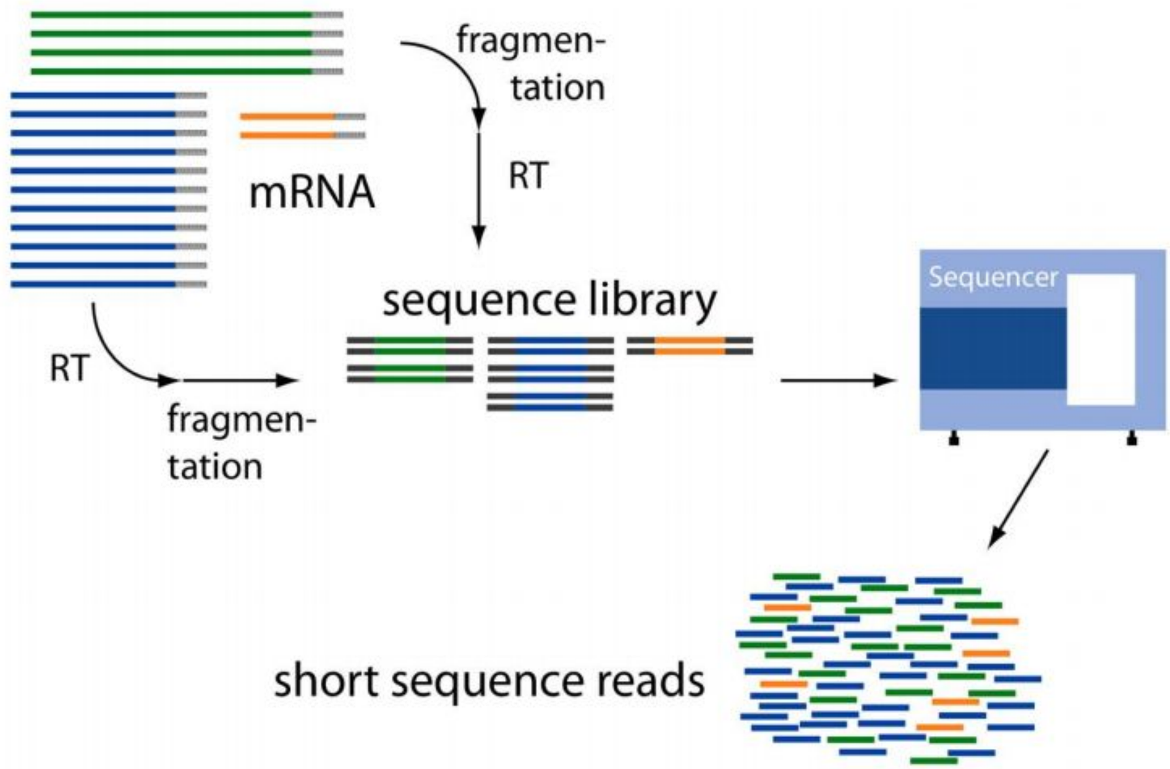
Transcriptomics

(Alternative) isoform

Splicing



Sequencing: overview



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

How to make cDNA libraries

- Extract RNA, convert to cDNA
- pass to next gen sequencer
- millions to billions of reads

make cDNA?

- Prime mRNA with random hexamers R6
- reverse transcriptase => cDNA first strand synthesis
- then second strand

=> illumina cDNA library

How to sequence (1)

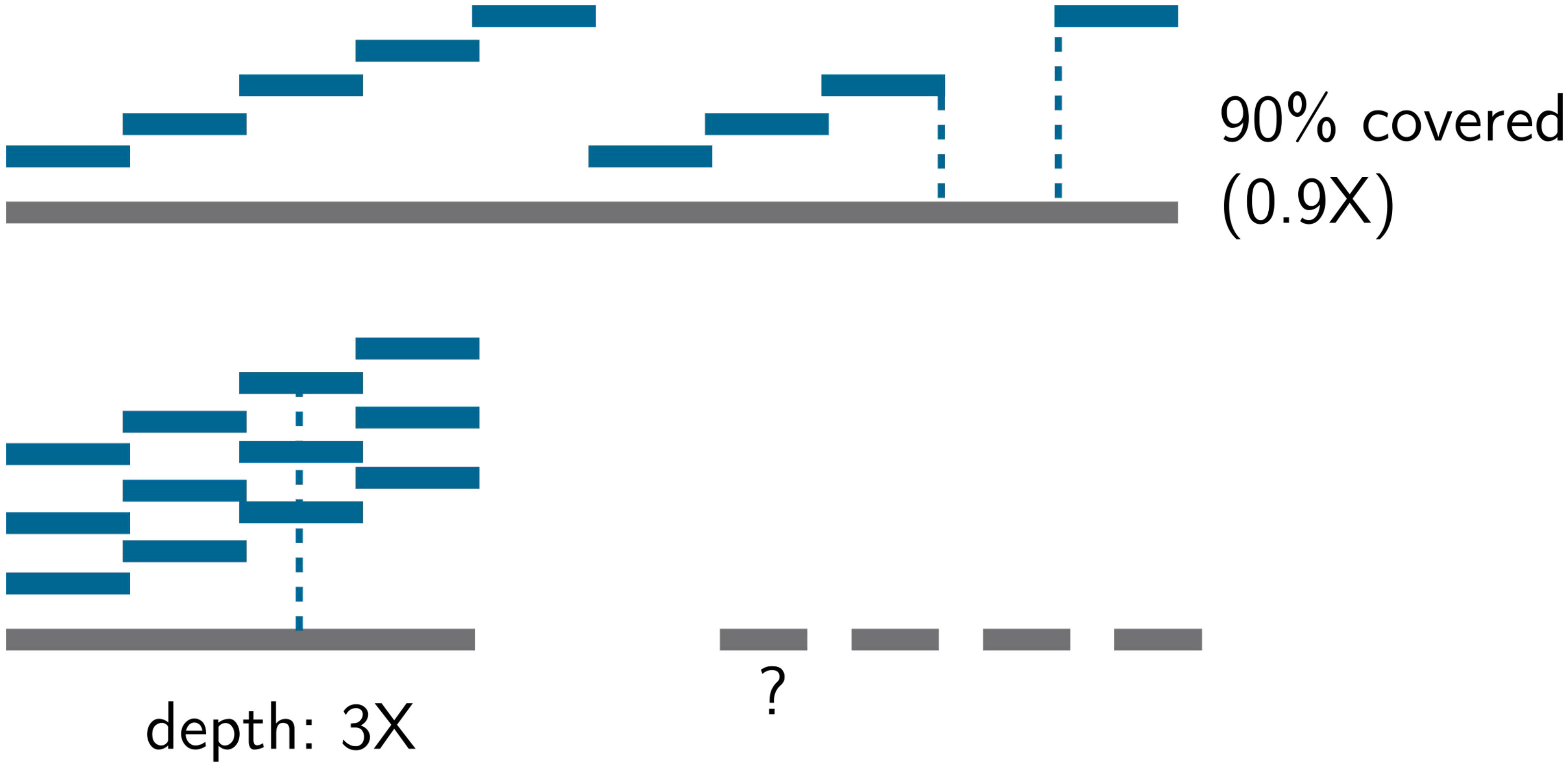
- polyA+
- Ribo-Zero (human, mouse, plants, bacteria, ...)

(ARN = 90% of ARNr, 1-2% of ARNm)

in prokaryotes: no polyA (= no capture), no splicing (= less complex)

- paired-end
- replicates

How to sequence (2)



RNA-seq

-reads around **150-200** bp

-the number of **detected transcripts increases with the sequencing depth**

-the **expression** measure is **more precise with more depth**

-5 millions reads can be enough to detect genes mildly-highly expressed in human

-100 millions must be preferred to detect lowly expressed genes (see for instance **saturation curves** in “Differential expression in RNA-seq: a matter of depth.” *Genome Res.* 2011)

- these numbers depends on the species/tissues (complex splicing...) §

-keep **replicates** in mind

There are plenty of protocols...

Méthode	Description	Référence
mRNA-seq	Identification les ARN messagers.	[Mortazavi et al., 2008]
miRNA-seq	Identification les micro ARN.	[Ruby et al., 2006]
GRO-Seq (Global Run-On Sequencing), PRO-Seq (Precision Run-On Sequencing) et NET-Seq (Native elongation transcript sequencing)	Sélection et séquençage uniquement les ARNs en cours de transcription par l'ARN polymérase II.	[Core et al., 2008] [Kwak et al., 2013] [Churchman and Weissman, 2011]
Ribo-Seq (Ribosome profile sequencing) et TRAP-Seq (Targeted purification of polysomal mRNA sequencing)	Identification les ARNs messagers en cours de traduction.	[Ingolia et al., 2009] [Reynoso et al., 2015]
RIP-Seq (RNA immunoprecipition sequencing), CLIP-Seq (Cross-linking and immunoprecipitation sequencing), PAR-CLIP (Photoactivatable-ribonucleoside-enhanced cross-linking and immunoprecipitation) et iCLIP (individual-nucleotide resolution CLIP)	Détermination des régions d'ARN liées à une protéine d'intérêt.	[Cloonan et al., 2008] [Chi et al., 2009] [Hafner et al., 2010] [Huppertz et al., 2014]
ChIRP-Seq (Chromatine isolation by RNA purification)	Identification des régions du génome qui interagissent avec l'ARN.	[Chu et al., 2011]
PARE-Seq (Parallel analysis RNA ends sequencing)	Etude des sites de clivage des micro-ARNs ainsi que de la dégradation des ARNs.	[German et al., 2009]

from Clara Benoit Pilven's PhD thesis

Resources: genomes, transcriptomes, annotations

Common databases

e!Ensembl

UCSC

National Center for Biotechnology Information
NCBI

Specific databases

FlyBase

EcoCyc

MicroScope

GENCODE

SGD Saccharomyces GENOME DATABASE

AmoebaDB
Amoeba Genomics Resource

VectorBase
Bioinformatics Resource for Invertebrate Vectors of Human Pathogens

From Rachel Legendre (Institut Pasteur)

FASTA/Q formats

FASTA format:

```
>61DFRAAXX100204:1:100:10494:3070/1
AAACAACAGGGCACATTGTCACTCTT
GTATTTGAAAAACACTTTCCGGCCAT
```

FASTQ format:

```
@61DFRAAXX100204:1:100:10494:3070/1
AAACAACAGGGCACATTGTCACTCTTGTATTTGAAAAACACTTTCCGGCCAT
+
ACCCCCCCCCCCCCCCCCCCCCCCCCCCCCBC?CCCCCCCCC@@CACCCCCA
```

FASTA/Q formats

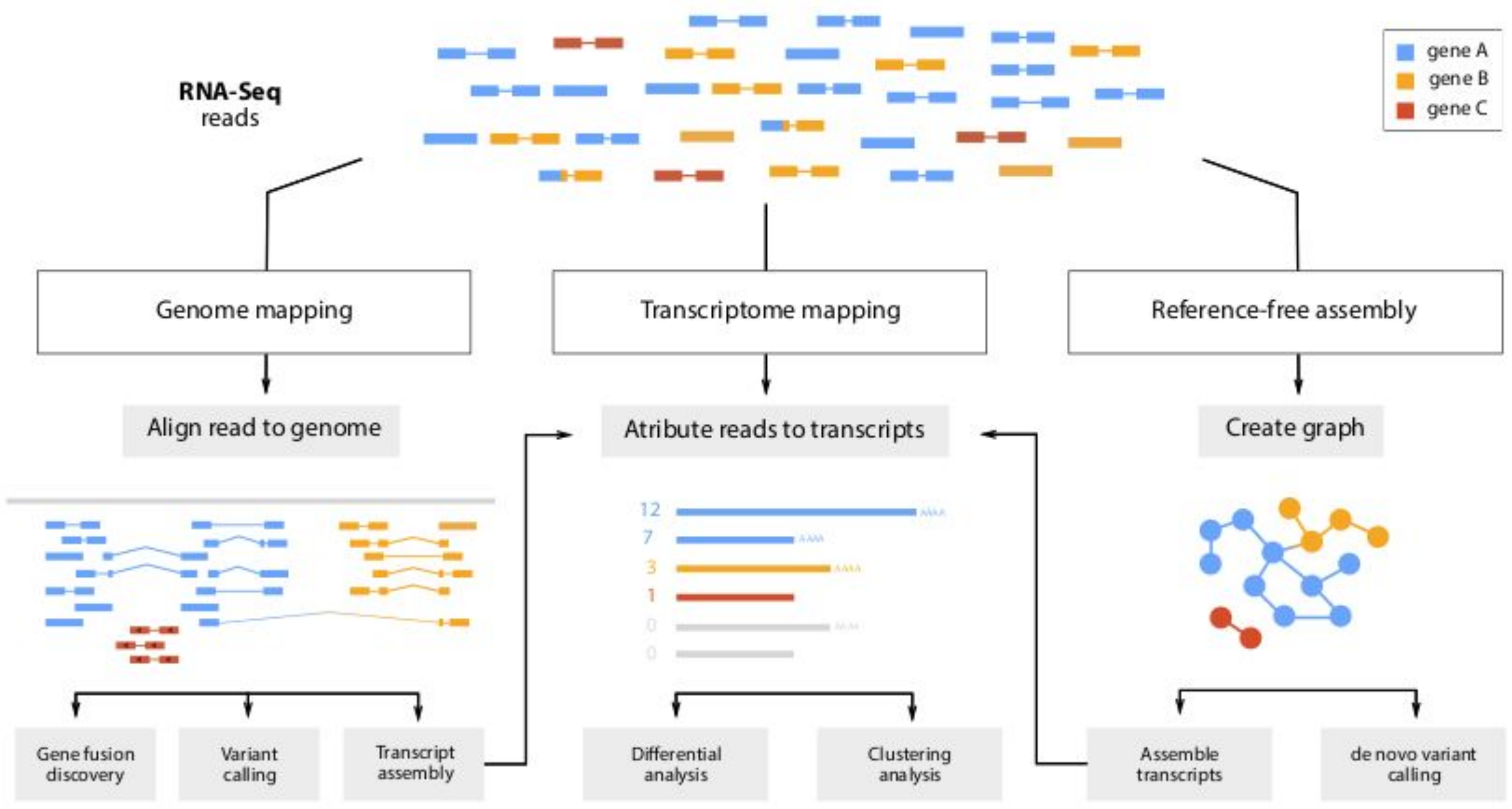
```
SSSSSSSSSSSSSSSSSSSSSSSSSSSSSSSSSSSSSSSSS.....................................................
..........................XXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXX......................
...............................IIIIIIIIIIIIIIIIIIIIIIIIIIIIIIIIIIIIIIIII......................
.................................JJJJJJJJJJJJJJJJJJJJJJJJJJJJJJJJJJJJJJJJ......................
LLLLLLLLLLLLLLLLLLLLLLLLLLLLLLLLLLLLLLLLLLL....................................................
!"#$%&'()*+,-./0123456789:;<=>?@ABCDEFGHIJKLMNOPQRSTUVWXYZ[\]^_`abcdefghijklmnopqrstuvwxyz{|}~
|                         |    |        |                              |                     |
33                        59   64       73                            104                   126
0........................26...31.......40
                         -5....0........9.............................40
                               0........9.............................40
                                  3.....9..............................41
0.2......................26...31........41

S - Sanger        Phred+33,  raw reads typically (0, 40)
X - Solexa        Solexa+64, raw reads typically (-5, 40)
I - Illumina 1.3+ Phred+64,  raw reads typically (0, 40)
J - Illumina 1.5+ Phred+64,  raw reads typically (3, 41)
    with 0=unused, 1=unused, 2=Read Segment Quality Control Indicator (bold)
    (Note: See discussion above).
L - Illumina 1.8+ Phred+33,  raw reads typically (0, 41)
```

$$Q_{\text{sanger}} = -10 \log_{10} p$$

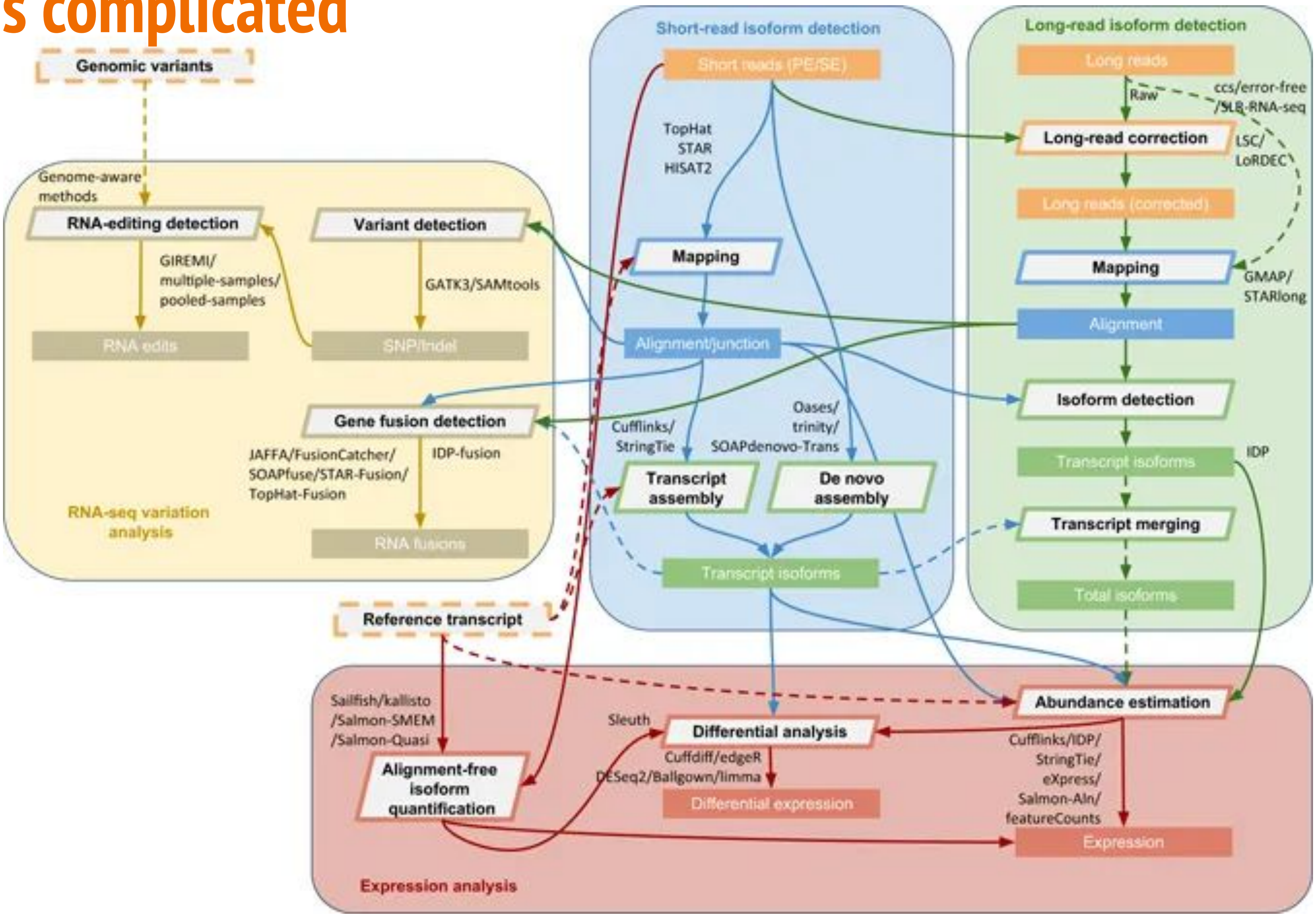
Quality	Error rate
10	10%
20	1%
30	0.1%
40	0.01%

What people do with their RNA-seq



From J. Audoux's PhD thesis

It’s complicated



Nature Communications **8**, Article number: 59 (2017)

Outcomes of RNA-seq studies

- gene annotation
- protein/function prediction
- gene/splicing quantification
- isoform discovery/fusion transcripts/lncRNA...
- variant calling
- methylations
- RNA structures
-

Cleaning - Preprocessing

Known biases in RNA-seq

?

Known biases in RNA-seq

Biological sample:

- presence of pre-mRNA
- 3’ bias over-represented (RNA degradation)
- contaminations

Library preparation:

- DNAse fail
- pcr bias
- variable insert size (smaller than sequencing length)
- reads with no inserts

Sequencing:

- quality drops at the end of reads

Quality Control (QC)

Quality Control (QC) is important to:

- Check if your sample sequencing went well
- Know when you need to sequence again (sequencing platform QC fail)
- Identify potential problems that can be fixed, or not
- Follow the impact of preprocessing steps

⇒ FastQC (https://www.bioinformatics.babraham.ac.uk/projects/fastqc/)

+ MultiQC (https://multiqc.info/) when comparing multiple datasets

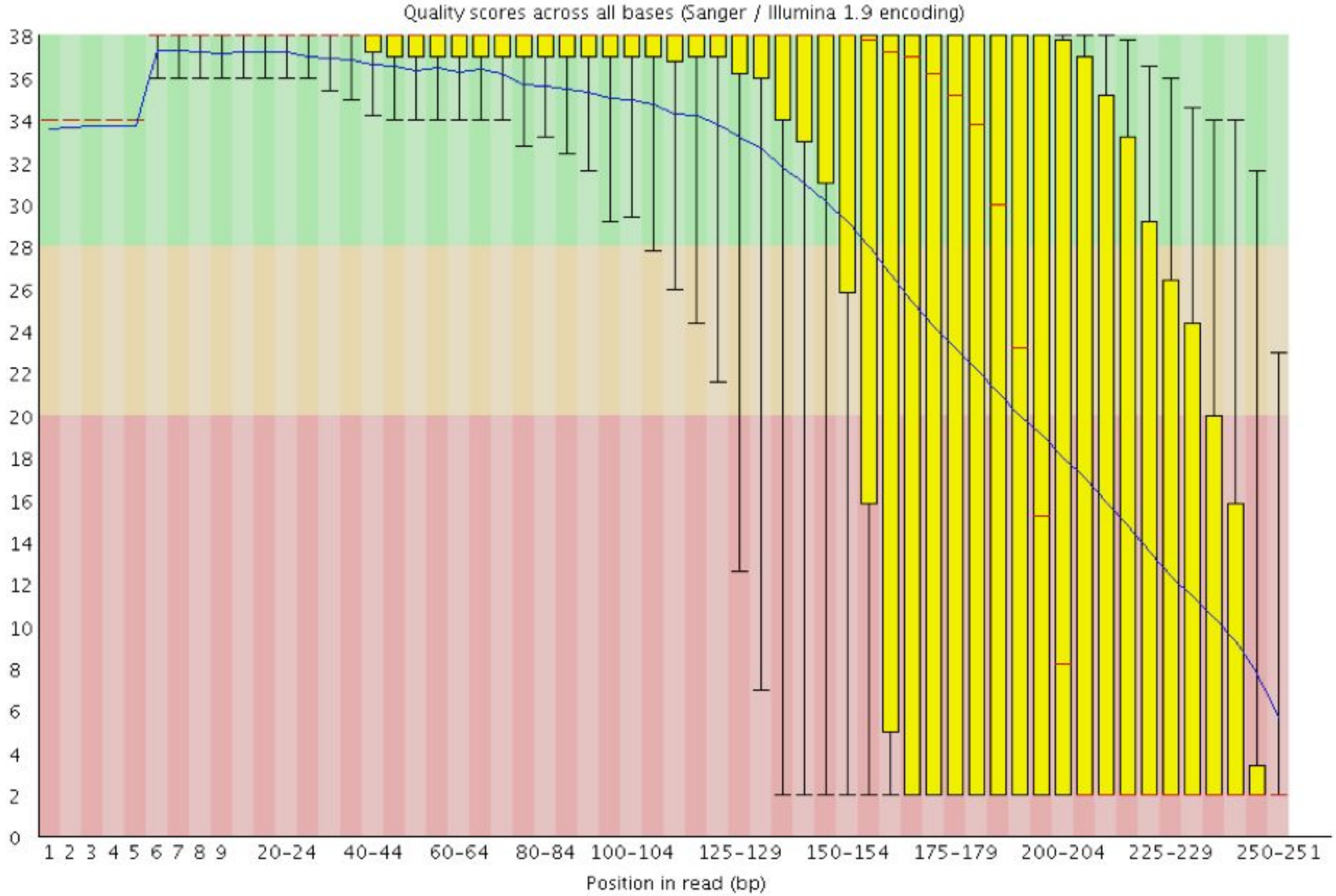
Practical: Quality Control (QC)

Open Galaxy

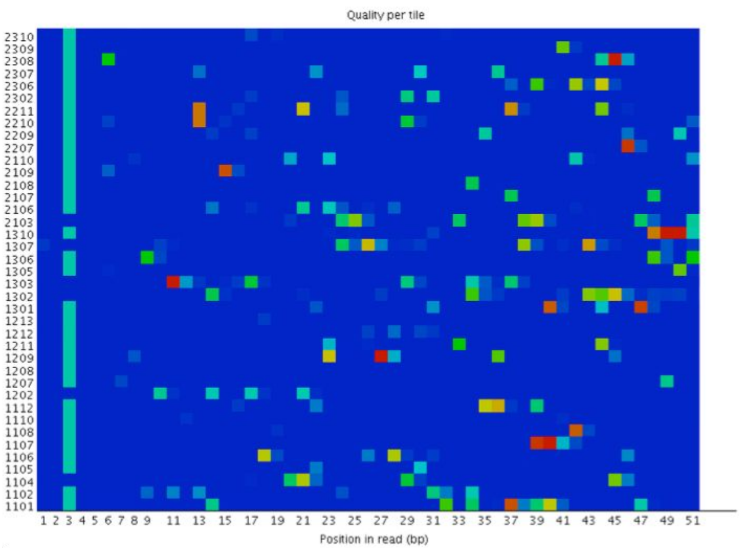
GTN Practical: [Reference-based RNA-seq data analysis]

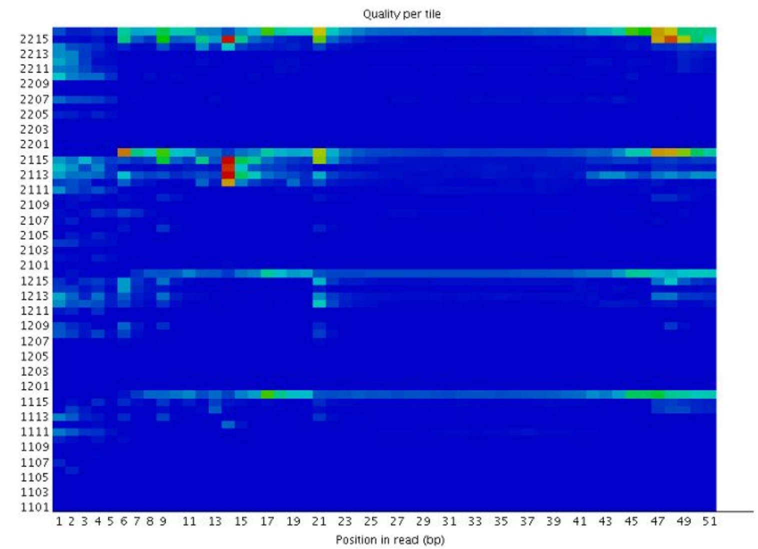
Loss of base call accuracy with increasing sequencing cycles

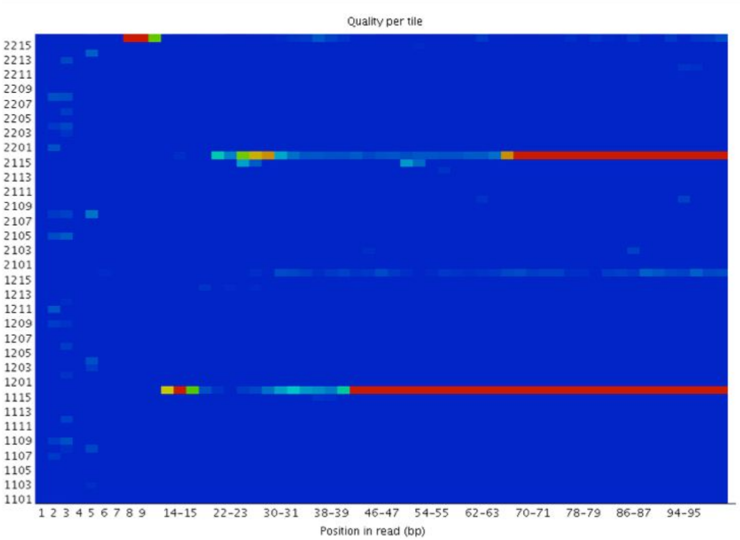
Source: https://sequencing.qcfail.com

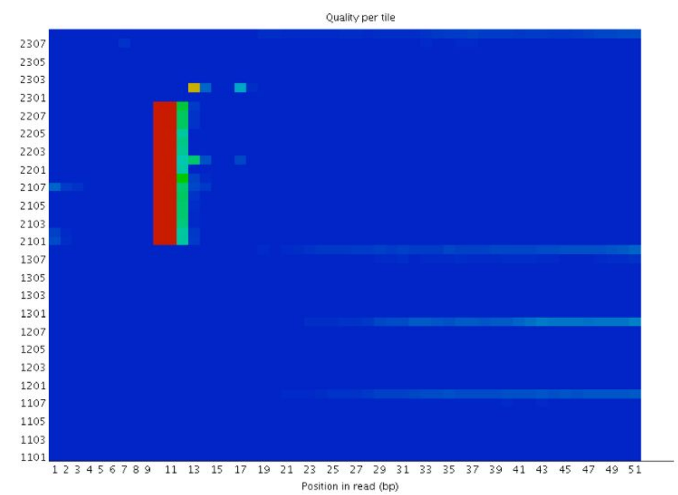


Position specific failures of flowcells





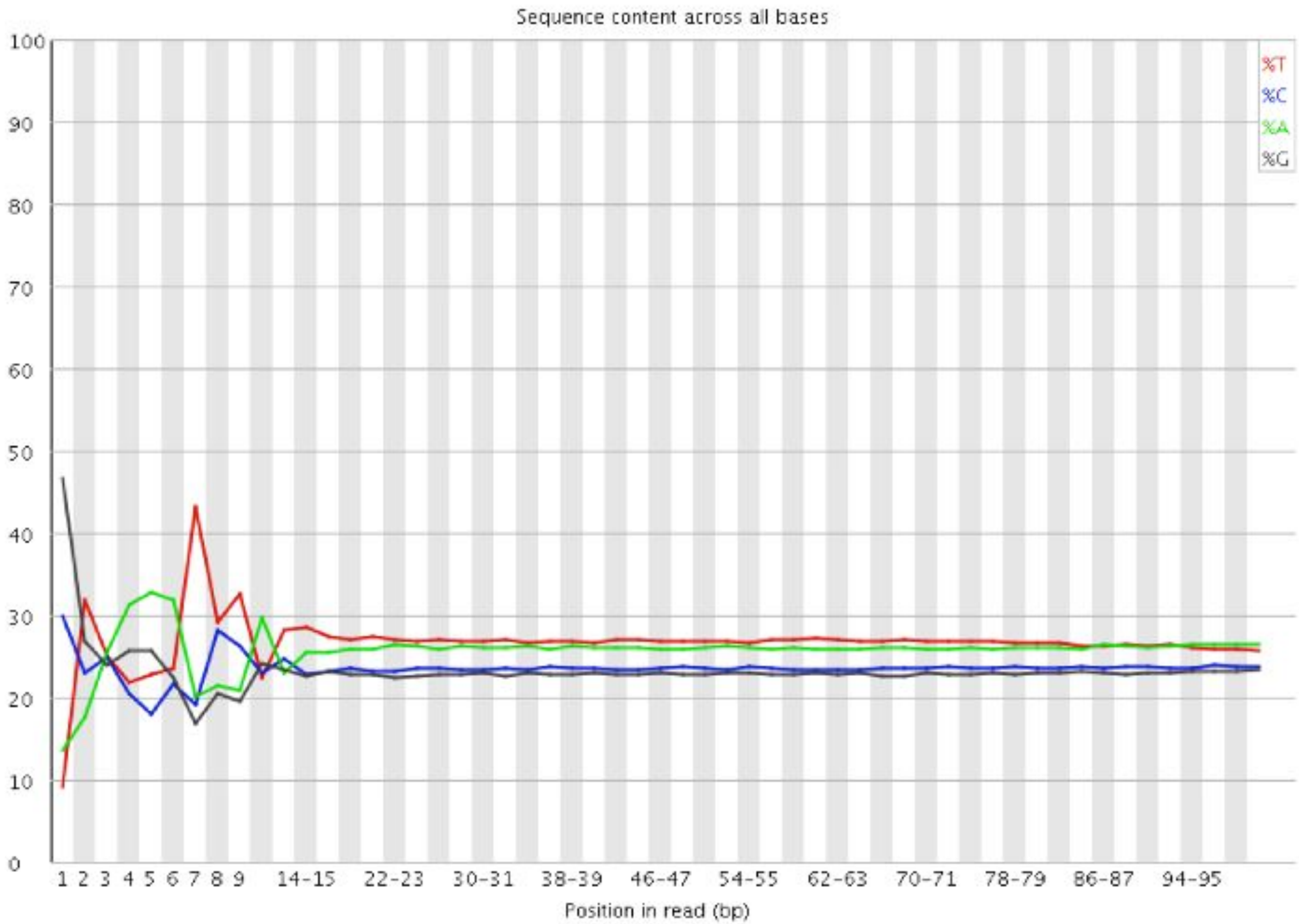




Source: https://sequencing.qcfail.com

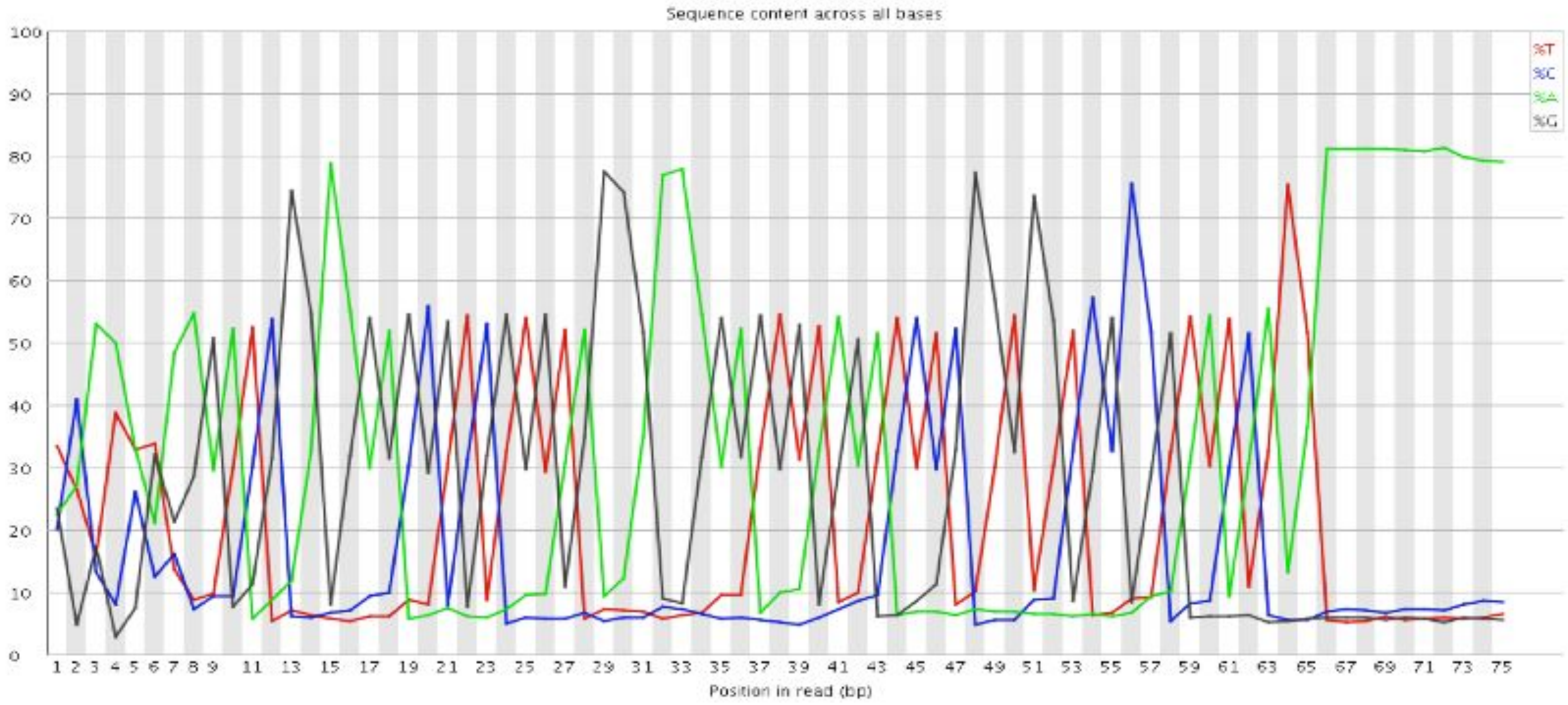
Positional sequence bias in random primed libraries

Source: https://sequencing.qcfail.com



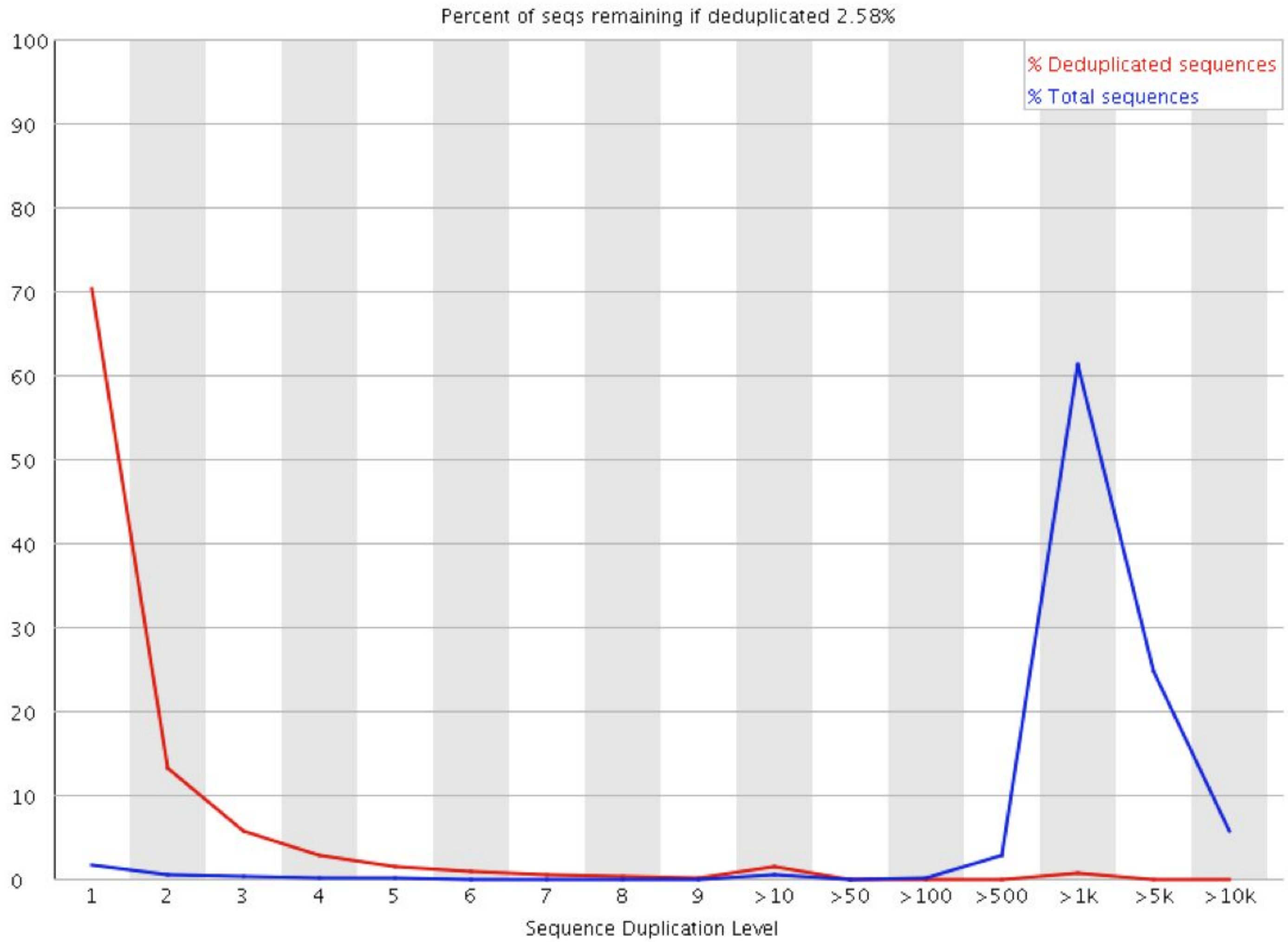
Contamination with adapter dimers

Source: https://sequencing.qcfail.com

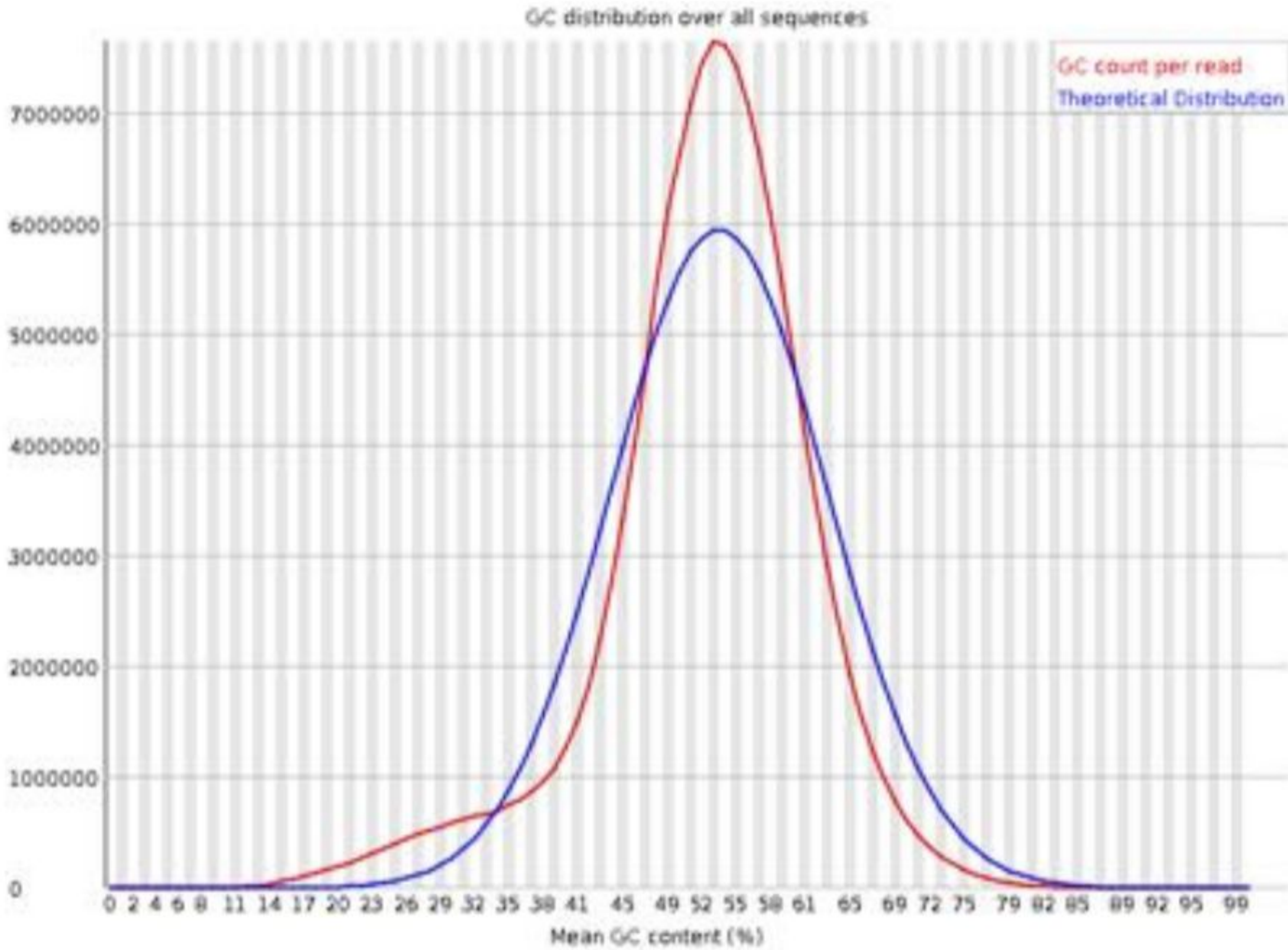


Libraries contain technical duplication

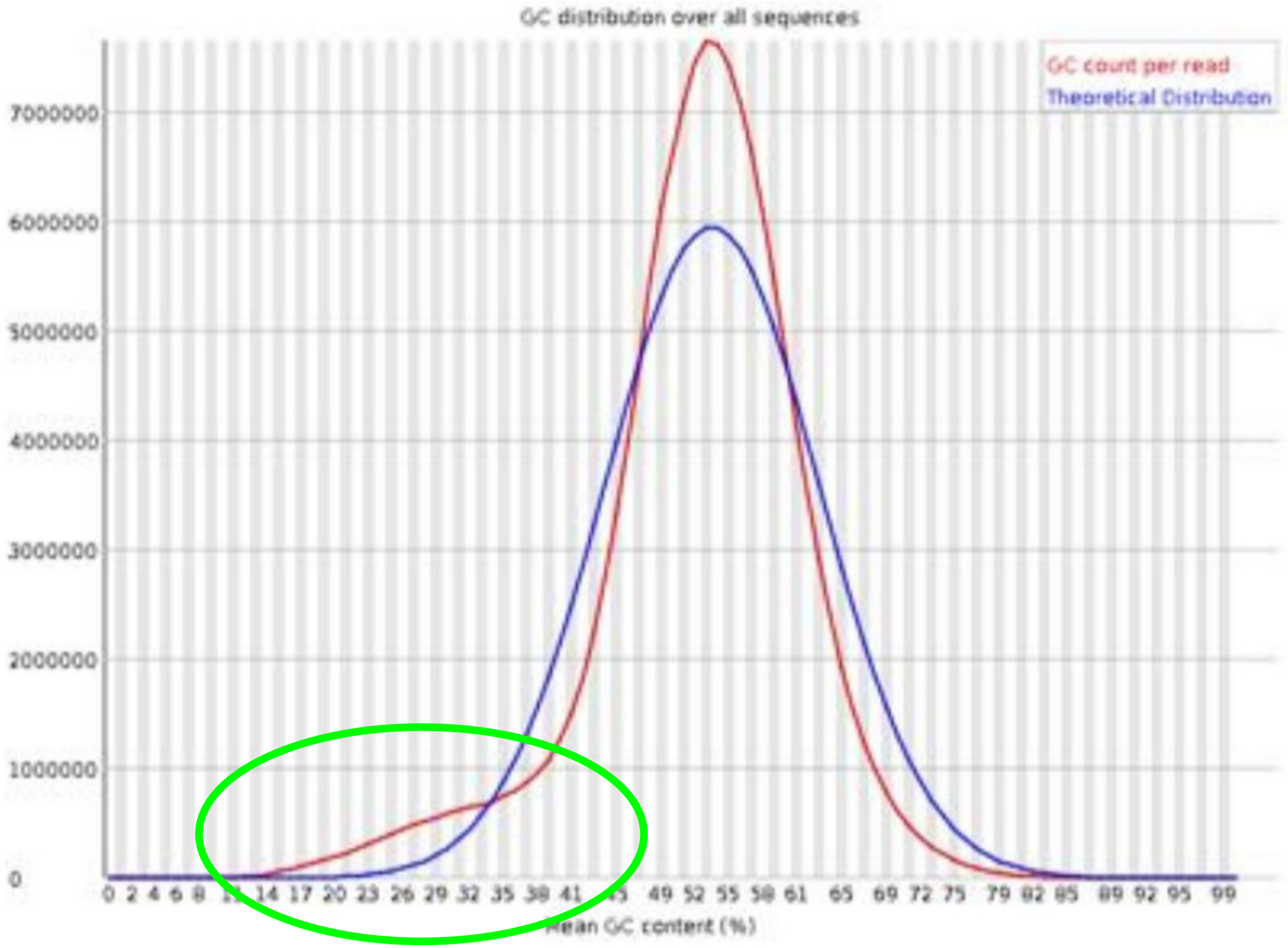
Source: https://sequencing.qcfail.com



GC content / Contamination ?



GC content / Contamination ?



Cleaning - Preprocessing

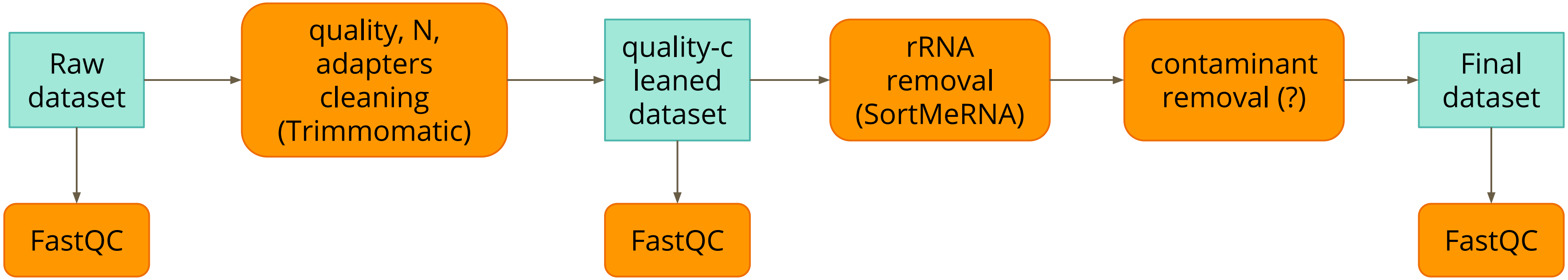
Cleaning has to be done in the reverse order that errors were generated.

1. Sequencing errors: quality trimming and filtering, Ns removal
2. Library preparation: adapters removal
3. Sample contamination: rRNA, mito, other contaminants

Note 1: step 1 (quality trimming) is not considered critical anymore and could even hinder downstream tools/algorithms.

Note 2: If the reads are going to be aligned against a reference genome, this whole process can be skipped or applied very lightly

Cleaning - Preprocessing



To map or not to map ?

With reference RNA-seq

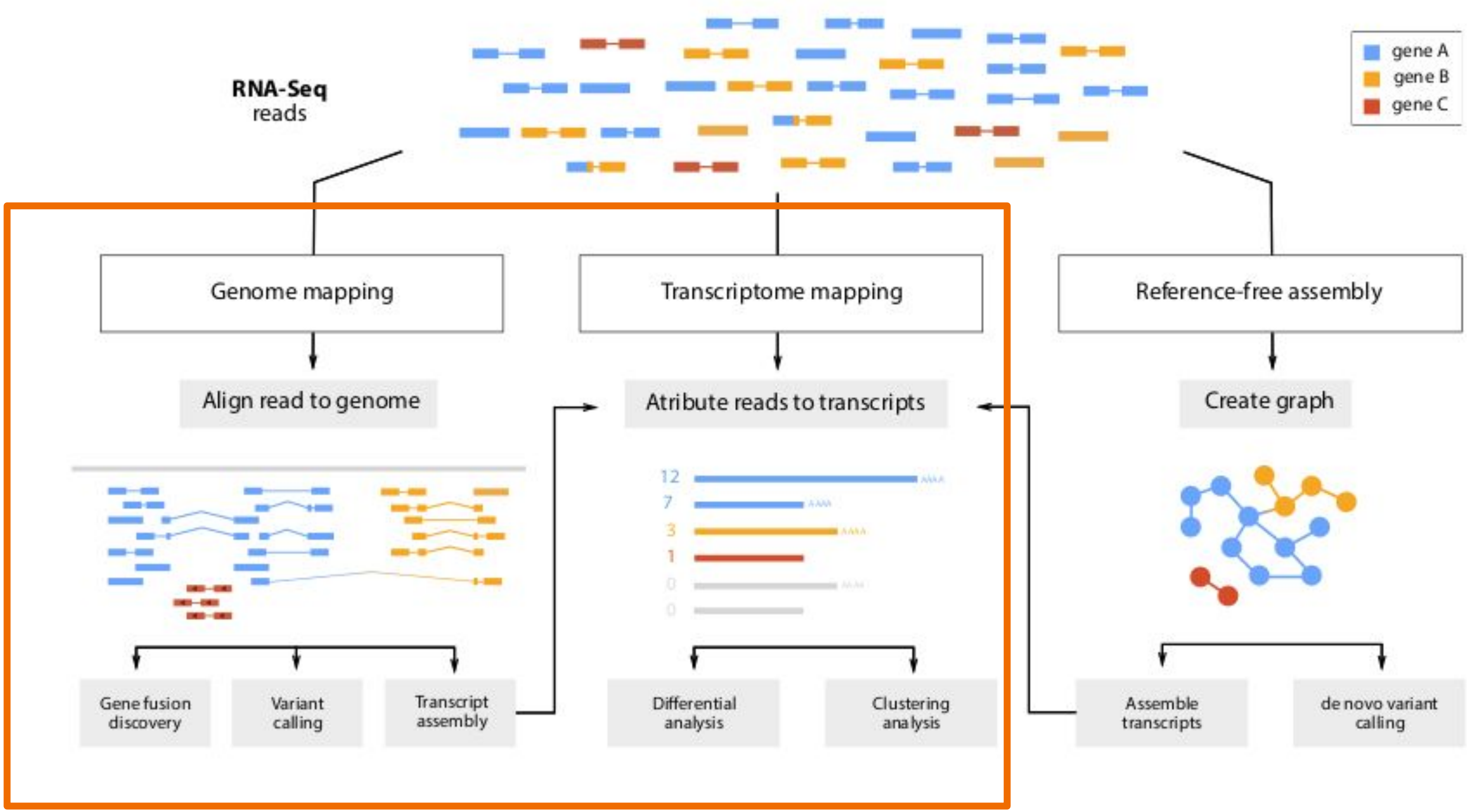
W/ reference RNA-seq. For what purpose ?

Mainly:

- Differential expression
  - between genes
  - between transcripts/isoformes

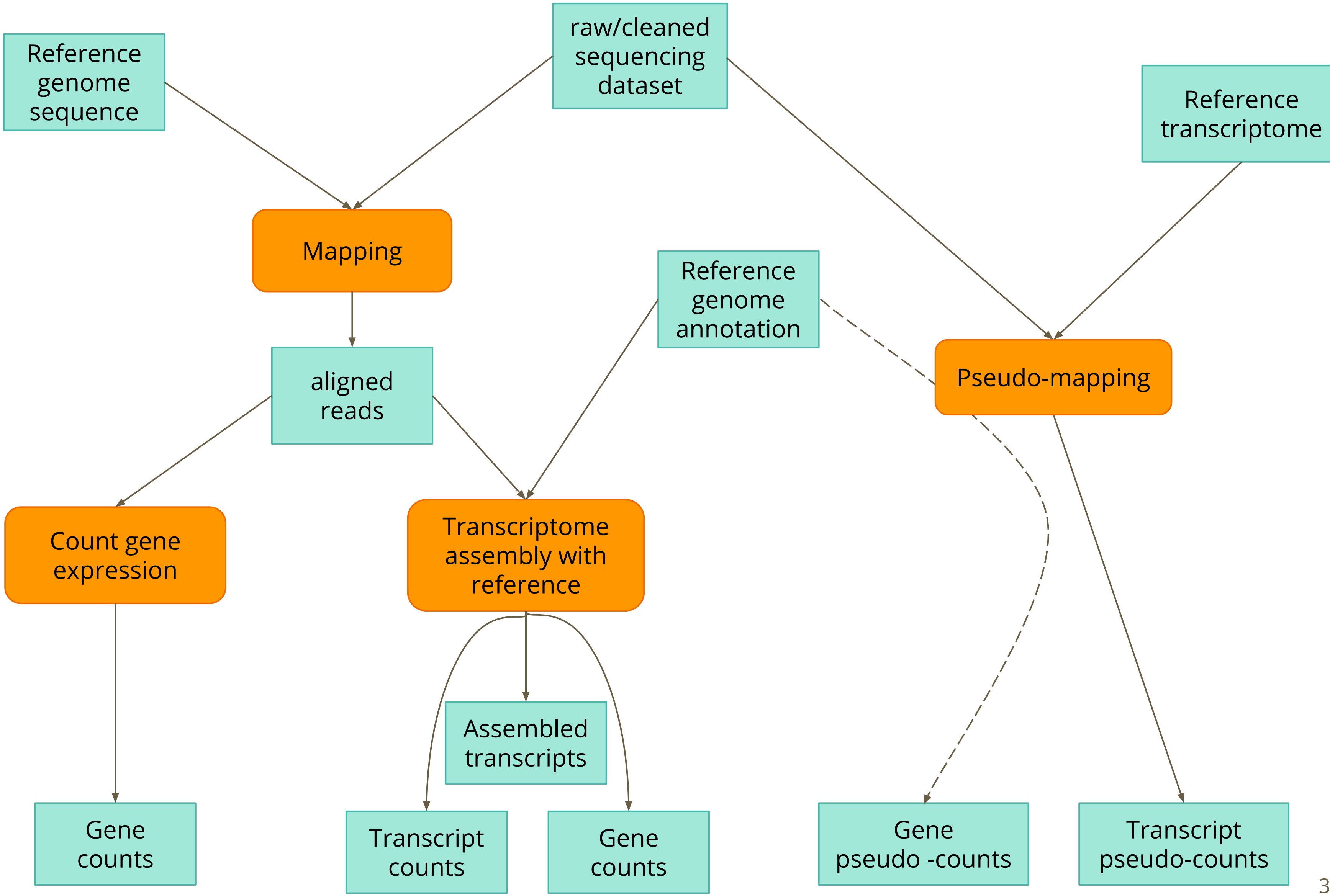
- Transcriptome assembly
  - variant calling
  - isoforme discovery

What people do with their RNA-seq

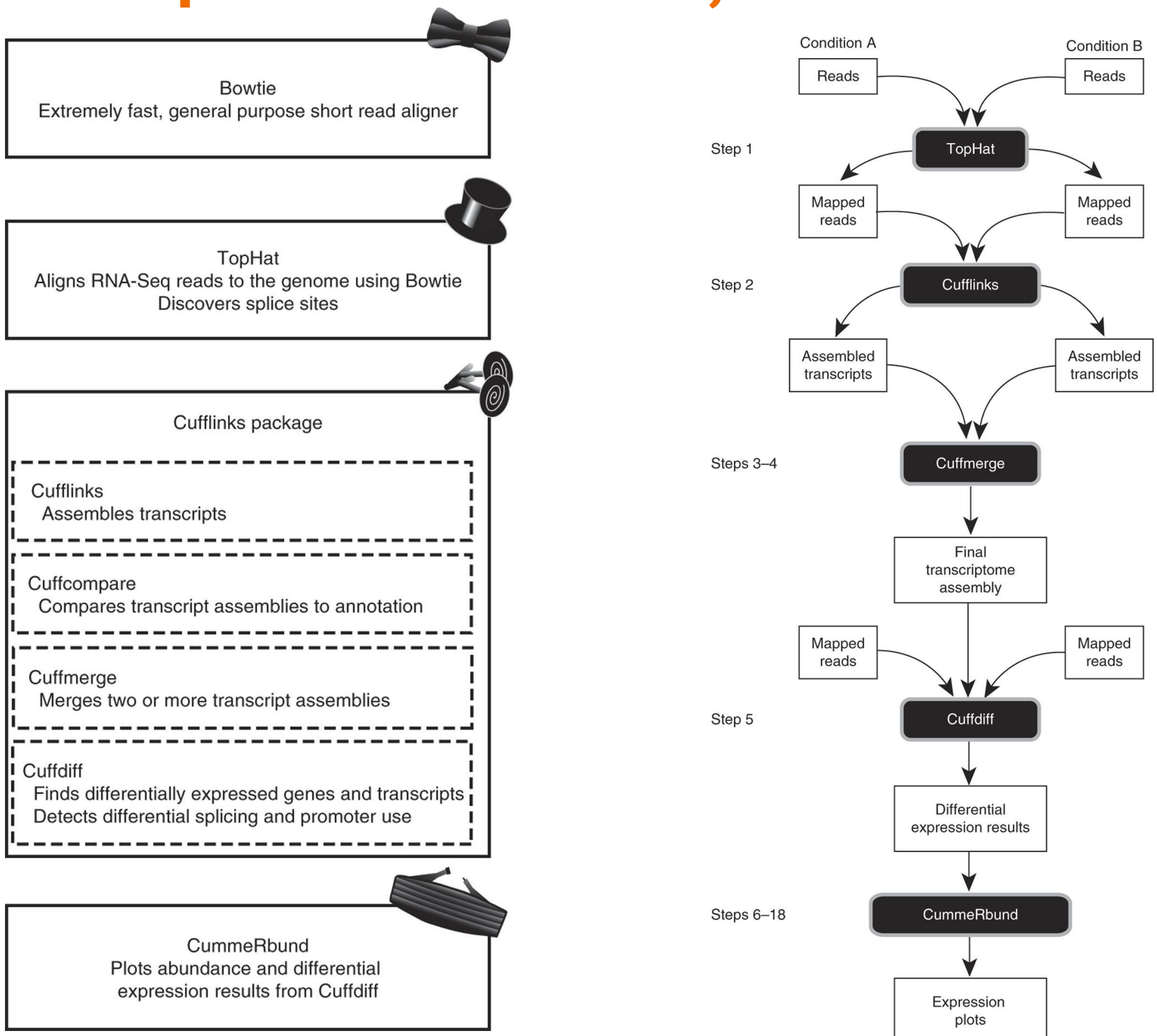


From J. Audoux's PhD thesis

RNA-seq w/ ref

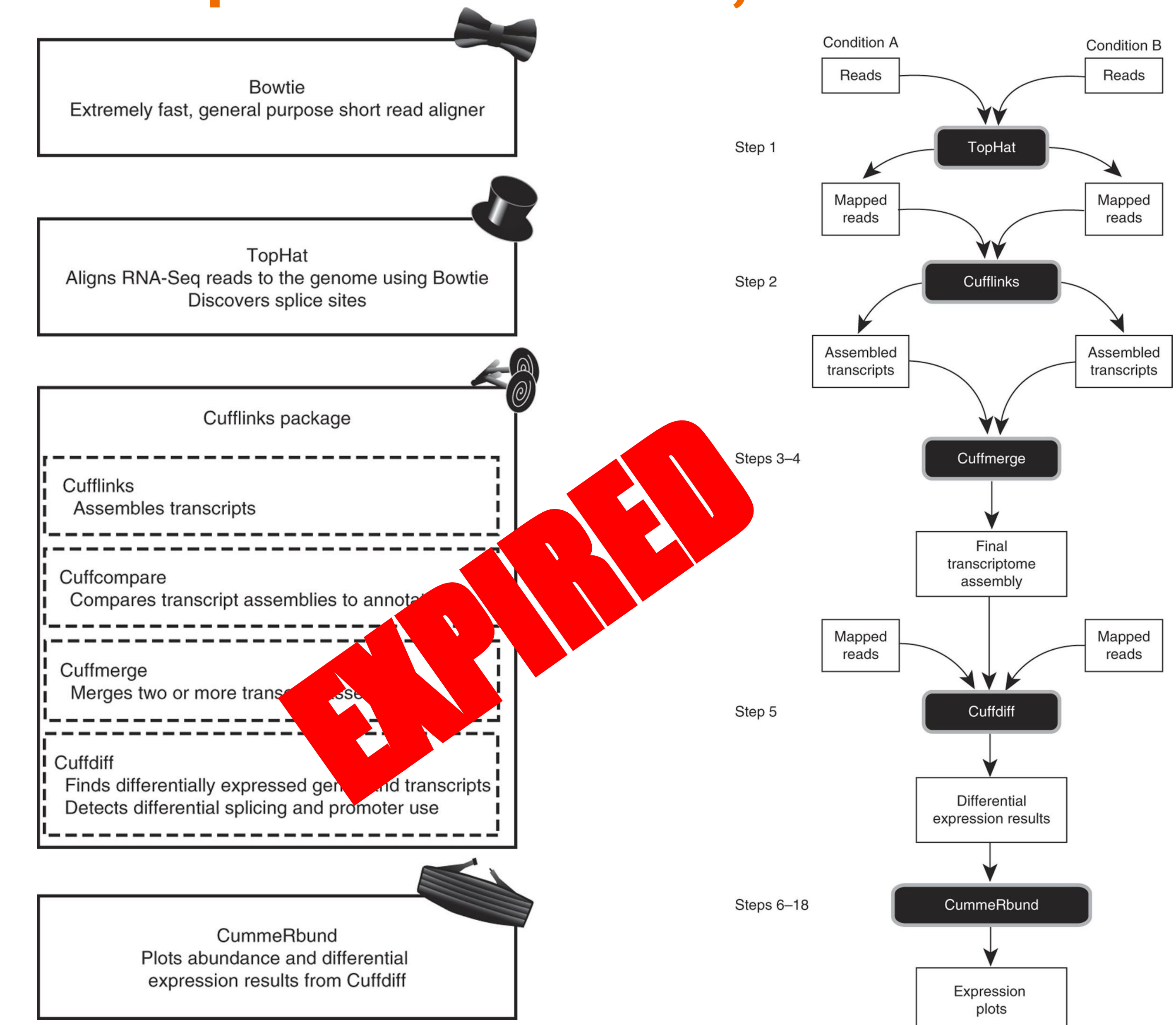

Reference genome sequence
raw/cleaned sequencing dataset
Reference transcriptome
Mapping
Reference genome annotation
aligned reads
Pseudo-mapping
Count gene expression
Transcriptome assembly with reference
Assembled transcripts
Gene counts
Transcript counts
Gene counts
Gene pseudo -counts
Transcript pseudo-counts

The champion: Tuxedo Suite, “Classic” version



Nat Protoc. 2012;7(3):562–578. doi:10.1038/nprot.2012.016

The champion: Tuxedo Suite, "Classic" version



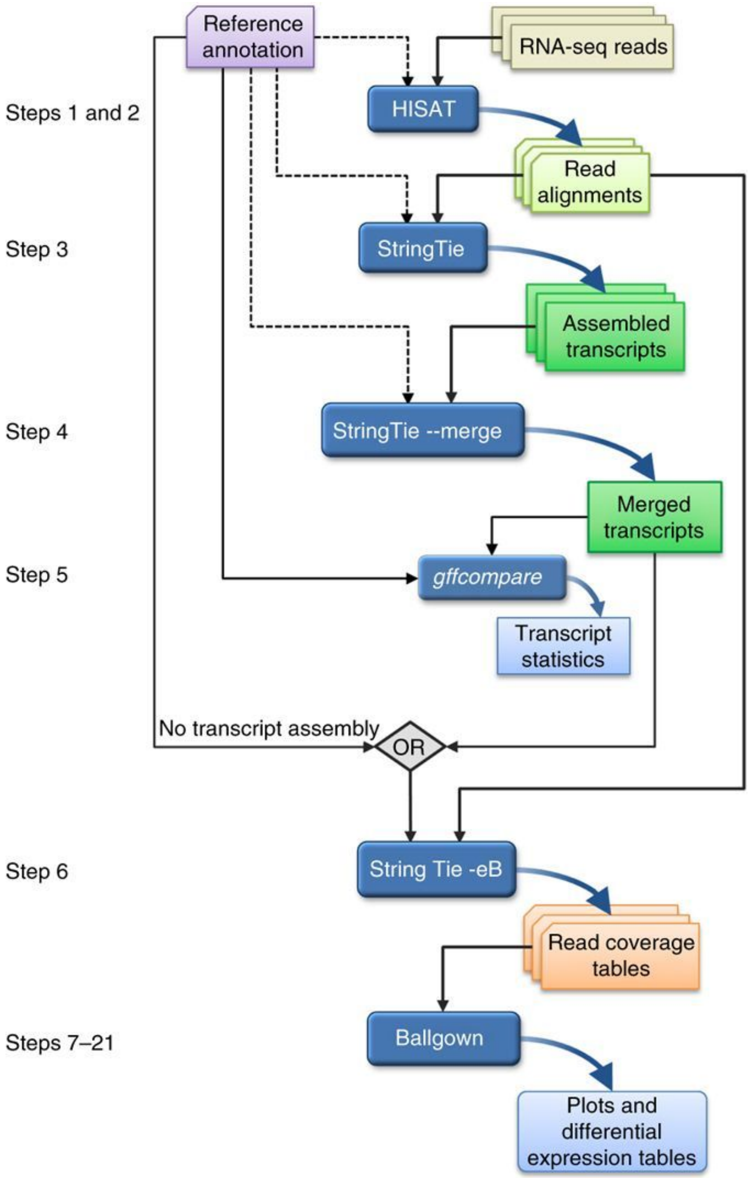
Nat Protoc. 2012;7(3):562–578. doi:10.1038/nprot.2012.016

The champion: Tuxedo Suite, New version

HISAT/HISAT2: splice aware aligner

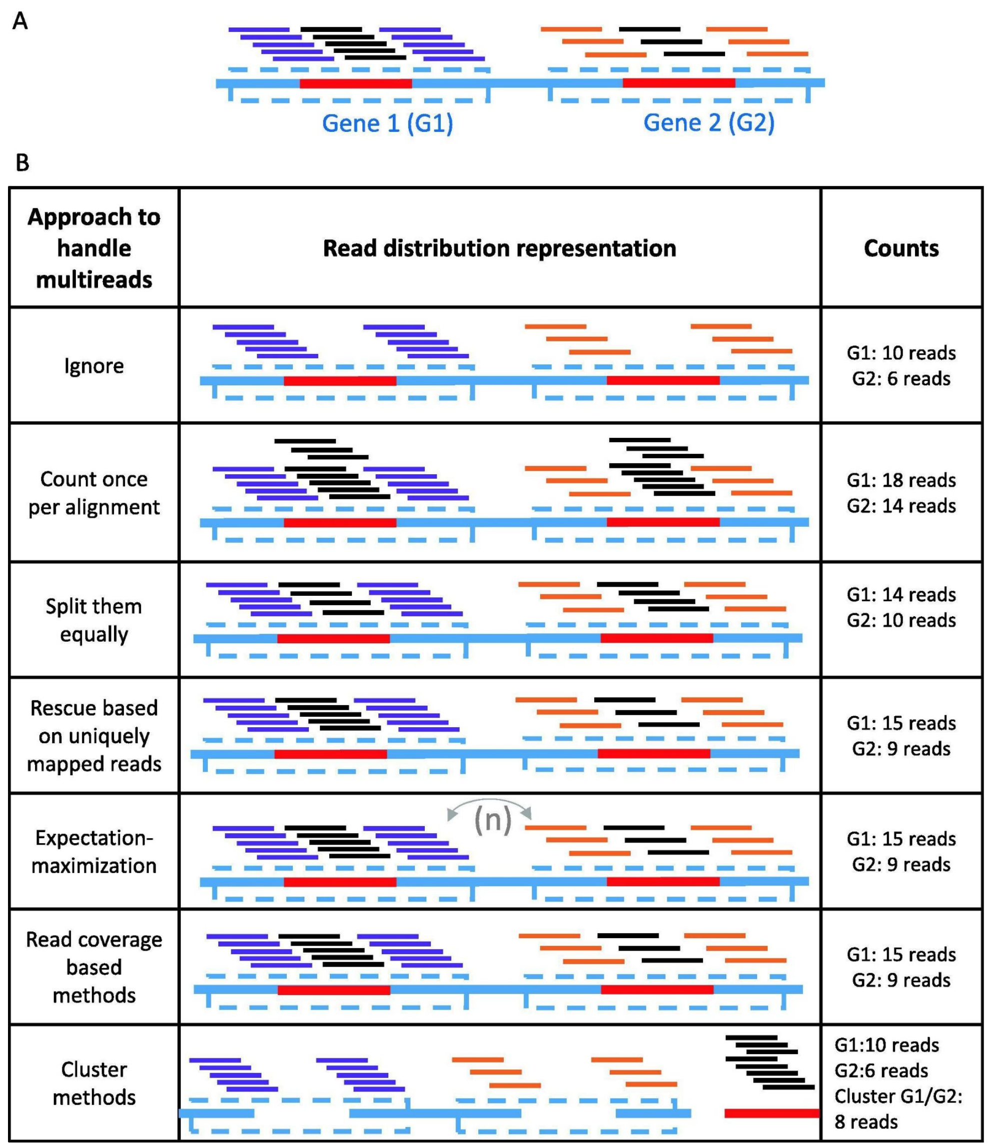
StringTie: Transcriptome assembler

Ballgown: Differential expression analysis



Nat Protoc. 2016;11(9):1650–1667. doi:10.1038/nprot.2016.095

Counting gene expression from alignments



Deschamps-Francoeur, et al. 2020. doi:10.1016/j.csbj.2020.06.014

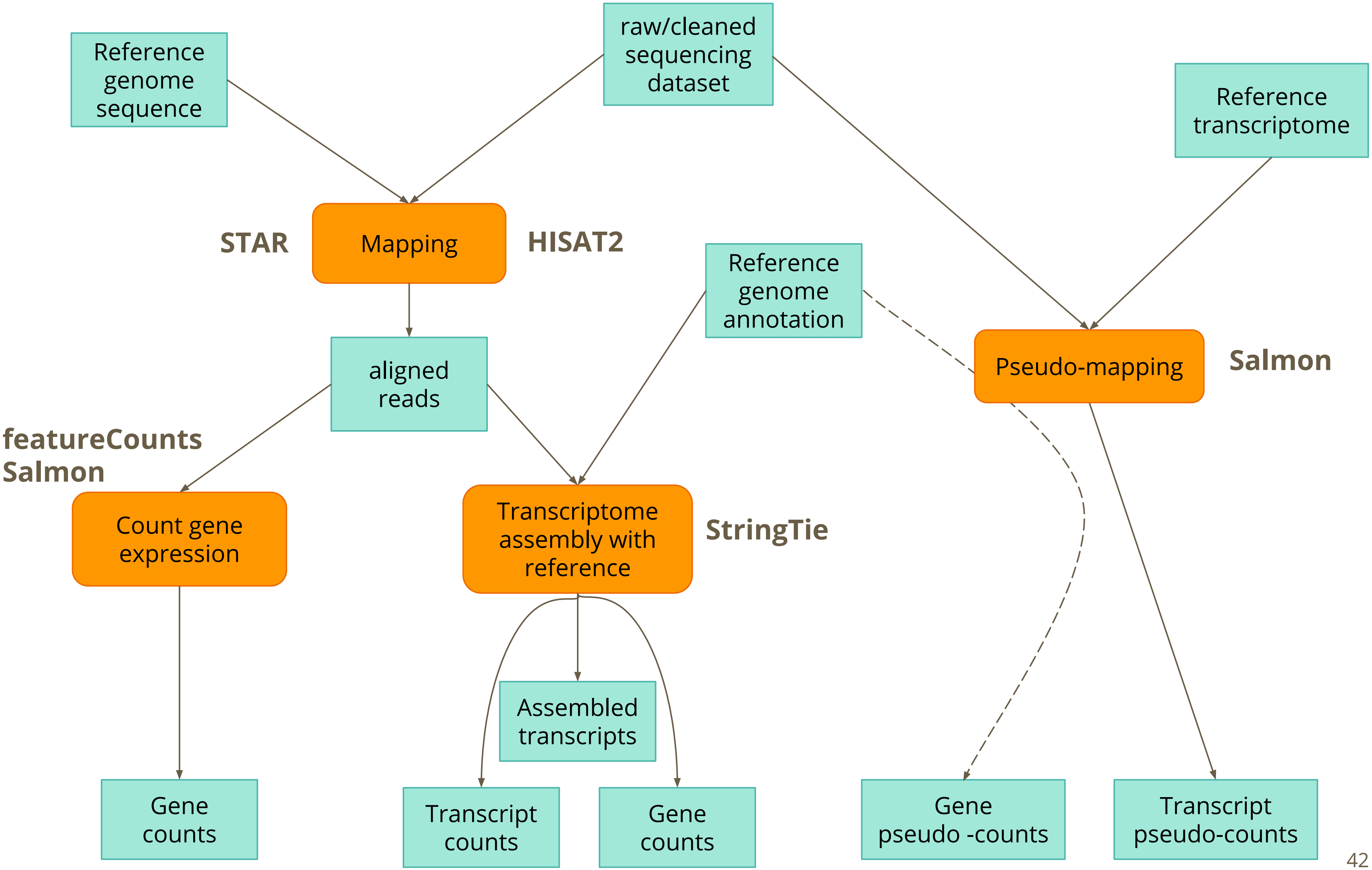
Counting gene expression from alignments

Table 1
Computational strategies and methods that handle multi-mapped reads.

Tool	Quantification level	Input	Strandedness can be specified	Count type	Strategy	Paired end	Confidence level	Focus
HTSeq-count	Gene	BAM	Y	Counts	Ignore	Y	N	Long RNA
STAR geneCounts	Gene	Fastq	Y	Counts	Ignore	Y	N	Long RNA
Cufflinks	Transcript	BAM	Y	RPKM	Split equally, Rescue	Y	N	Long RNA
featureCounts	Gene	BAM	Y	Counts	Ignore, count all, split equally	Y	N	Long RNA
CoCo	Gene	BAM	Y	Counts, CPM, TPM	Rescue	Y	N	Small RNA Long RNA
ERANGE	Transcript	BAM	N	RPKM	Rescue	Y	N	Long RNA
EMASE	Transcript	BAM	N	Counts, TPM	EM	Y	N	Long RNA
IsoEM2	Both	SAM	Y	FPKM, TPM	EM	Y	Confidence intervals	Long RNA
Kallisto	Transcript	Fastq	Y	TPM	EM	Y	Bootstrap values	Long RNA
RSEM	Both	Fastq, BAM	Y	Counts, TPM, FPKM	EM	Y	95% credibility intervals	Long RNA
Salmon	Transcript	Fastq	Y	Counts, TPM	EM	Y	Bootstrap values	Long RNA
MMR	N/A	BAM	Y	N/A	Read coverage	Y	N/A	Long RNA
MuMRescueLite	Genomic loci	Custom format	N	Counts	Read coverage	N	N	Short sequence tags
Rcount	Gene	BAM	Y	Counts	Read coverage	N	N	Long RNA
ShortStack	Gene	Fastq, BAM	N	Counts, RPM	Read coverage	N	N	Small RNA
mmquant	Gene	BAM	Y	Counts	Gene Clustering	Y	N	Small RNA Long RNA
SeqCluster	Gene	BAM	N	Counts	Gene clustering	N	N	Small RNA
Fuzzy method	Gene	Custom format	N	Fuzzy counts	Fuzzy sets	N	Fuzzy counts	Small RNA Long RNA
geneQC	Gene	SAM	Y	NA	ML	Y	Mapping uncertainty level	Small RNA Long RNA

Deschamps-Francoeur, et al. 2020. doi:10.1016/j.csbj.2020.06.014

RNA-seq w/ ref



Reference genome sequence
raw/cleaned sequencing dataset
Reference transcriptome
STAR
Mapping
HISAT2
Reference genome annotation
aligned reads
Pseudo-mapping
Salmon
featureCounts
Salmon
Count gene expression
Transcriptome assembly with reference
StringTie
Assembled transcripts
Gene counts
Transcript counts
Gene counts
Gene pseudo -counts
Transcript pseudo-counts

Practical: Mapping and Quantification

Open Galaxy

GTN Practical: [Reference-based RNA-seq data analysis]

Recommended pipeline (as of Sept 2021)

- Transcriptome assembly: HISAT2 + StringTie (+ Ballgown ?)
- Transcript/Gene quantification with mapping: STAR + Salmon
- Mapping-less transcript quantification: Kallisto or Salmon

De novo RNA-seq

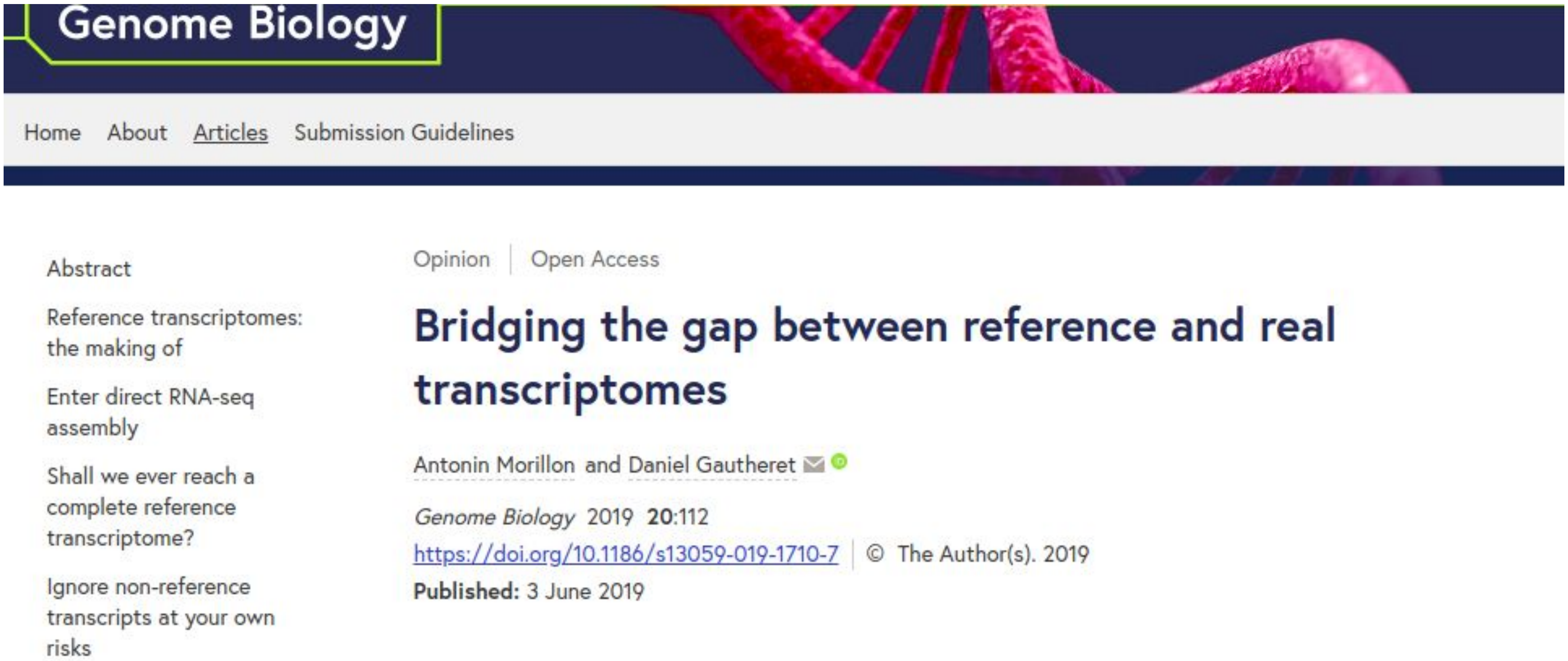
De novo approaches

- *De novo* methods are approaches that are **free from a reference** for producing results
- Reference-based approaches have limitations as **results depends on the quality of the reference**
- Sometimes we don’t even have a reference
- *De novo* and reference-based are **complementary**

Why do we need *de novo* approaches

Aren't references good enough?

- ❑ Disease-associated transcripts
- ❑ Genetic polymorphism in transcripts
- ❑ *de novo* methods are helping creating tomorrow's references


Genome Biology

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Bridging the gap between reference and real transcriptomes

Antonin Morillon and Daniel Gautheret

Genome Biology 2019 **20**:112

https://doi.org/10.1186/s13059-019-1710-7 |

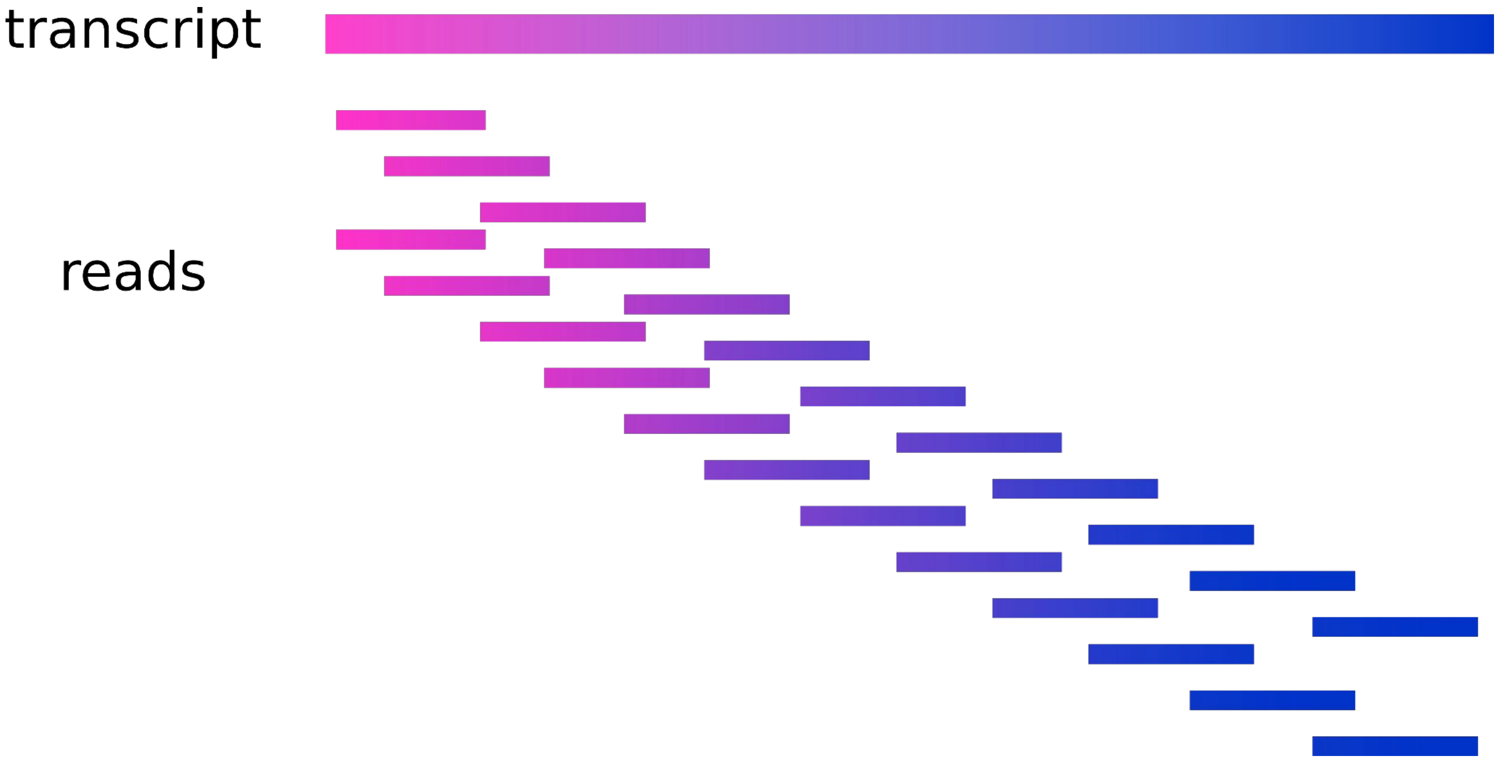
Published: 3 June 2019

The more novel and specific is your need, the more likely you need new bioinformatics (and *de novo*)

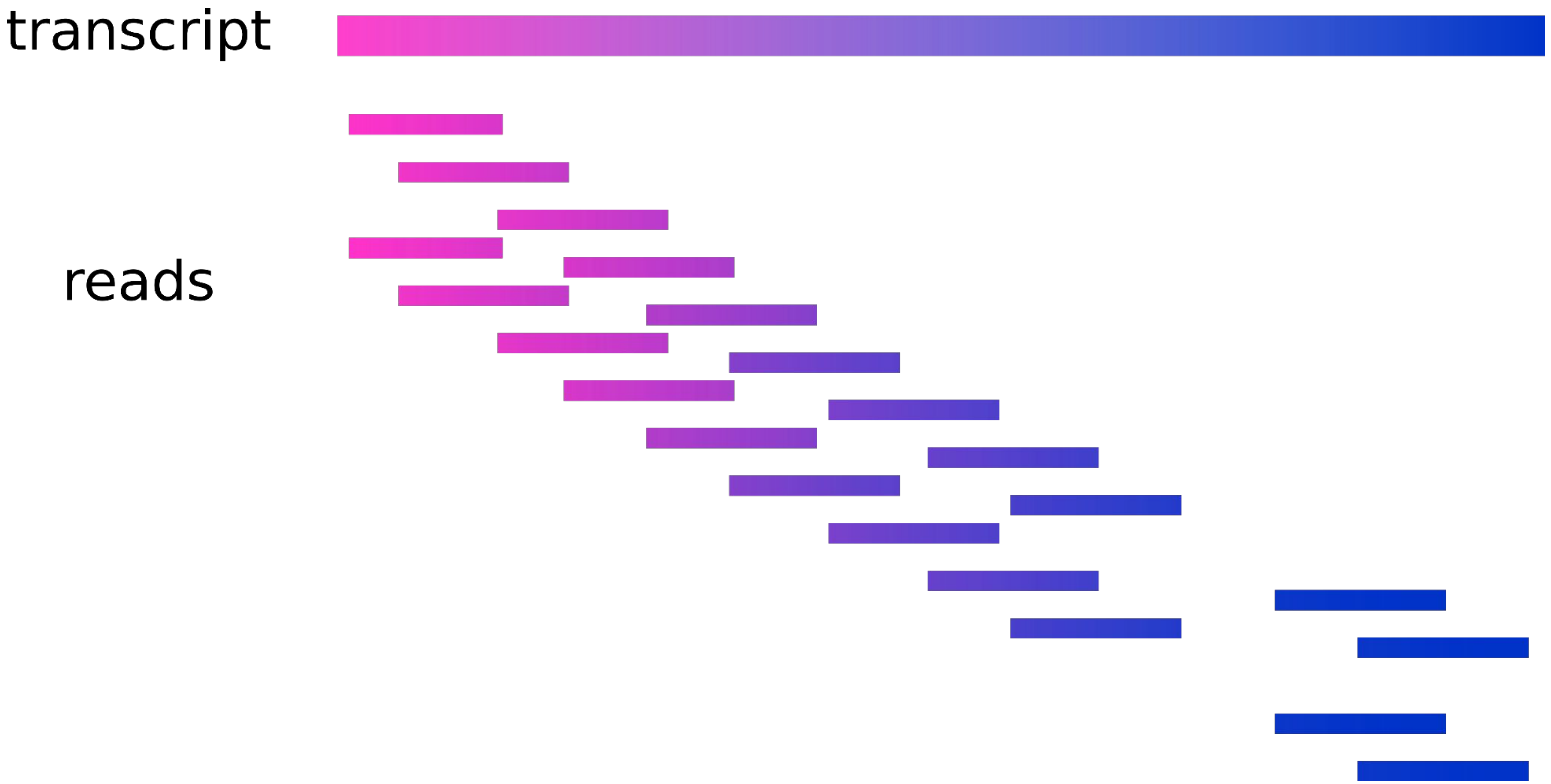
What can be done with *de novo* methods

- transcript assembly + quantification
- genetic polymorphism detection
- alternative transcript detection + quantification

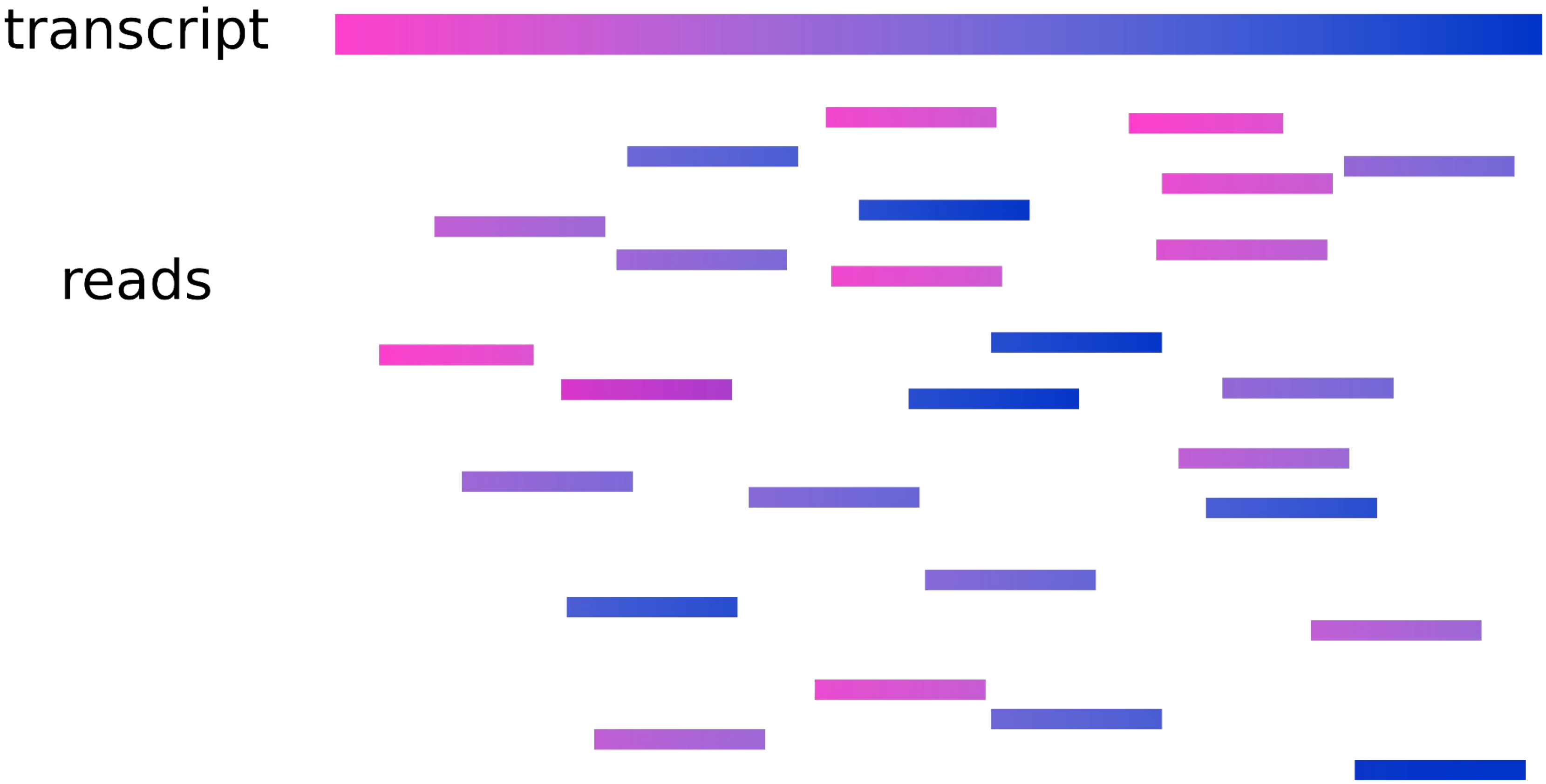
The *de novo* assembly challenge



The *de novo* assembly challenge



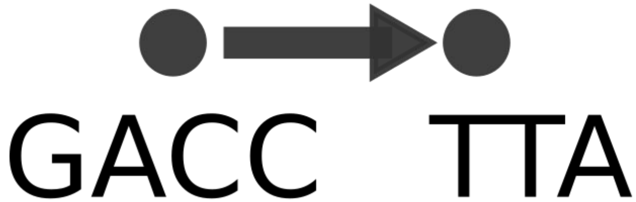
The *de novo* assembly challenge



Assembly recap

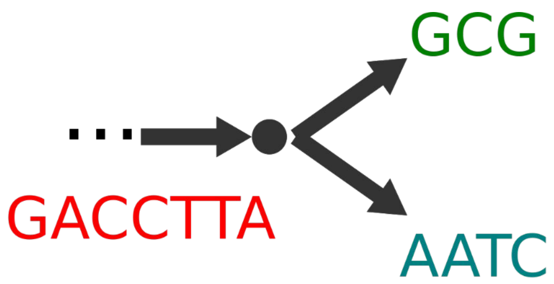
Assembly is like taking a step after another in a maze

One step is a group of nucleotides



Assembly recap

Until you have a choice to make :



why does this happen? check the reads:

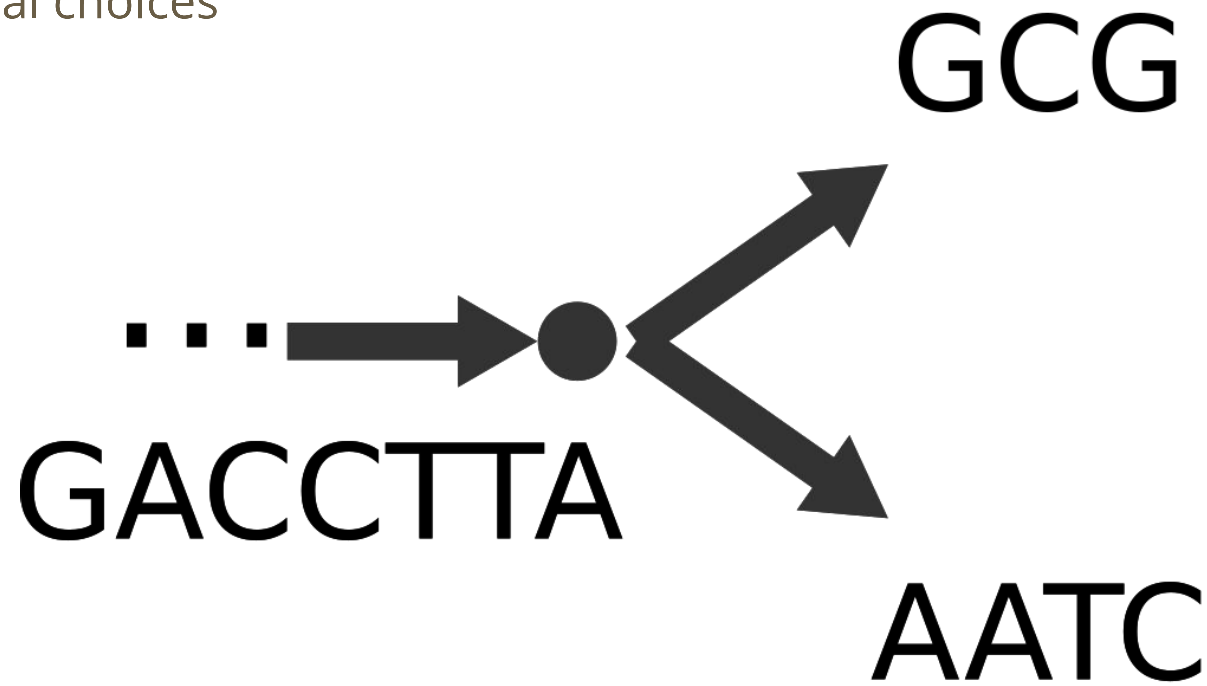
CTTAGCG

TTAAATC

and in the initial molecules, an exon is shared:

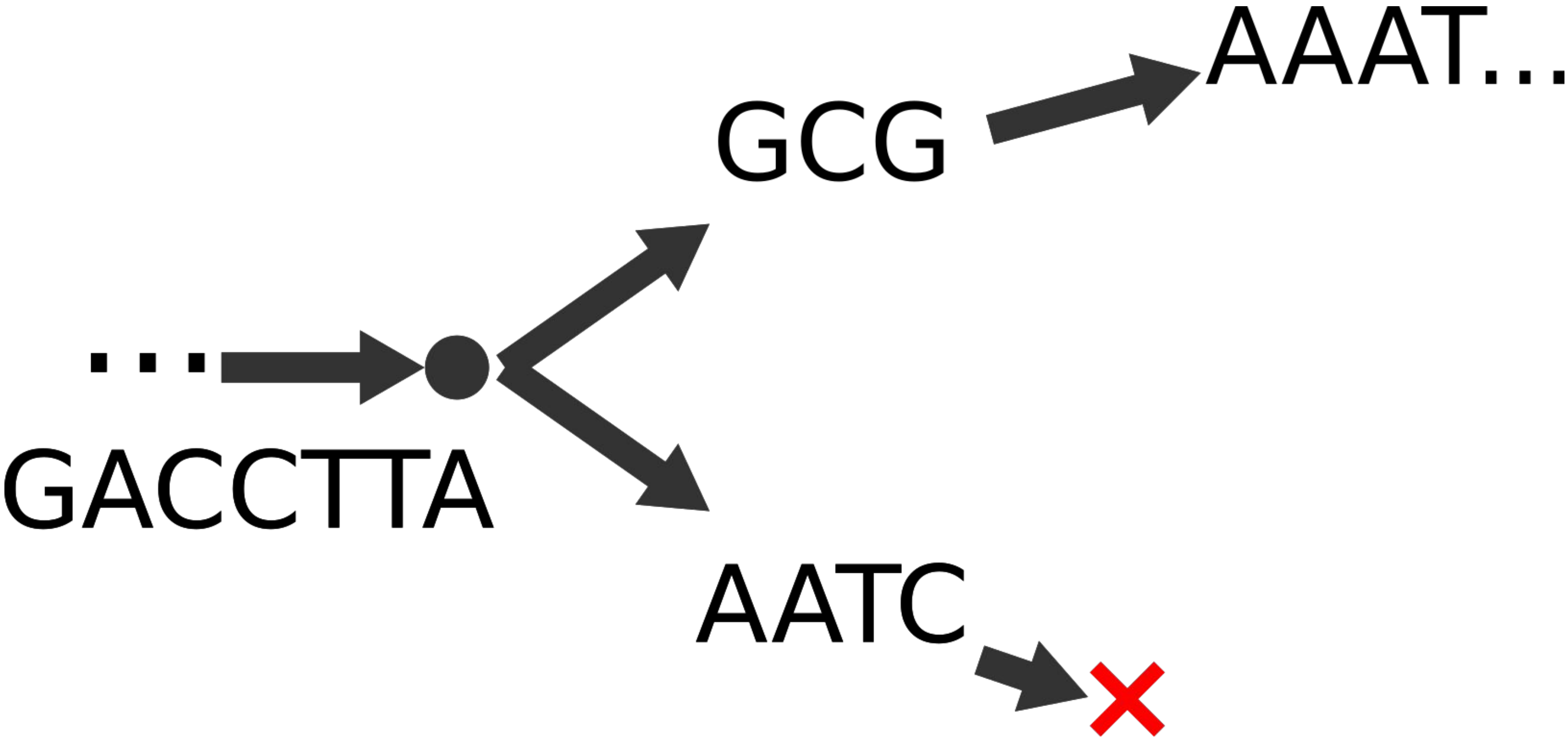
Greedy algorithms

local choices



Greedy algorithms

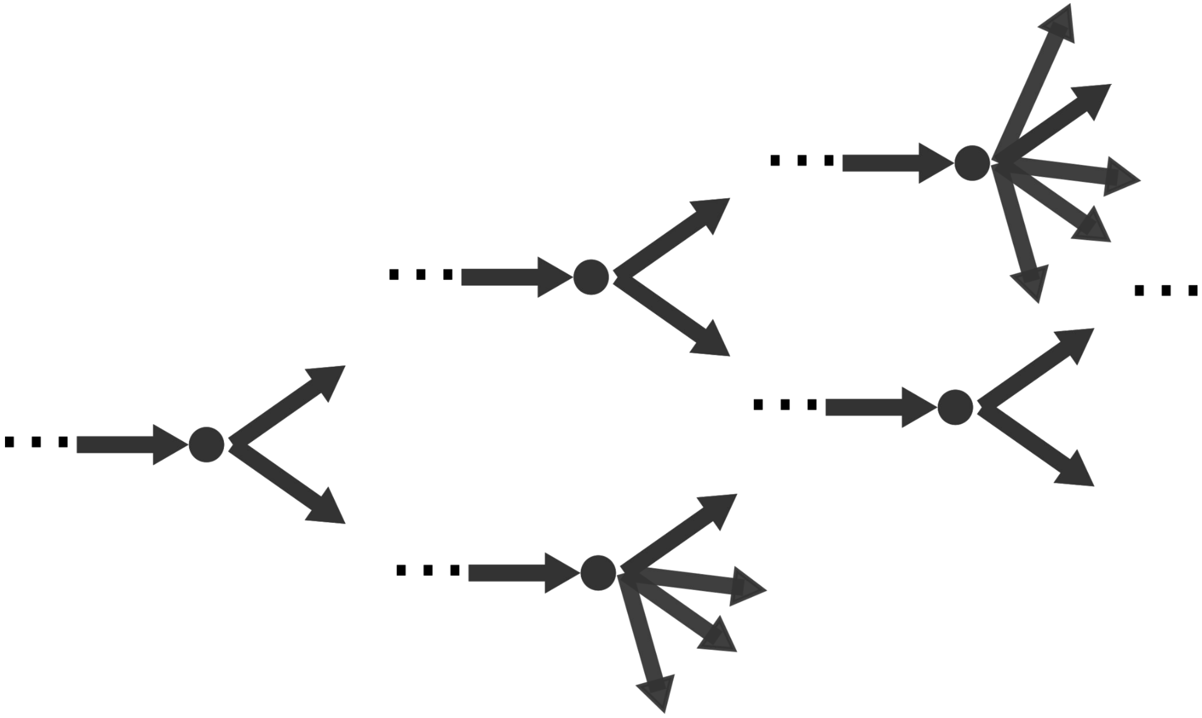
local choices can lead to bad decisions



All vs all overlaps algorithms

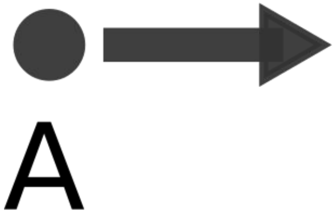
Have a global view of the possibilities in the “maze”

Ideal but... **quadratic**



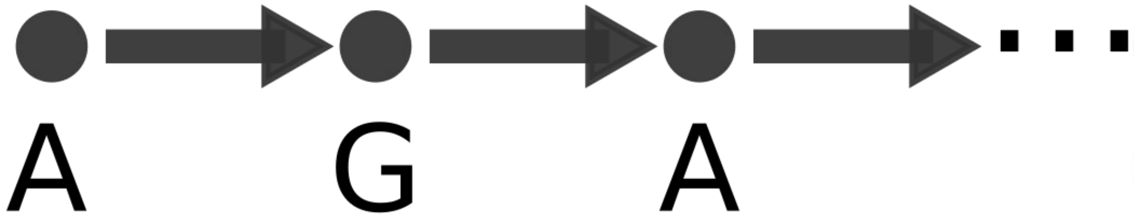
de Bruijn graph assembly

With de Bruijn graphs we walk in the maze nucleotide by nucleotide:



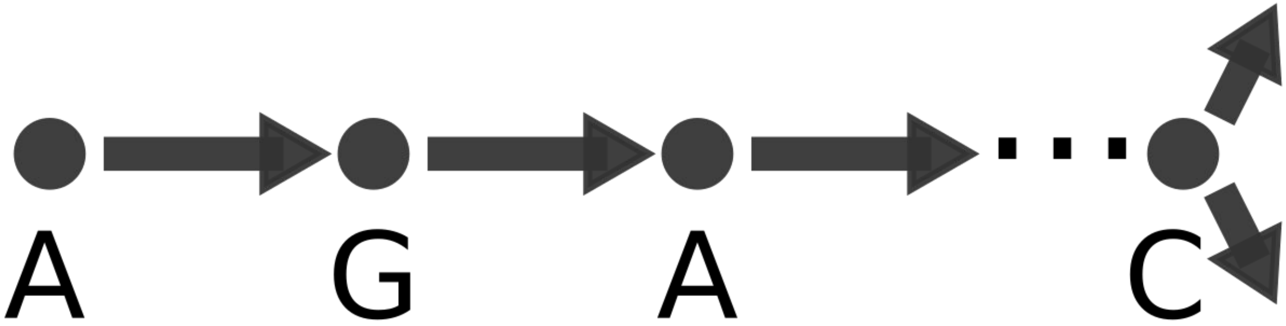
de Bruijn graph assembly

Your next step must correspond to the nucleotide that comes after in the original transcript



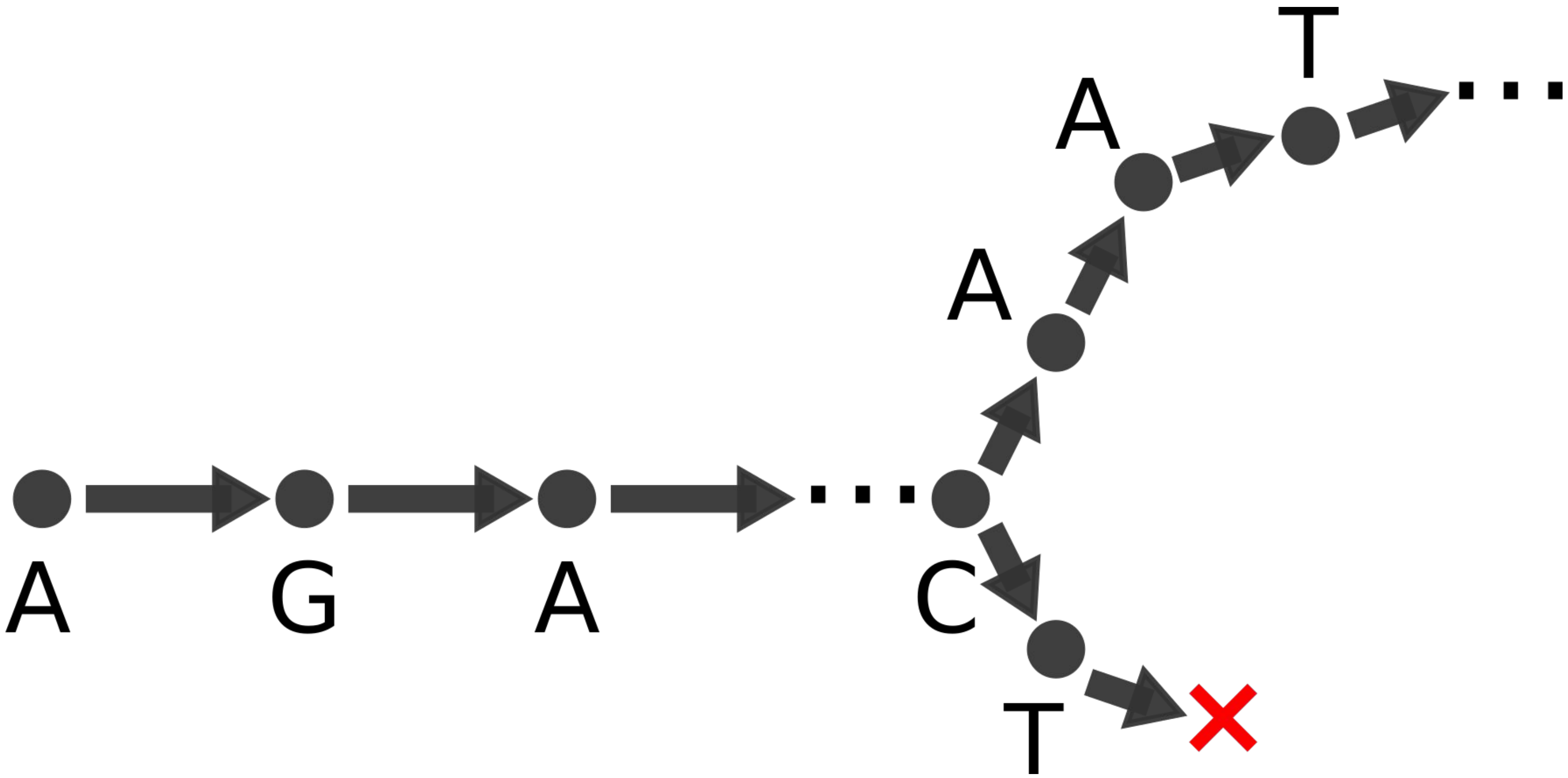
Result: concatenation of the nucleotides (AGA...)

de Bruijn graph assembly



de Bruijn graph assembly

Some dead ends and other bifurcations can be seen



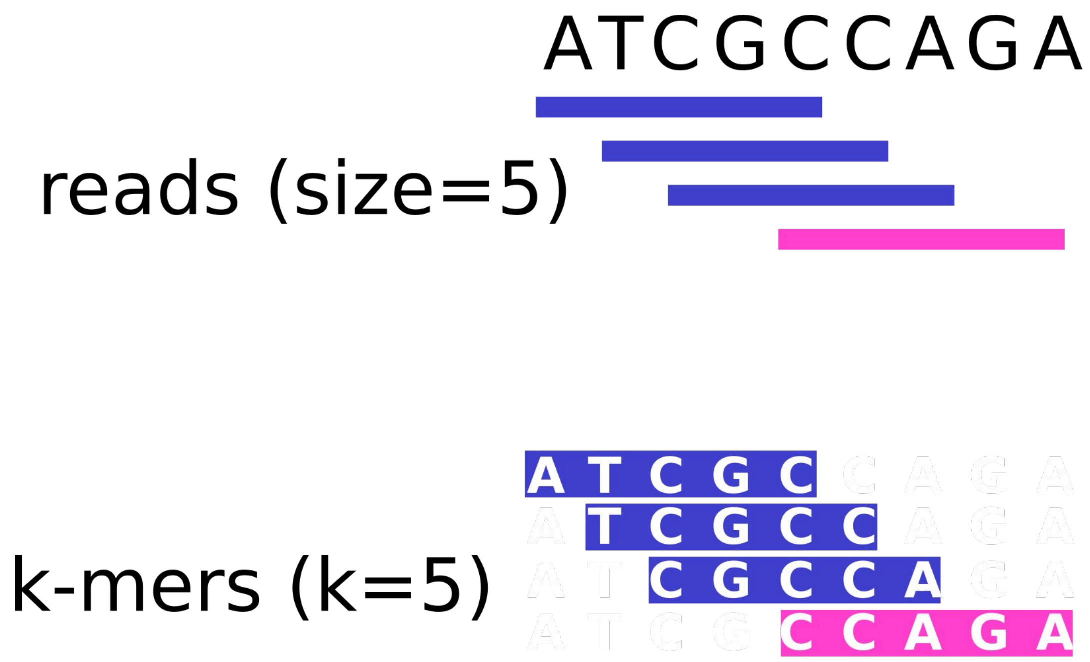
de Bruijn graph assembly

Store the “maze” in a graph structure (de Bruijn graph)

- helps with local choices
- cost efficient (RAM & runtime)

de Bruijn graph in practice: k-mers

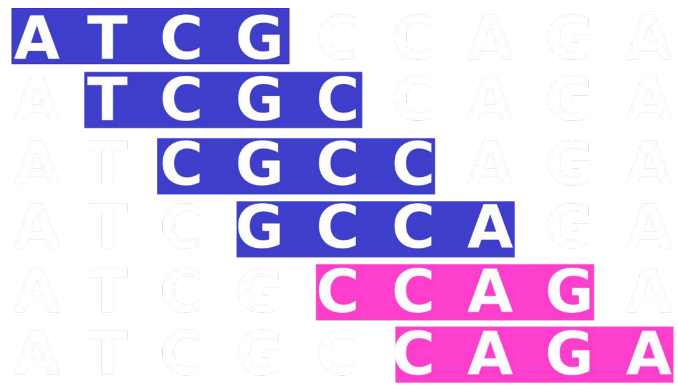
k-mers: why don't we use reads



result: ATCGCCA, CCAGA

de Bruijn graph in practice: k-mers

k-mers (k=4)



result: ATCGCCAGA

de Bruijn graph in practice: k-mers

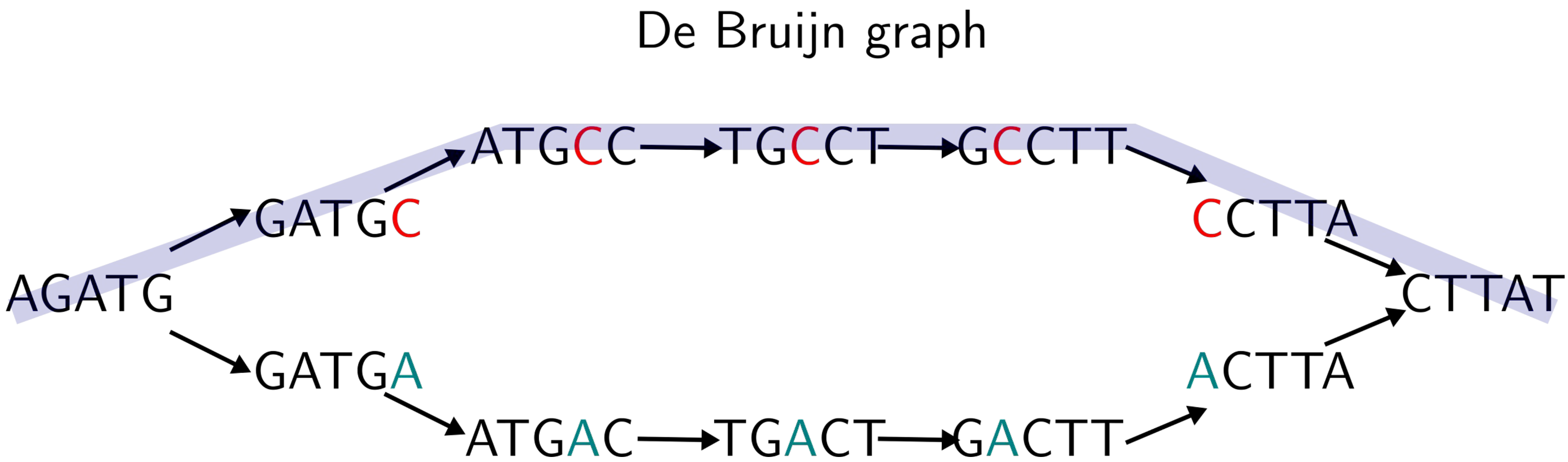
k-mers help bridging the assembly

they are key elements to work with the dBG

in practice implementations allow using several k sizes

tradeoff larger k: more conservative /smaller k: more gaps filled in the graph

Path in the De Bruijn graph



assembly : a set of gap-less sequences extracted from paths covering the graph (after some modifications to he graph...)

Vocabulary: bubbles/bulges

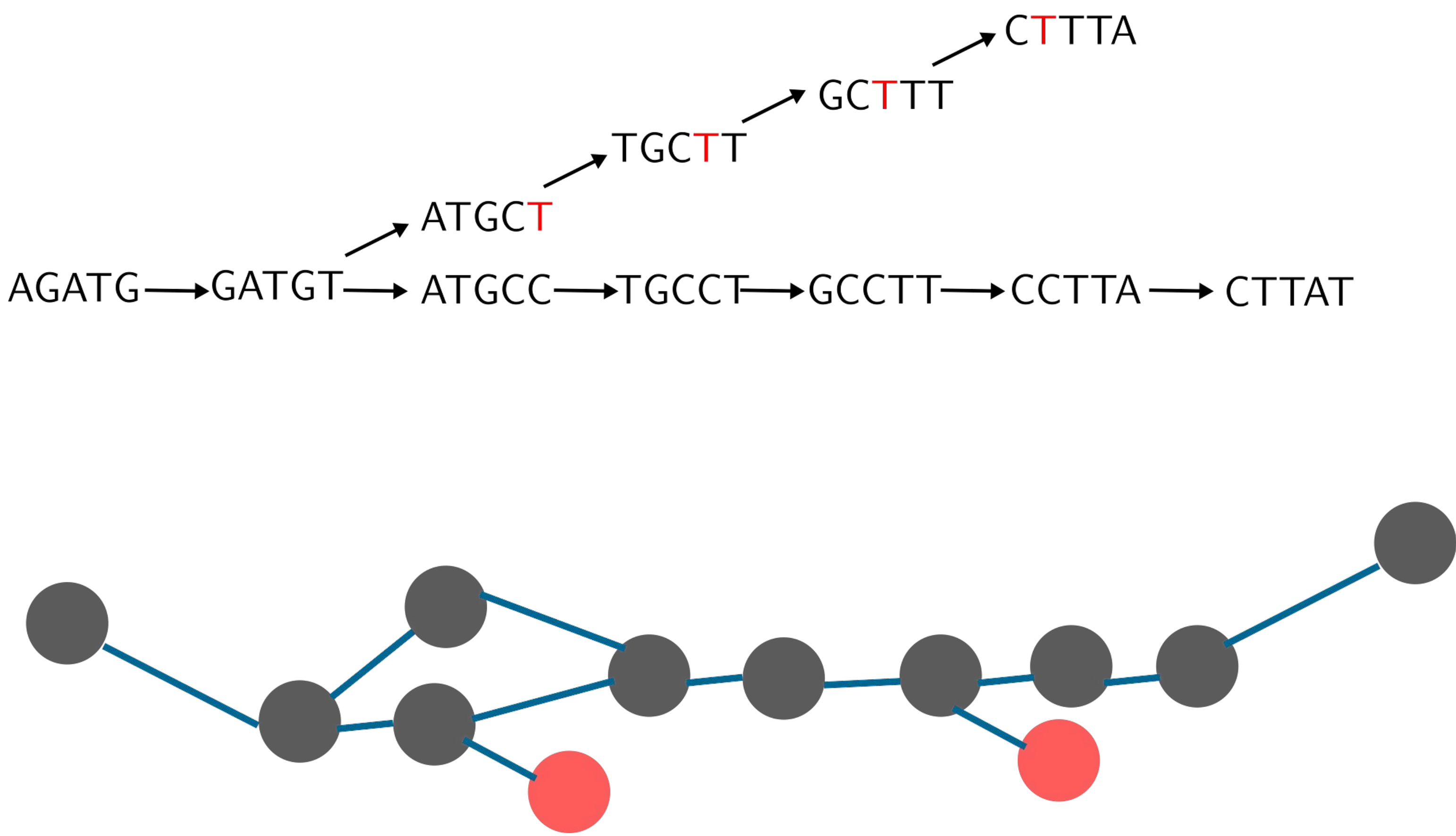
AGATGCCTTAT

AGATGCCTTAT
AGATGACTTAT

Vocabulary: tips/dead ends

reads

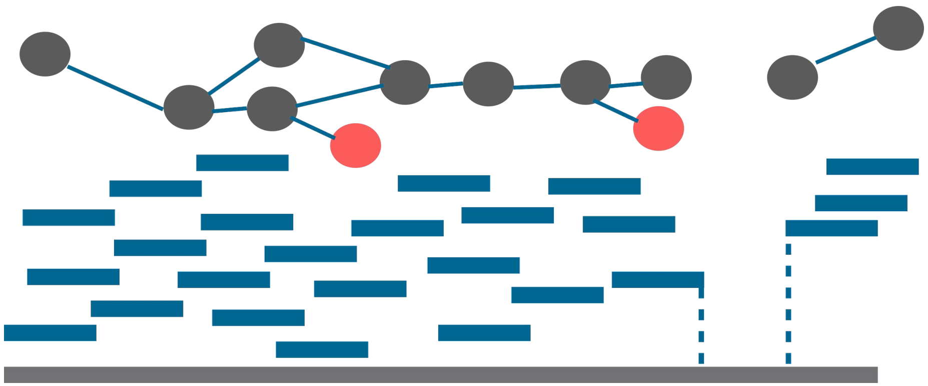
AGATGCCTTA
AGATGCTTTA
AGATGCCTTA
GATGCCTTAT
GATGCCTTAT



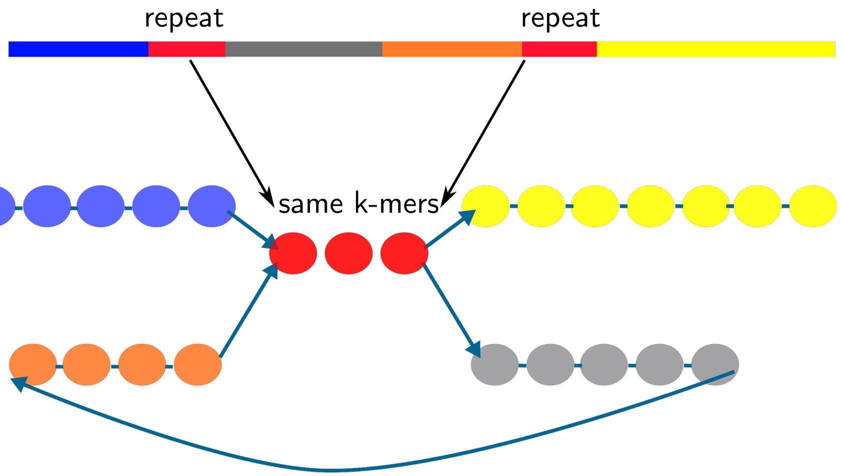
An assembly generally is

- smaller than the reference,
- fragmented

- missing reads create gaps



- repeats fragment assemblies
  and reduce total size

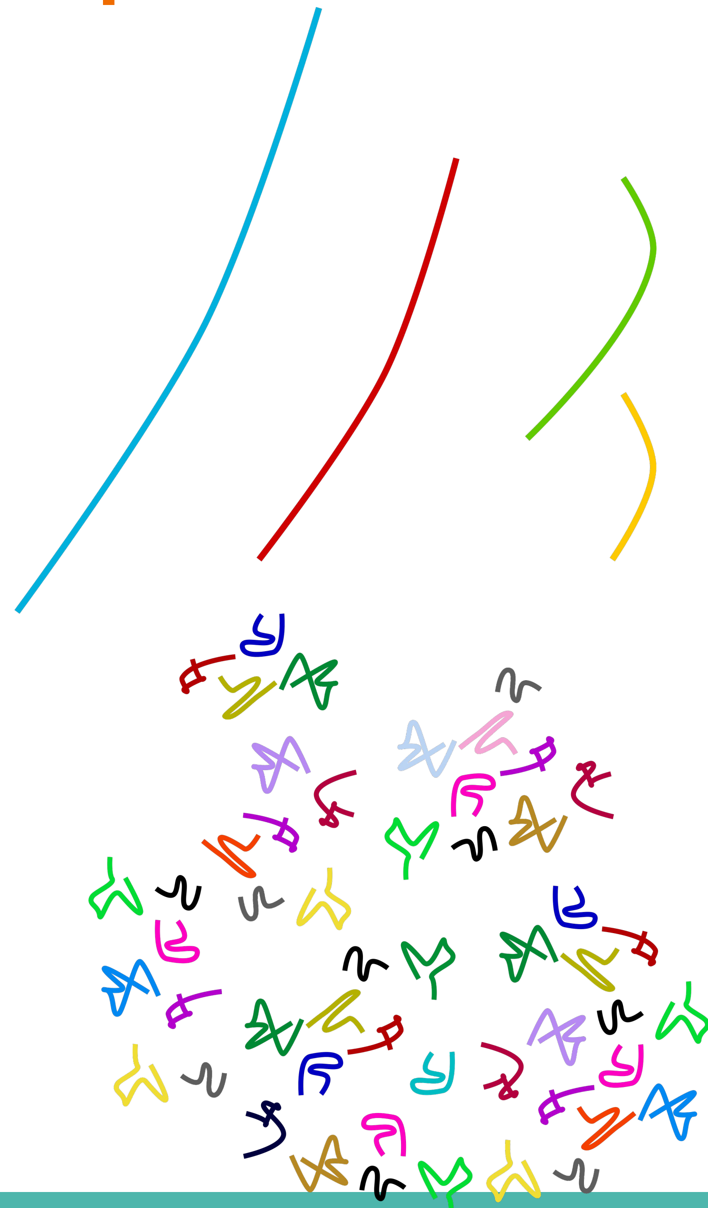


Contrasting genome and transcriptome assemblies

genome

-uniform coverage
-single contig per locus
-double stranded
-theory: one massive graph per chromosome
-practice: repeats aggregate, contigs
 smaller than chromosomes

transcriptome
-exponentially distributed coverage
-multiple contigs per locus
-strand specific
- theory: thousands of small disjoint
  graphs, one per gene
-practice: gene families, ALU & TE,
 low covered

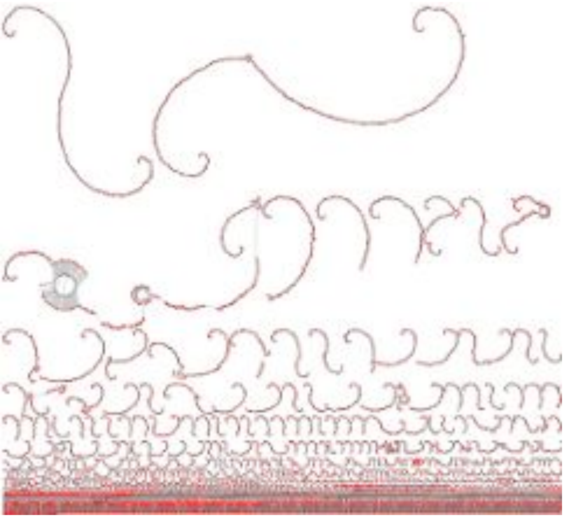


Contrasting genome and transcriptome assemblies

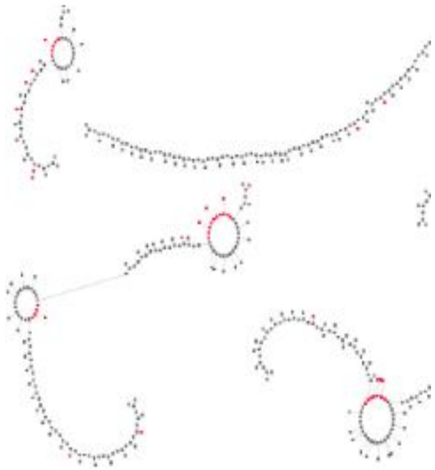
Despite these differences, DNA-seq assembly methods apply:

- Construct a de Bruijn graph (same as DNA)
- Output contigs (same as DNA)
- Allow to re-use the same contig in many different transcripts (new part)

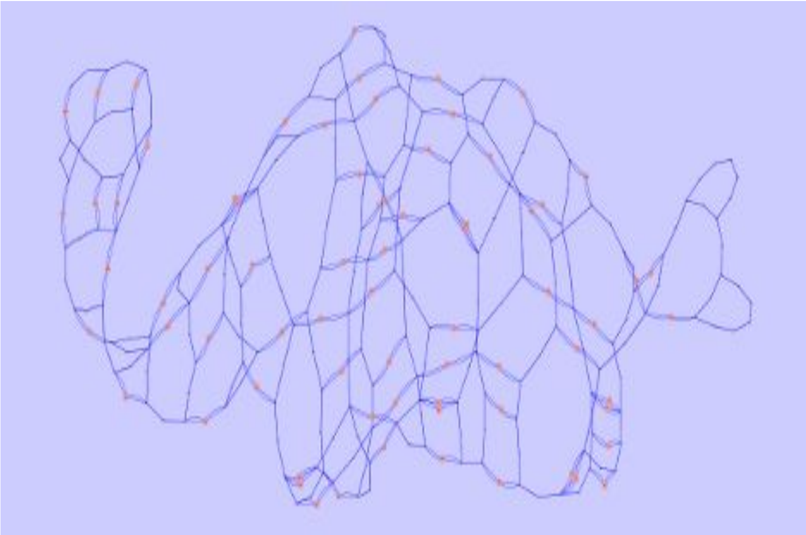
Real instance graphs



graph from shallow covered Drosophila dataset



zoomed-in bubbles (+ tips)



gene family

Credit: ERABLE team (Lyon)

There is no single solution for assembly...

Conclusions of the GAGE benchmark : in terms of assembly quality, there is no single best assembler. Applies to RNA-seq.

Main tools:

-**TransAbyss**, Robertson et al. *Nat. Met* 2010 https://github.com/bcgsc/transabyss

-**Bridger**, Chang et al. *Genome Biol.* 2015 https://github.com/fmaguire/Bridger_Assembler

-**SOAPdenovo-Trans**, Xie et al. *Bioinformatics* 2014 https://github.com/aquaskyline/SOAPdenovo2

-**Trinity**, Grabherr et al. *Nat. Biotechnol.* 2011 https://github.com/trinityrnaseq/trinityrnaseq/wiki

- **rnaSPAdes**, Bushmanov et al. *GigaScience* 2019 http://cab.spbu.ru/software/spades/

The main building blocks in theory

1. (optional) correct the reads (for instance BayesHammer in rnaSPAdes)
2. build a graph from the reads (remove k-mers seen once)
3. remove likely sequencing errors (tips)
4. remove known patterns (bubbles)
5. return simple paths (i.e. contigs), **allow nodes to be used several times**

Warning: what's in the paper is different than what's in the implementation...

J Comput Biol

2. Assembly in SPAdes: An Outline

Go to:

Below we outline the four stages of SPAdes, which deal with issues that are particularly troublesome in SCS: sequencing errors; non-uniform coverage; insert size variation; and chimeric reads and bireads:

(1) Stage 1 (assembly graph construction) is addressed by every NGS assembler and is often referred to as de Bruijn graph *simplification* (e.g., *bulge/bubble* removal in EULER/Velvet). We propose a new approach to assembly graph construction that uses the *multisized de Bruijn graph*, implements new bulge/tip removal algorithms, detects and removes chimeric reads, aggregates biread information into *distance histograms*, and allows one to backtrack the performed graph operations.

(2) Stage 2 (k-bimer adjustment) derives accurate distance estimates between *k*-mers in the genome (edges in the assembly graph) using joint analysis of distance histograms and paths in the assembly graph.

Trinity assembler

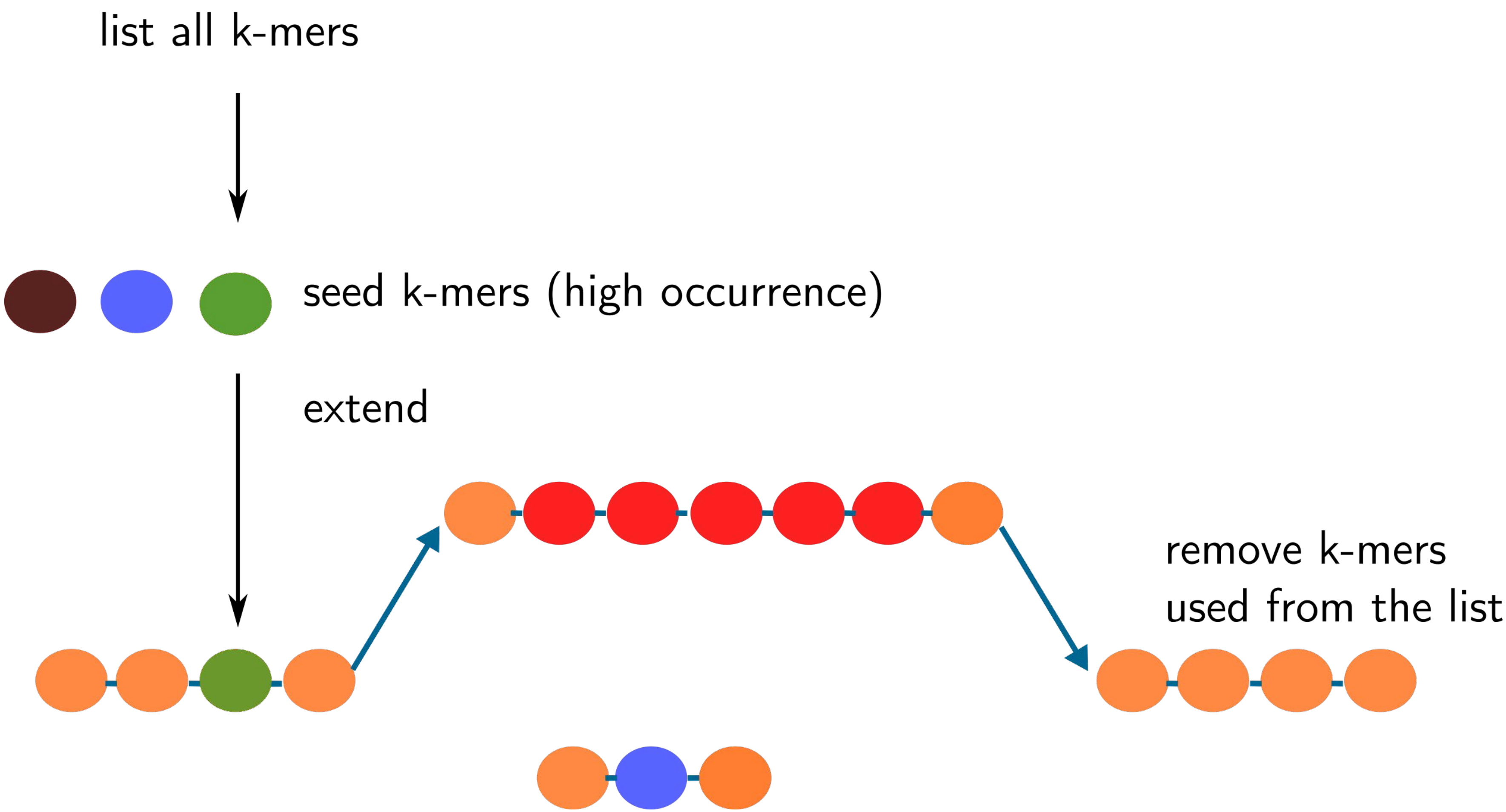
- Inchworm de Bruijn graph construction, part 1

- Chrysalis de Bruijn graph construction, part 2

- Butterfly Graph traversal using reads, isoforms enumeration

Trinity: detail

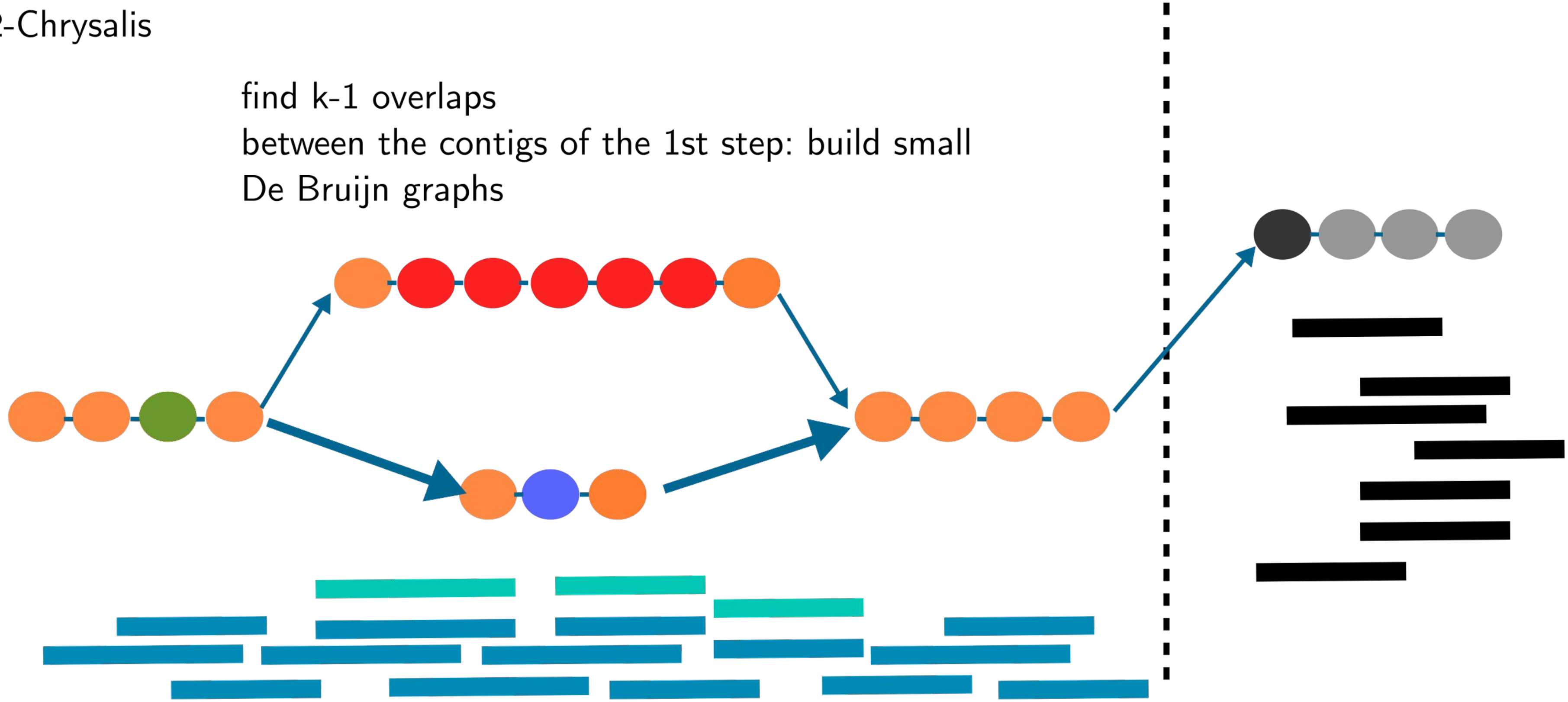
1-Inchworm



Trinity: detail

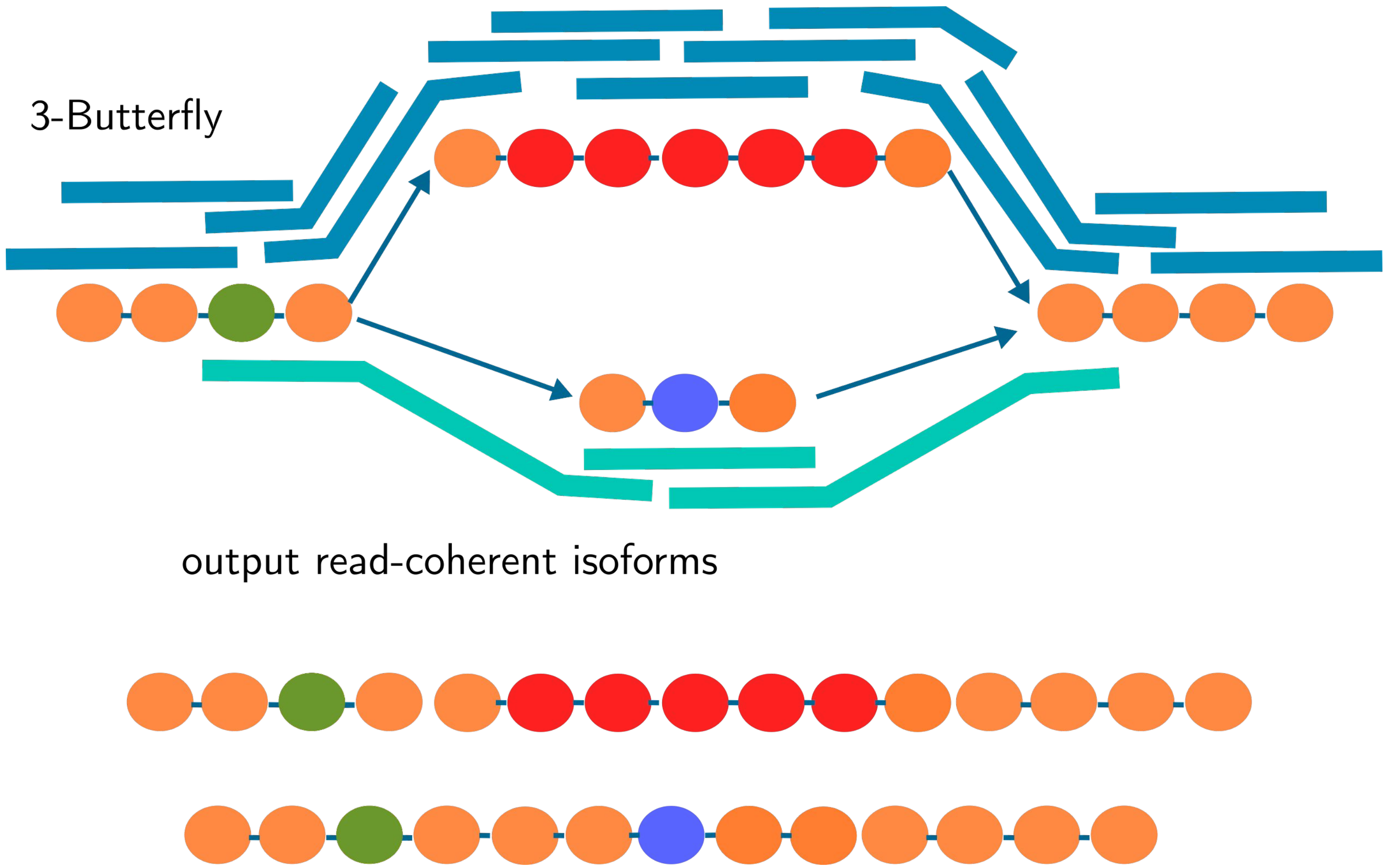
2-Chrysalis

find k-1 overlaps
between the contigs of the 1st step: build small
De Bruijn graphs



use read mapping information to separate clusters

Trinity: detail



Trinity output

```
>TRINITY_DN1000_c115_g5_i1 len=247 path=[31015:0-148 23018:149-246]
AATCTTTTTTGGTATTGGCAGTACTGTGCTCTGGGTAGTGATTAGGGCAAAAGAAGACAC
ACAATAAAGAACCAGGTGTTAGACGTCAGCAAGTCAAGGCCTTGGTTCTCAGCAGACAGA
AGACAGCCCTTCTCAATCCTCATCCCTTCCCTGAACAGACATGTCTTCTGCAAGCTTCTC
CAAGTCAGTTGTTCACAGGAACATCATCAGAATAAATTTGAAATTATGATTAGTATCTGA
TAAAGCA
```

-Trinity read cluster 'TRINITY_DN1000_c115'

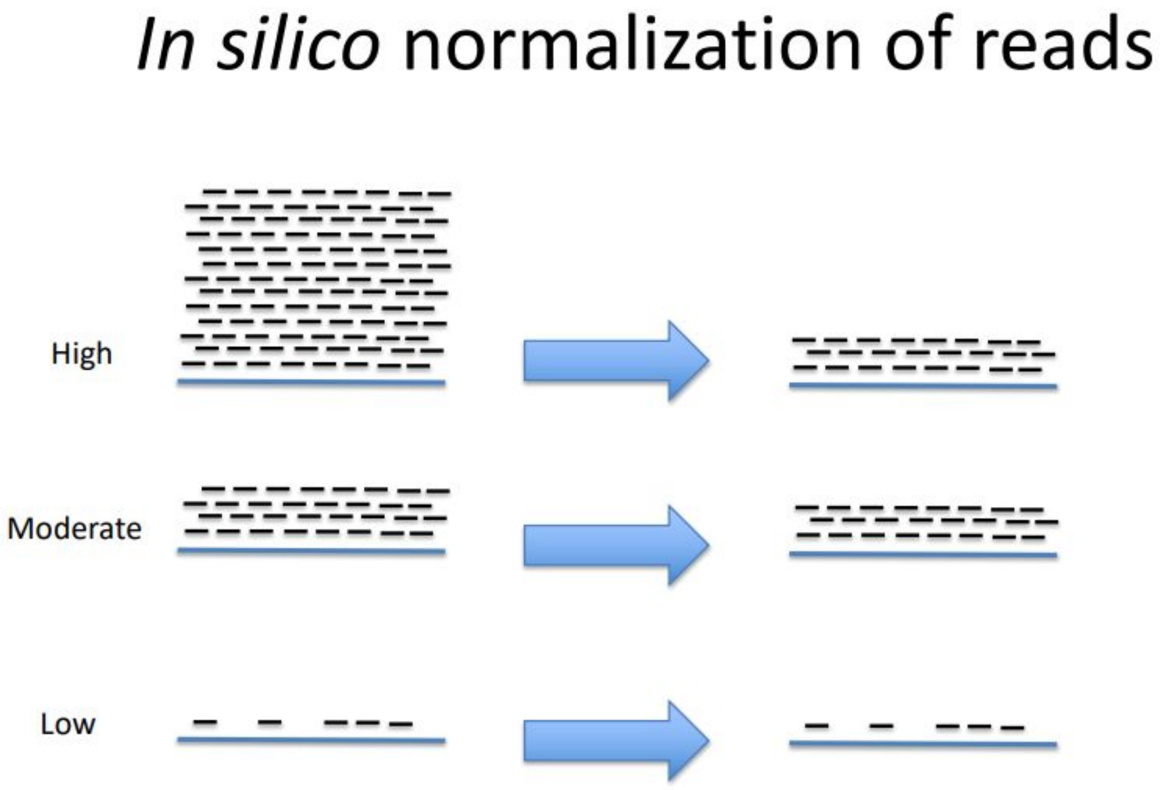
- gene 'g5'

- isoform 'i1'

-path=[31015:0-148 23018:149-246]") indicates the path traversed in the Trinity de Bruijn graph to construct that transcript

Normalization effects on assembly (example of Trinity)

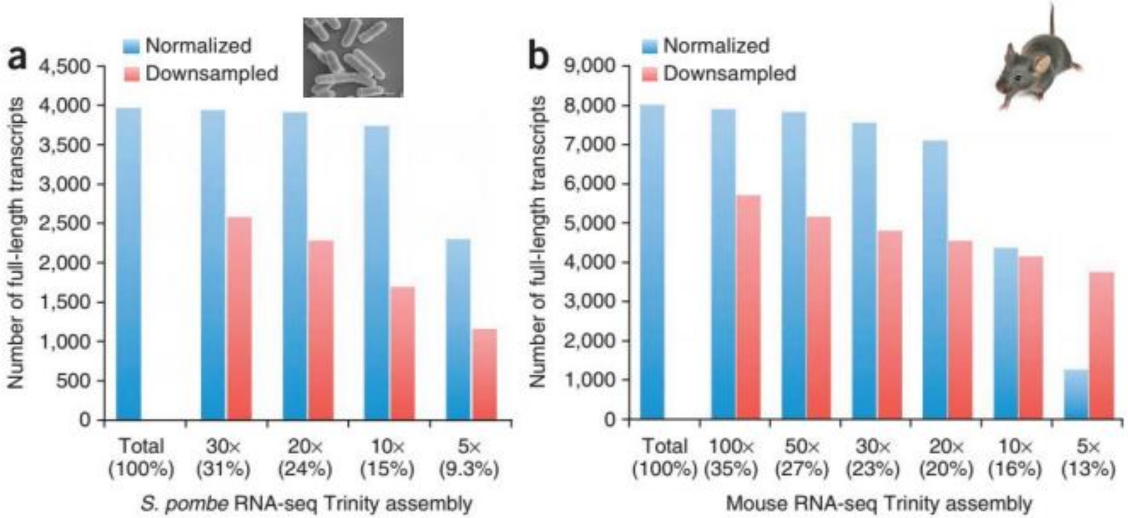
From Brian Haas



Normalization effects on assembly (example of Trinity)

From Brian Haas

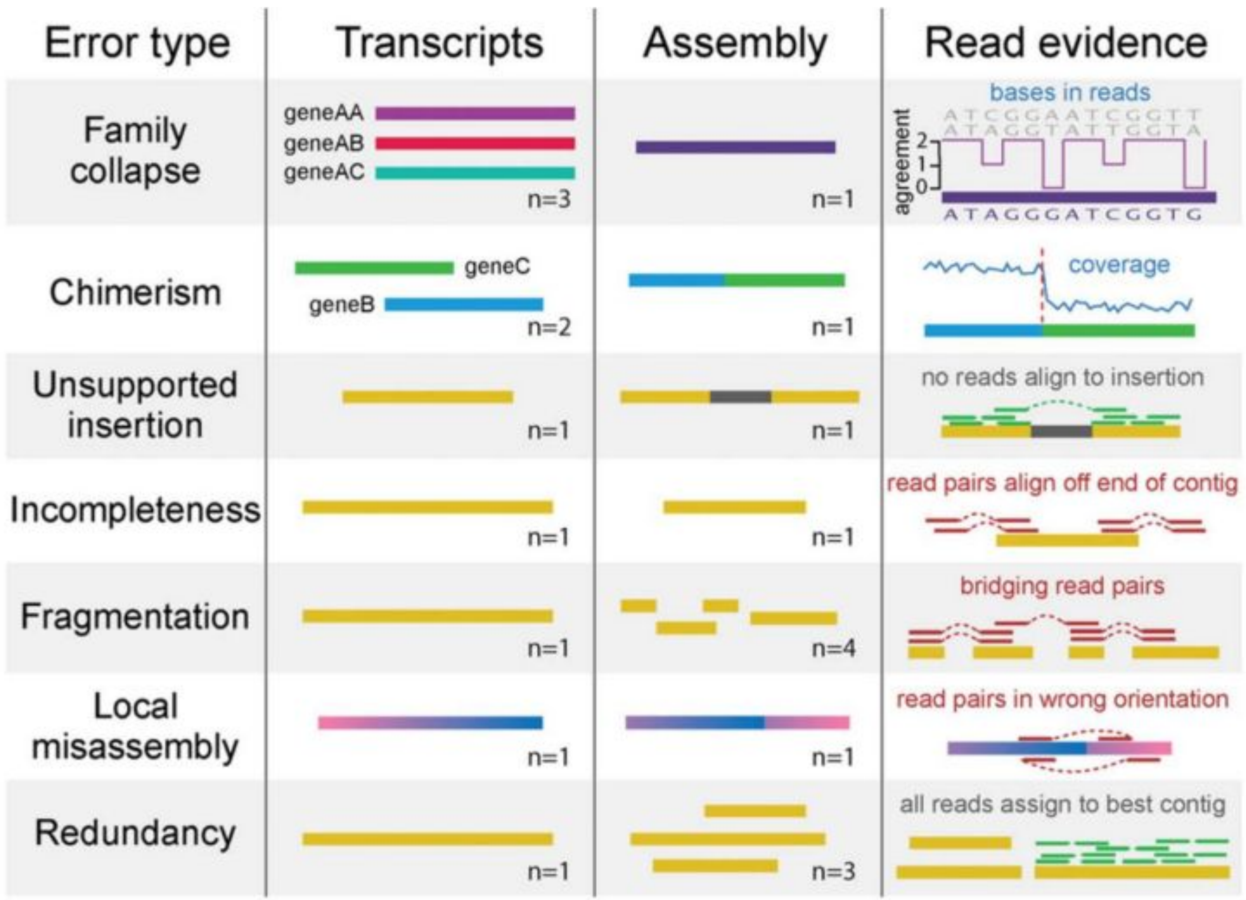
Impact of Normalization on *De novo* Full-length Transcript Reconstruction



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

Total (100%) 30× (31%) 20× (24%) 10× (15%) 5× (9.3%)
S. pombe RNA-seq Trinity assembly
Total (100%) 100× (35%) 50× (27%) 30× (23%) 20× (20%) 10× (16%) 5× (13%)
Mouse RNA-seq Trinity assembly

Errors made by assemblers



Smith-Unna et al. Genome Research, 2016

Assembly quality assessment

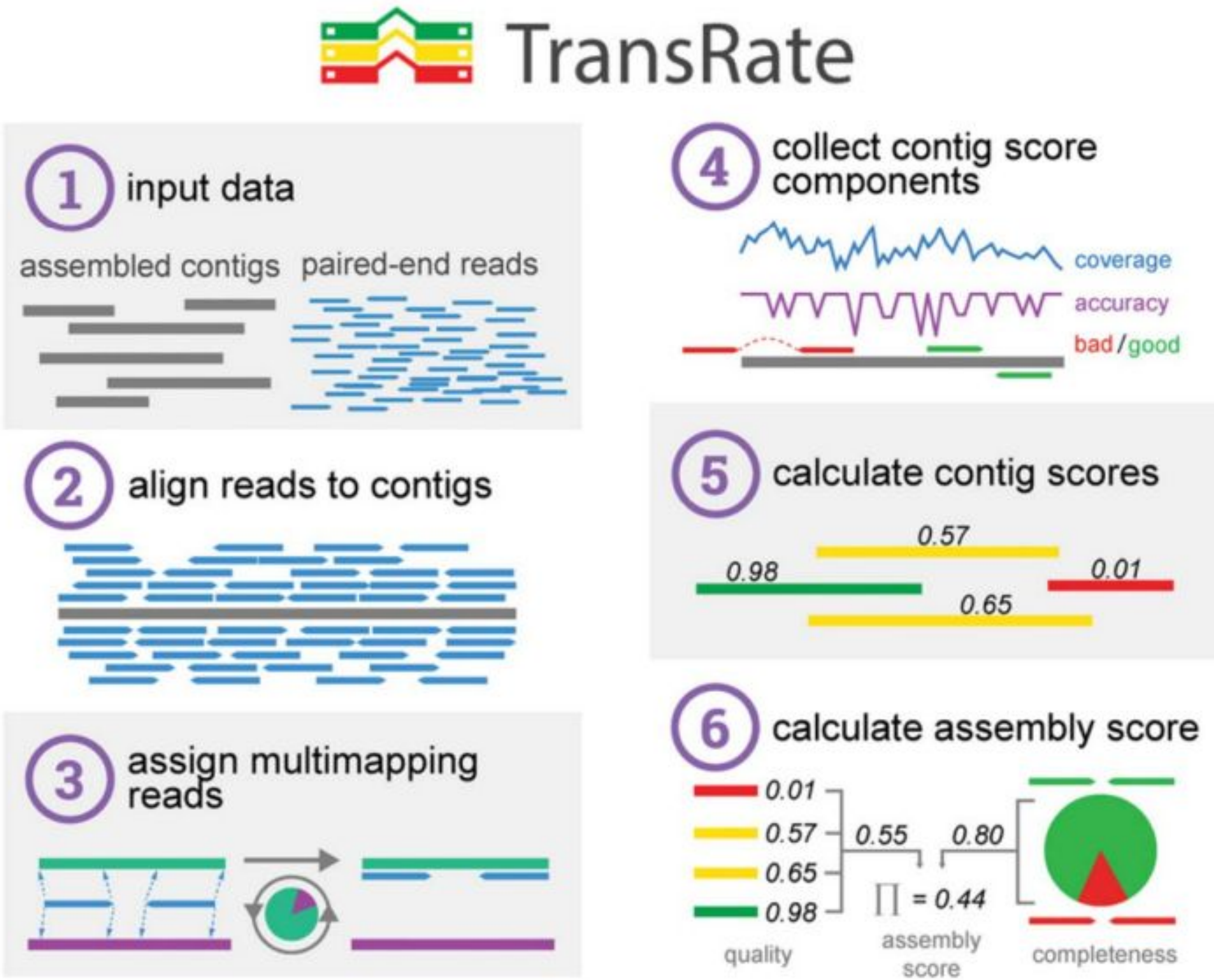
In transcriptome assemblies

- N50 is not very useful.
  - unreasonable isoform annotation for long transcripts drives higher N50
  - very sensitive reconstruction for short lowly expressed transcripts leads to lower N50

95%-assembled isoforms statistics
reference-free evaluation must be preferred
read remapping

Main tools:

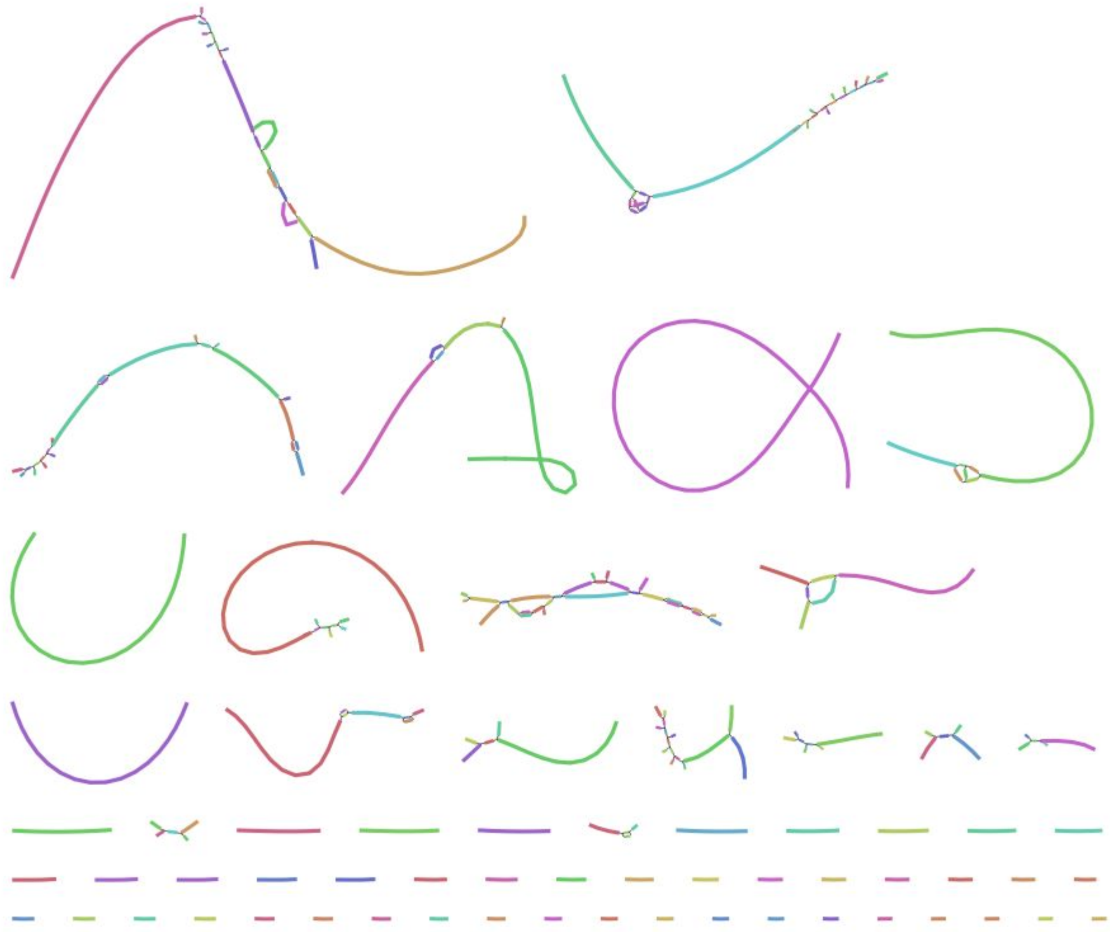
- rnaQuast http://cab.spbu.ru/software/rnaquast/
- Transrate http://hibberdlab.com/transrate/



Smith-Unna et al. Genome Research, 2016

Visualization: Bandage

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



Meta-practices

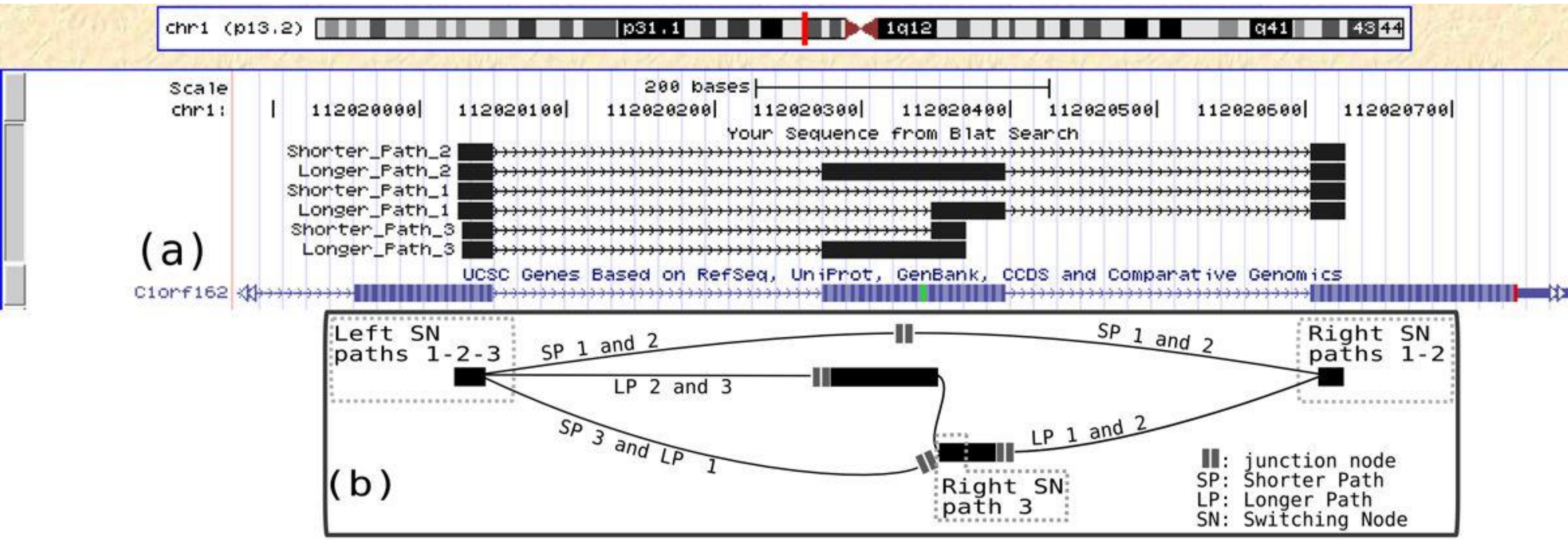
1- Read surveys, Twitter, blogs
2. Pick two assemblers
3. Run each assembler at least two times (different parameters)
4. Compare assemblies
5. If possible, visualize them

An assembly is not the absolute truth, it is a mostly complete, generally fragmented and mostly accurate hypothesis

Currently, Trinity, RNASpades and TransAbyss could be pointed as the most trustworthy/qualitative
(for known species. Not one tool for all issues).

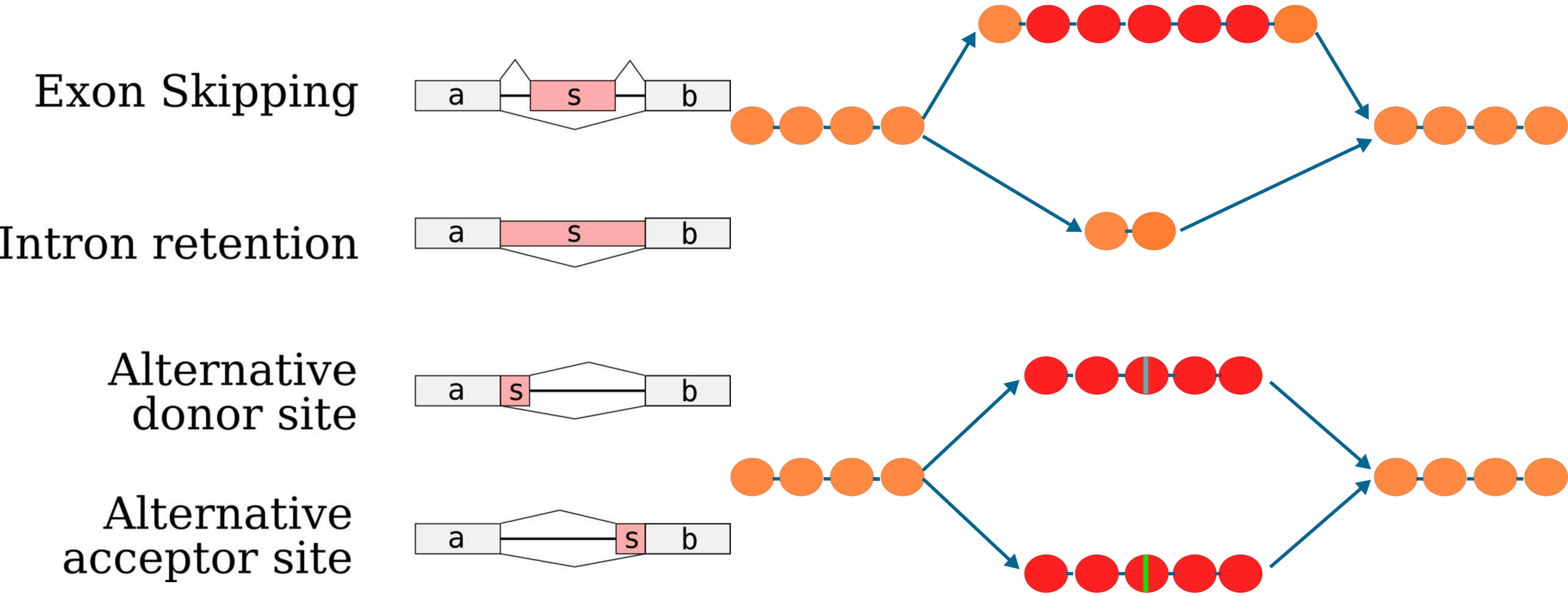
Practical: Trinity assembly

Assembly does not output all variants

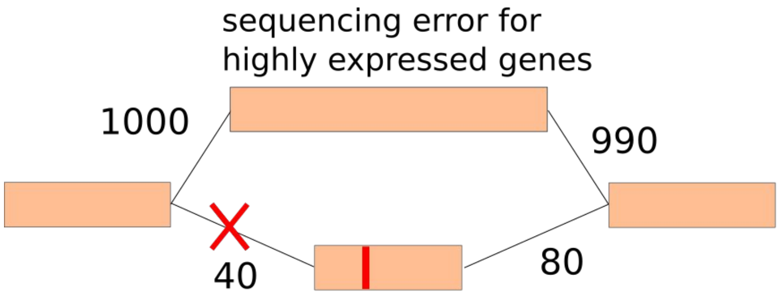


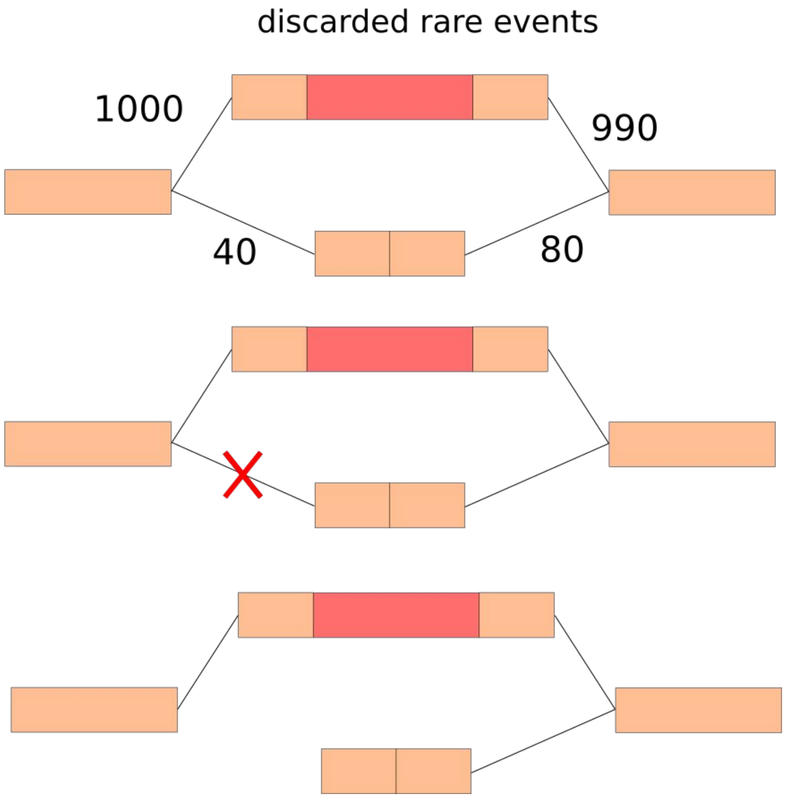
KISSPLICE

Goal: instead of assembling full-length transcripts, KISSPLICE (Sacomoto et al. 2012) focuses on assembling ONLY the **bubbles** that contain events and **enumerate** the maximum of them



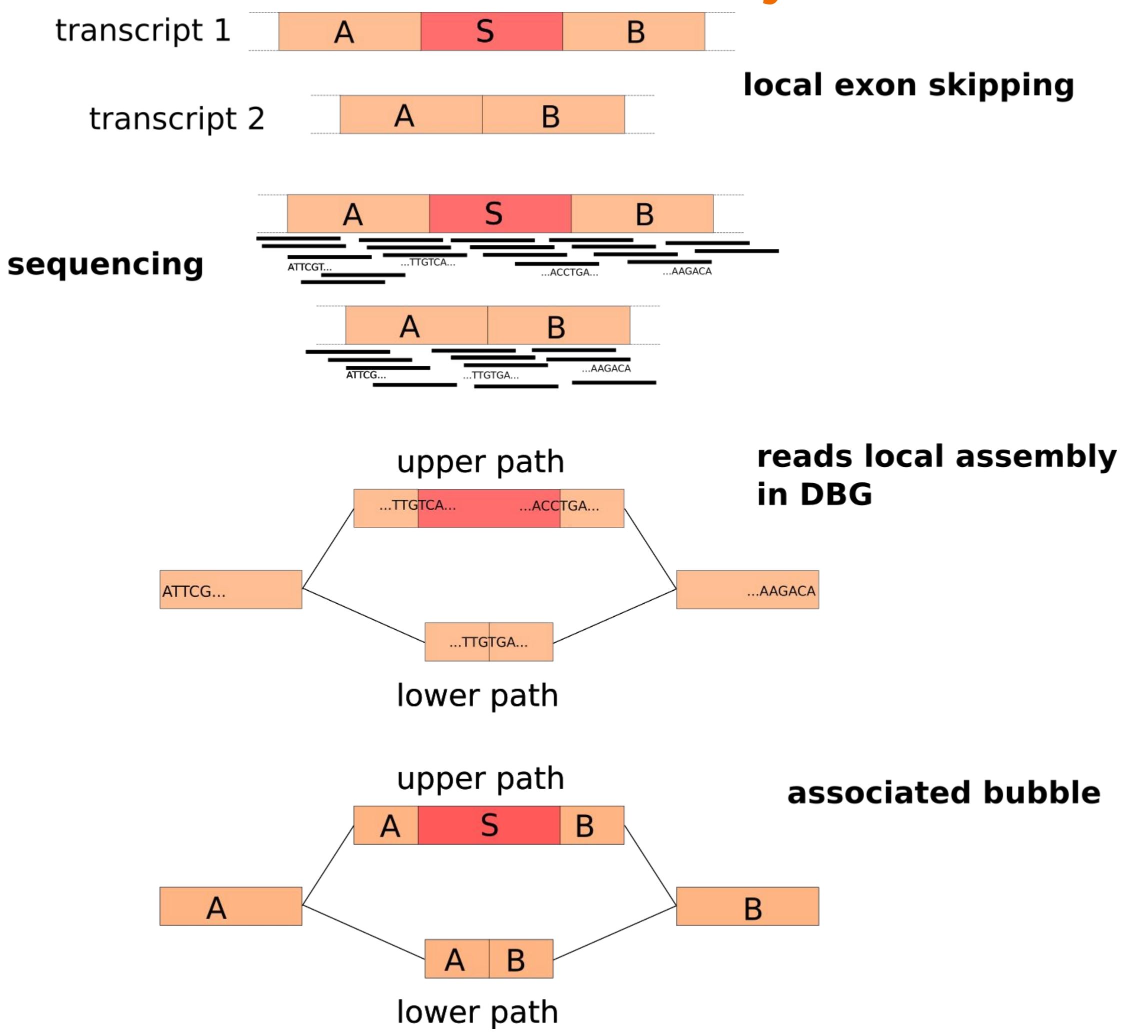
KISSPLICE: graph cleaning + local assembly





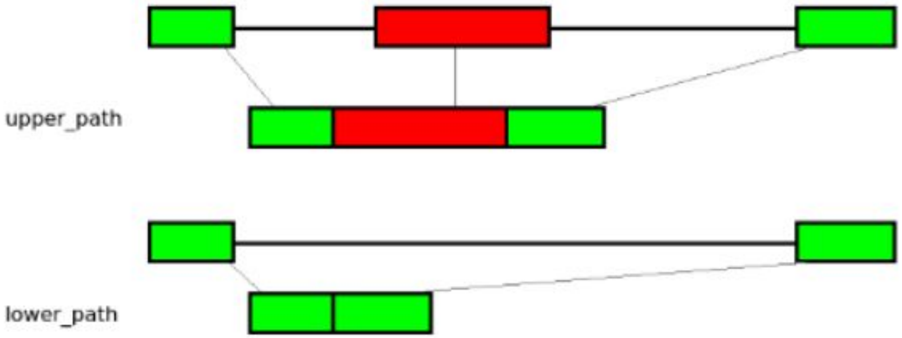
example: discard if ratio is
<0.05

Variants in local assembly



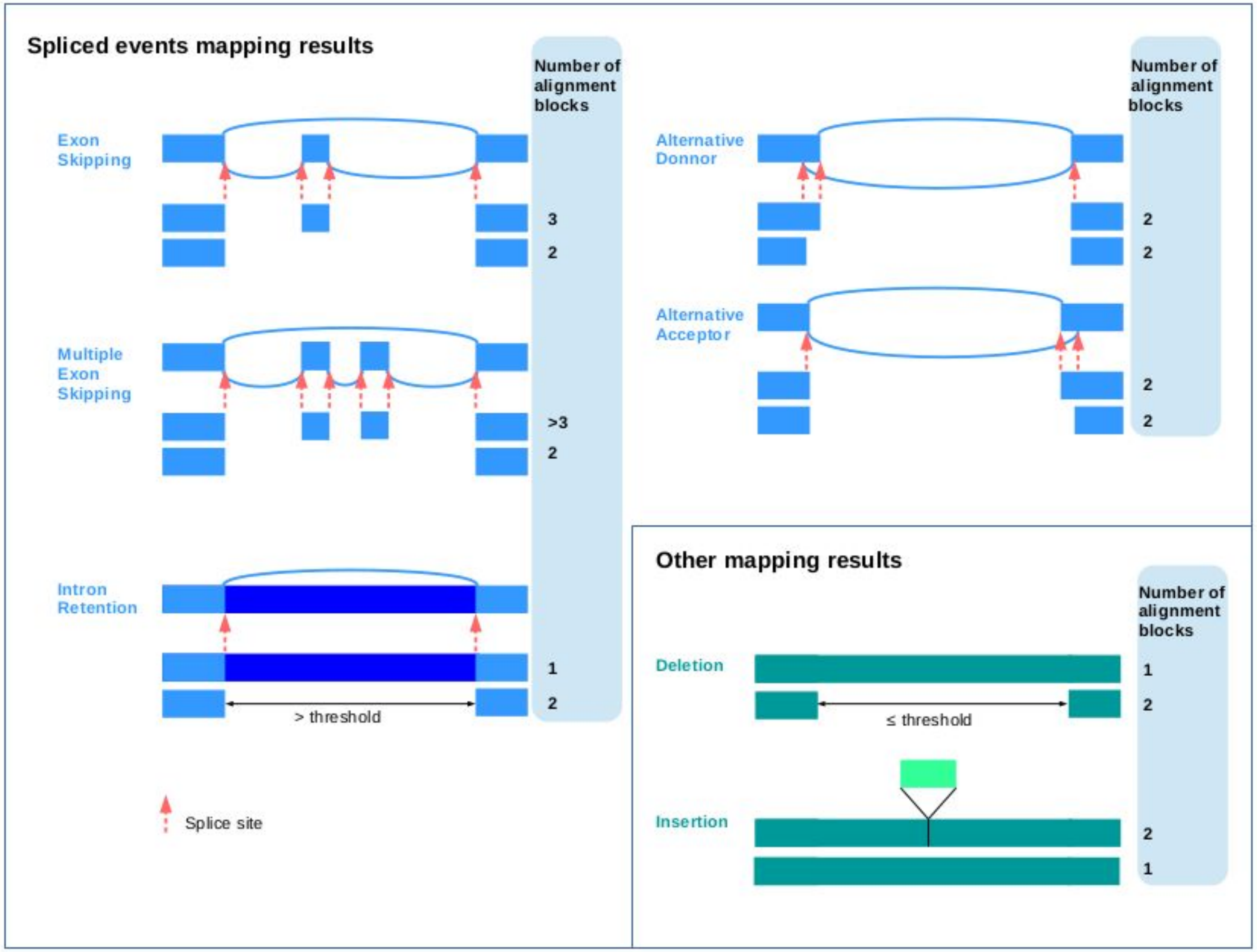
KISSPLICE’s output

```
>bcc_89|Cycle_0|Type_1|upper_path_length_122|C1_0|C2_1|C3_2|C4_1|rank_0.55097
CCCTGATGGCCTCAGAGGAGGAGTAAATGTGGGGACCTAGAGGAGGAGCTGAAAATTGTTACCAACAACTTGAAATCCCTGGAGGCCCAGGCGGACAAGTATTCCACCAAAGAAGATAAATA
>bcc_89|Cycle_0|Type_1|lower_path_length_46|C1_0|C2_0|C3_2|C4_6|rank_0.55097
CCCTGATGGCCTCAGAGGAGGAGTATTCCACCAAAGAAGATAAATA
```



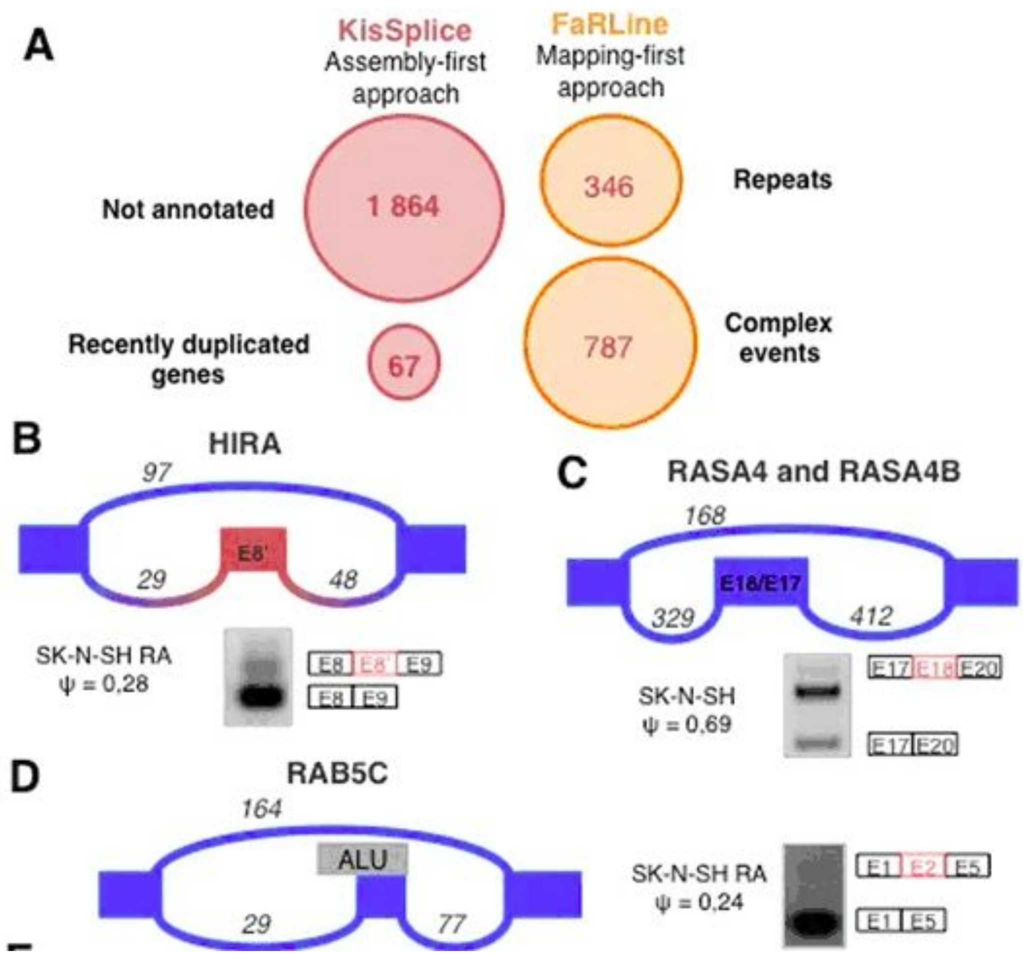
Post-processings

What do I have?	What I can use	
I have a reference genome	KisSplice2refgenome	differential analysis: kissDE
I have no reference genome	KisSplice2refTranscriptome	



for quantification only
see de-Kupl
Audoux et al. 2017

KISSPLICE case studies



Discover splicing events: Benoit Pilven et al. 2018

Farline: mapping
B found only by Kissplice (not annotated)
C found only by Kissplice (paralog)
D found only by mapping (Alu repeat)

Discover SNPs in pooled RNA-seq: Lopez-Maestre et al. 2016

Practical: Kissplice

Long reads : the ~~future~~ present of transcriptomics

Long reads overview

Possibilities & pipelines

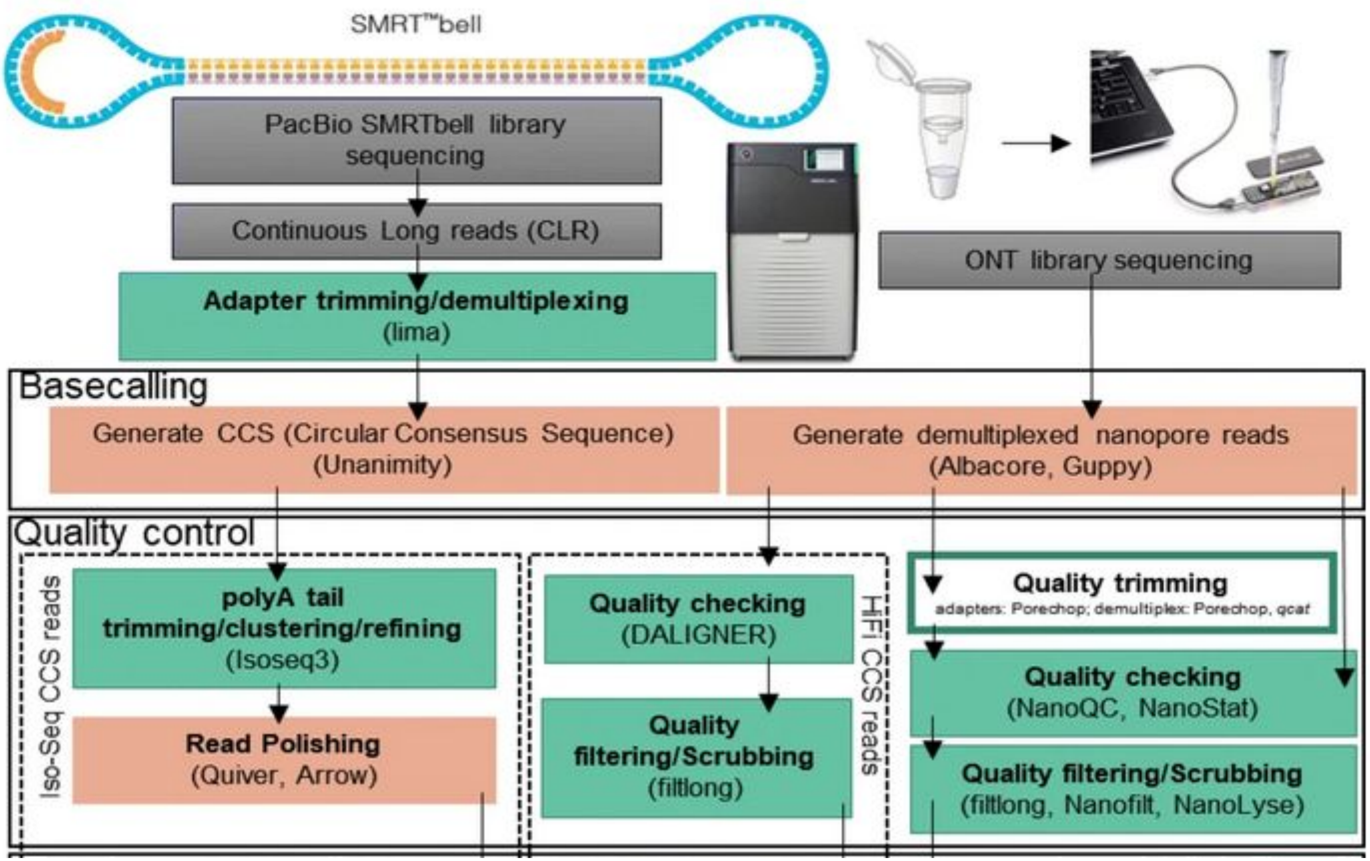
Limitations of short reads

- recent studies suggest that our reference transcriptomes **miss isoforms**
- in particular in the context of **alternative splicing**
- *de novo* assembly of species with unknown/hardly known transcriptomes is still a challenge
- the mandatory cDNA step in short reads protocols implies **bias**

Long reads technologies

- sequencing of long (>10kb) molecules is possible
  - **full RNAs !**
- with a higher (~1-5% to 14%) **error rate**
- **error profile** is different from SR: indels in **homopolymers**
- some allow to sequence directly RNA (reduced bias, epitranscriptomics)

Long reads technologies



from Shanika L. Amarasinghe et al. Genome Biol. 2020

Pacific Biosciences (Pacbio)

- in the case of RNA, a fragment is **read several times** and a consensus is computed
- read length limited by the longevity of the polymerase
- circular consensus sequence quality = *f*(fragment length, pol longevity)
- 4 passes : 1% error (0.1% reached after 9 passes)
- bias for indels in homopolymers

Pacific Biosciences (Pacbio)

- the protocol is better suited for studying **isoform identification only** (not quantification)
  - initial overrepresentation of shorter molecules lead to size selection which introduces a bias
  - mitigation solutions still in progress

Oxford Nanopore technologies (ONT)

- no limit to read length
- the fragment is read only once in the pore
- read quality depends on the speed of the fragment through the pore
  - **quality decreases in the late stages** of sequencing
- error rate >5%
- bias for **indels in homopolymers**

Oxford Nanopore technologies (ONT)

- 1D sequencing protocol : **single pass** of strands
- $1D^2$ protocol: sequence the **complementary strand immediately after** the forward strand and compute a consensus
- accuracy over homopolymers is in progress (from R10 chemistry)

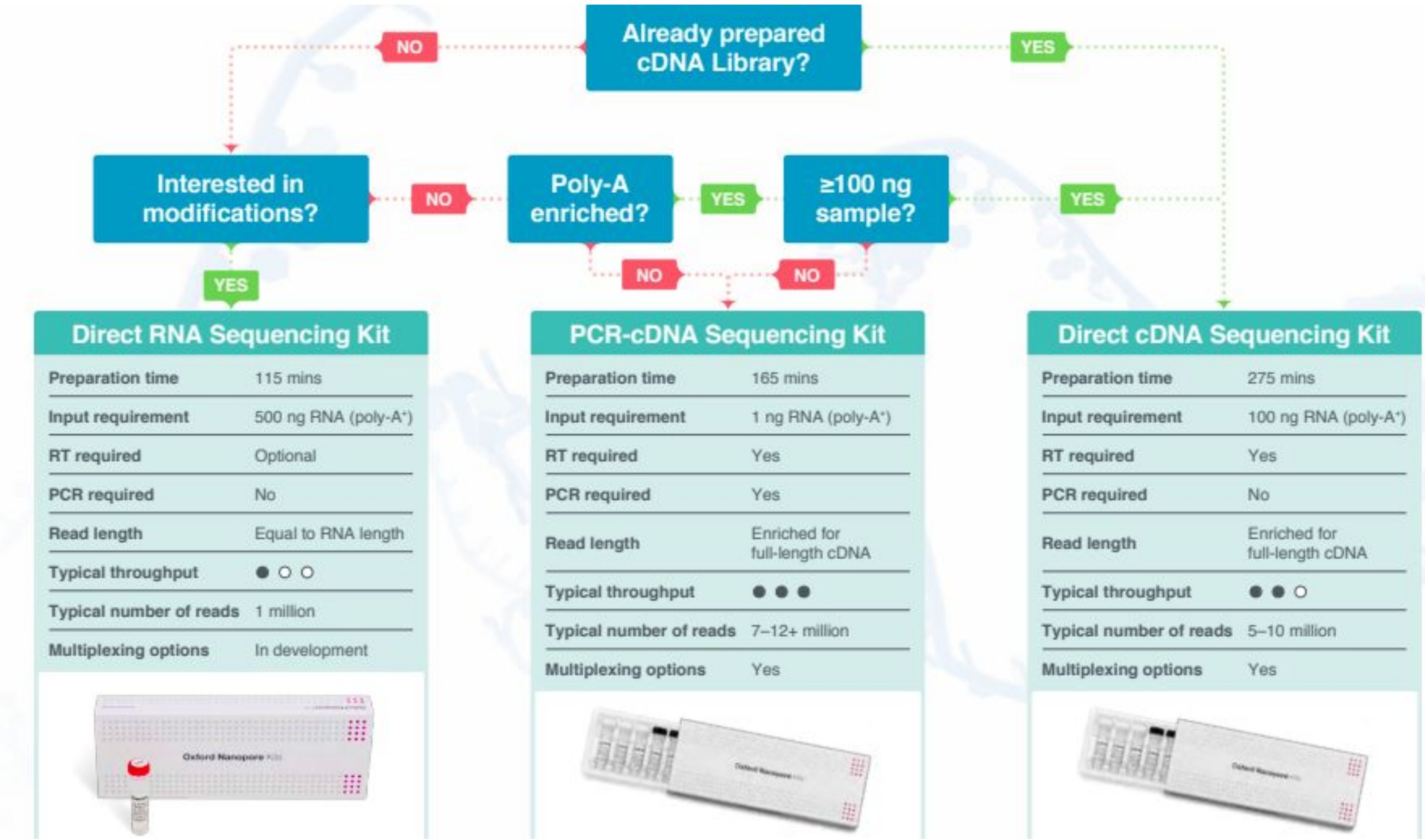
Oxford Nanopore technologies (ONT)'s RNA direct

Methods based on reverse transcription:

- ❏ Template switching and artifactual splicing
- ❏ Loss of strandedness information
- ❏ Loss of base modifications
- ❏ Propagation of error due to PCR

Direct RNA

- ❏ no bias due to PCR
- ❏ possible to study some RNA modifications
- ❏ as of today not adequate for quantification (too much material is required)



	Direct RNA Sequencing Kit	PCR-cDNA Sequencing Kit	Direct cDNA Sequencing Kit
Preparation time	115 mins	165 mins	275 mins
Input requirement	500 ng RNA (poly-A⁺)	1 ng RNA (poly-A⁺)	100 ng RNA (poly-A⁺)
RT required	Optional	Yes	Yes
PCR required	No	Yes	No
Read length	Equal to RNA length	Enriched for full-length cDNA	Enriched for full-length cDNA
Typical throughput	● ○ ○	● ● ●	● ● ○
Typical number of reads	1 million	7–12+ million	5–10 million
Multiplexing options	In development	Yes	Yes

material from Oxford Nanopore

What has been studied with long reads so far

Near mature:

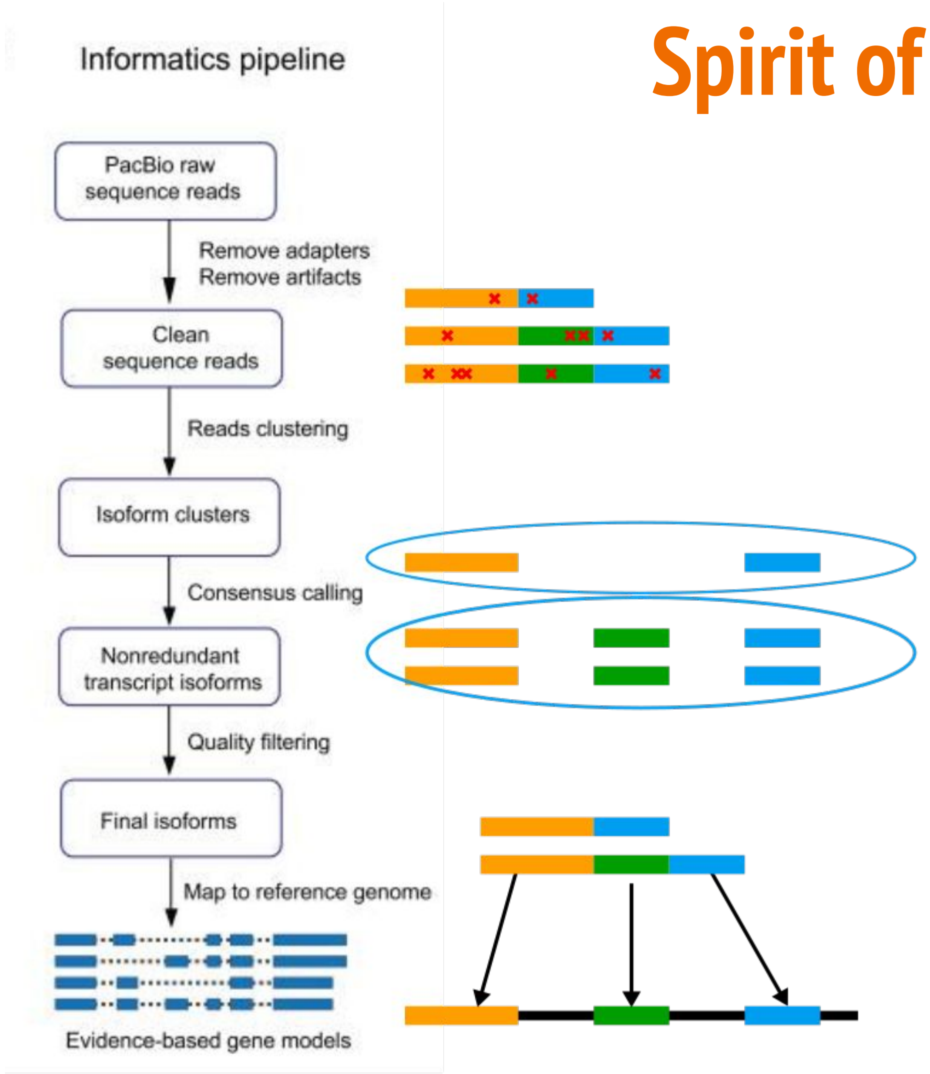
- **quantification** of already **known genes** and isoforms
- **quantification** of of **novel isoforms** from known genes ex
- **detection and characterization** of the different isoforms and **genes exon structure without quantification** (PacBio's "Iso-Seq" method)

What has been studied with long reads so far

Exploratory:

- ❏ RNA of paralogous genes (Dougherty et al., 2018, Chen et al., 2017)
- ❏ fusion transcripts (Nattestad et al., 2018).
- ❏ allele-specific expression (Tilgner et al., 2014), avelier et al., 2015).

Spirit of most analysis pipelines



reads
comparison all vs all

clusters:
isoform detection
compute consensus

report non redundant
polished transcript sequences

alignment to genome
(Minimap2, GraphMap2, GMAP...)

report genes/isoforms
quantify

adapted from Gordon et al. 2015

Isoform detection: PacBio's Iso-Seq3 + ToFU/Cupcake

https://github.com/ylipacbio/IsoSeq3/

- will tend to **merge alternative transcripts** (heavily depends on the reference quality)
- computationally expensive
- tailored to **Pacbio reads only**
- scripts for exon-junction description and quantification

Alternative isoforms detection pipelines

Specialized for Pacbio

- ❑ SQANTI (reference genome, gff)
- ❑ ToFu (reference genome & limited *de novo*)
- ❑ TAPIS (reference genome)
- ❑ IsoCon (*de novo* correction and detection of different transcripts at the base level, targeted data)

Specialized for Nanopore

- ❑ FLAIR (reference genome)

Technology agnostic

- ❑ TALON (input = alignments to ref)
- ❑ MANDALORION
- ❑ TrackCluster (*de novo*)

Pipelines focused on quantification

- developed by Nanopore (based on alignment + Salmon)
  https://github.com/nanoporetech/pipeline-transcriptome-de
- LIQA (truncated reads treated using an EM algorithm)

Application example

frontiers in Genetics

Front Genet. 2021; 12: 683408.
Published online 2021 Jul 15. doi: 10.3389/fgene.2021.683408

PMCID: PMC8321248
PMID: 34335690

PacBio Iso-Seq Improves the Rainbow Trout Genome Annotation and Identifies Alternative Splicing Associated With Economically Important Phenotypes

Ali Ali,[1] Gary H. Thorgaard,[2] and Mohamed Salem[1,*]

Long-read cDNA sequencing identifies functional pseudogenes in the human transcriptome

Robin-Lee Troskie, Yohaann Jafrani, Tim R. Mercer, Adam D. Ewing, Geoffrey J. Faulkner & Seth W. Cheetham

Genome Biology **22**, Article number: 146 (2021) | Cite this article

Long reads miscellaneous

specific alignment tools start to emerge (uLTRA, Sahlin et al. 2021)

cleaning for spliced sites (with ref) TranscriptClean , FLAIR

reference-free correction might become a standard in the years to come (isONcorrect, Sahlin et al. 2021) (/!\ generally, do not use reference free correction methods tailored for genomic long reads)

de novo assembly using short+long reads+ref: StringTie2

a website that lists long reads tools: https://long-read-tools.org/table.html

Next challenges with long reads

- guarantee full-length RNA or cDNA libraries
- sequence all different RNAs (not only poly-A)
- allele-specific transcripts
- acquire knowledge about 3' and 5' ends, polyA tails (homopolymers)
- new steps toward full de novo pipelines

What was not viewed during this session

-bacterial RNA

-genome-guided assembly

-metatranscriptomics

-single cell RNA

-tools specialized for ncRNAs, smallRNAs

-tools specialized for fusion transcripts

-transcript annotation (https://busco.ezlab.org/ for instance)

- ...

-**up next**: differential study (statistics for RNA-seq)