RNA-Seq Analysis Workshop

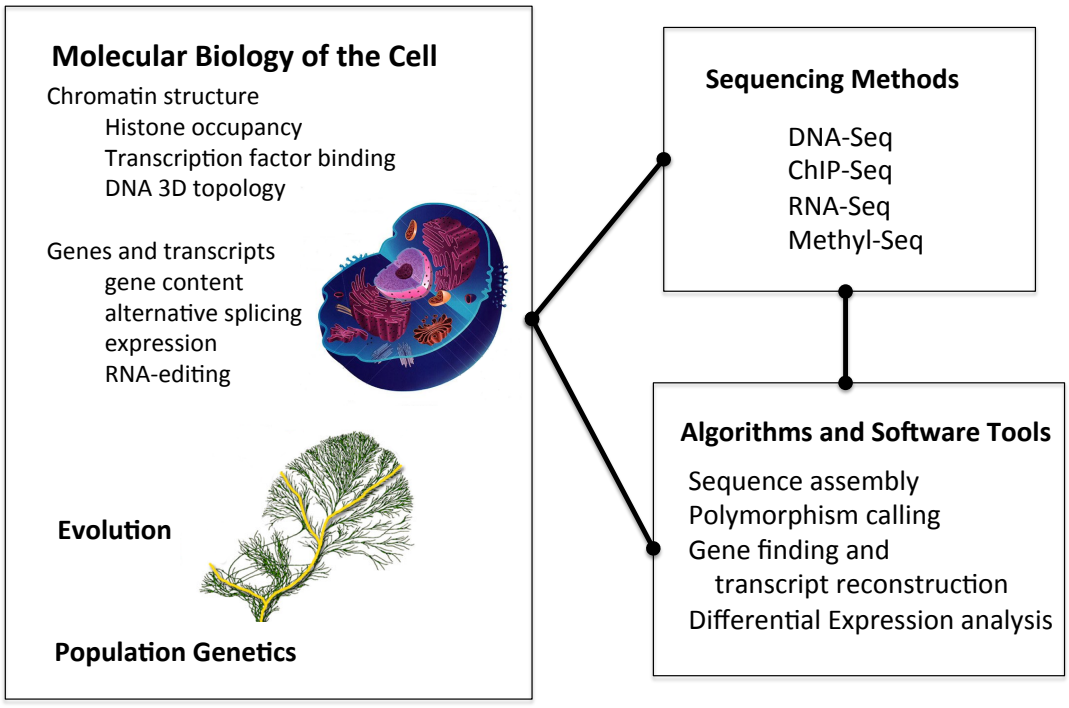
Tuxedo and Trinity for Next-Gen Transcriptome Studies

CSHL 2012-10

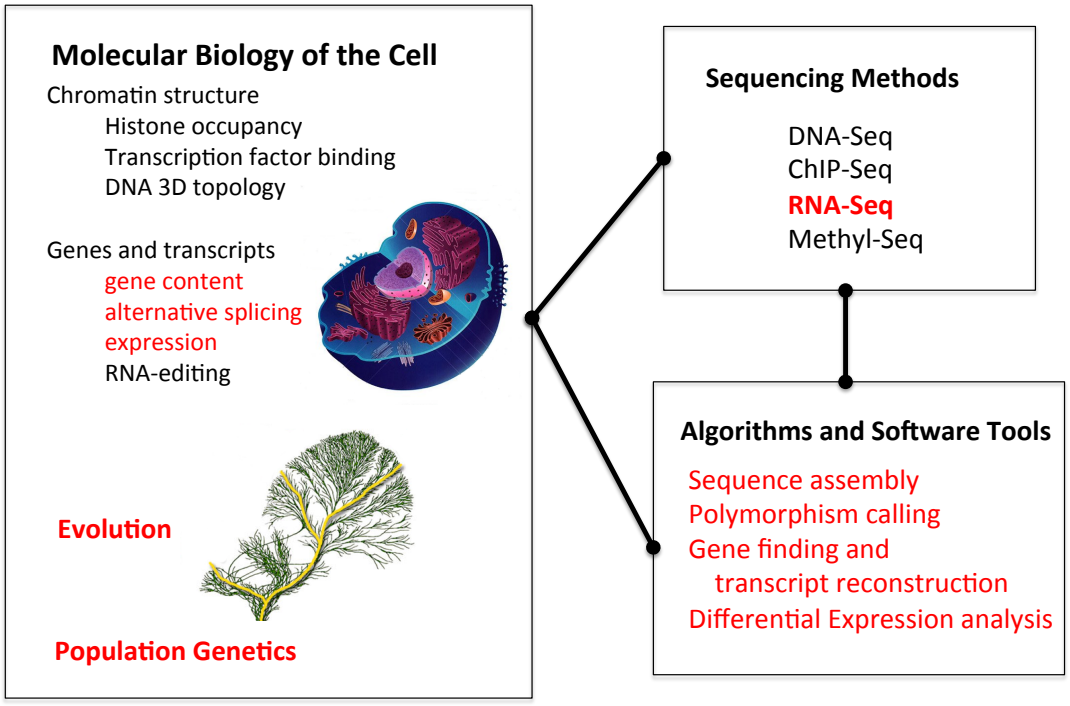
Brian Haas

Broad Institute

Next-gen Sequencing Transforming Modern Science



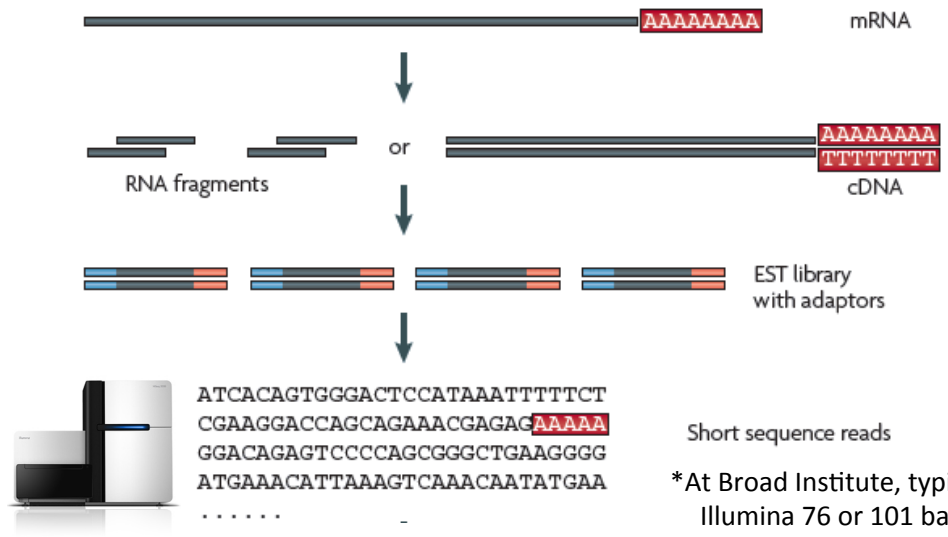
RNA-Seq as a Driving Technology



Outline

- RNA-Seq basics
- Analysis paradigms
- Genome-based rna-seq studies
- Data formats and visualization
- De novo transcriptome-based rna-seq studies
- Transcript Quantification
- Differential Expression Analysis

RNA-Sequencing Methodology



*At Broad Institute, typically Illumina 76 or 101 base reads as paired ends

*Adapted from Wang, Gerstein, and Snyder, Nature Reviews Genetics, 2009

Common Data Formats for RNA-Seq

FastA format:

```
>61DFRAAXX100204:1:100:10494:3070/1
AAACAACAGGGCACATTGTCACTCTTGTATTTGAAAAACACTTTCCGGCCAT
```

FastQ format:

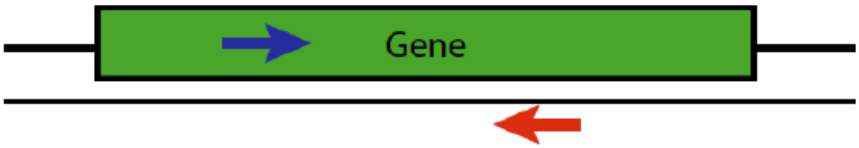
```
@61DFRAAXX100204:1:100:10494:3070/1
AAACAACAGGGCACATTGTCACTCTTGTATTTGAAAAACACTTTCCGGCCAT
+
ACCCCCCCCCCCCCCCCCCCCCCCCCCCCCBC?CCCCCCCCC@@CACCCCCA
```

AsciiEncodedQual= -10 * log10(Pwrong) + 30

Ascii ('C') = 64

So, Pwrong('C') = 10^((64-30) / (-10)) = 10^-3.4 = **0.0004**

Paired-end Sequences

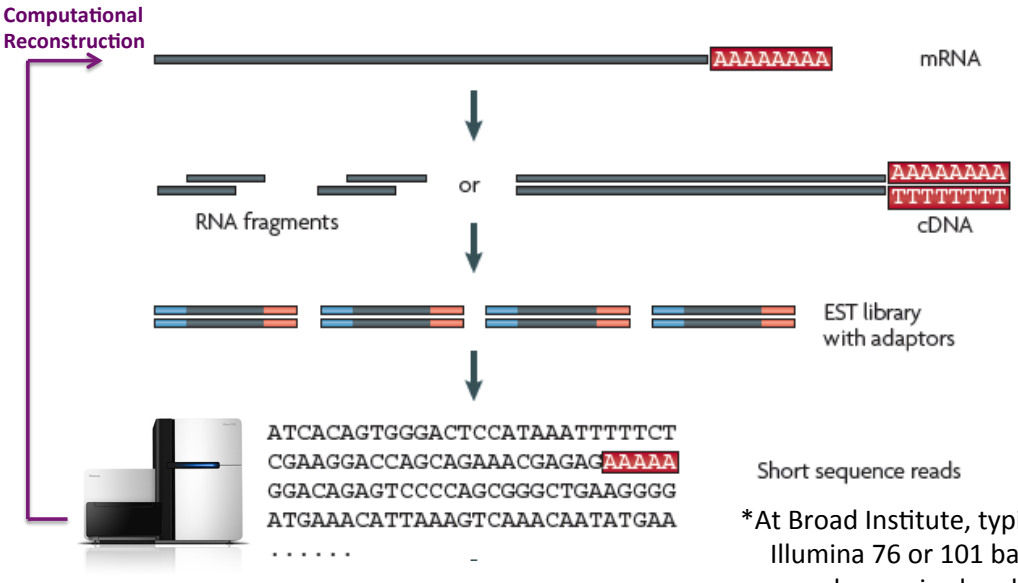


Two FastQ files, read name indicates left (/1) or right (/2) read of paired-end

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

```
@61DFRAAXX100204:1:100:10494:3070/2
CTCAAATGGTTAATTCTCAGGCTGCAAATATTCGTTCAGGATGGAAGAACA
+
C<CCCCCCCACCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCBCCCC
```

RNA-Sequencing Methodology



*At Broad Institute, typically Illumina 76 or 101 base reads as paired ends

*Adapted from Wang, Gerstein, and Snyder, Nature Reviews Genetics, 2009

Transcript Reconstruction from RNA-Seq Reads

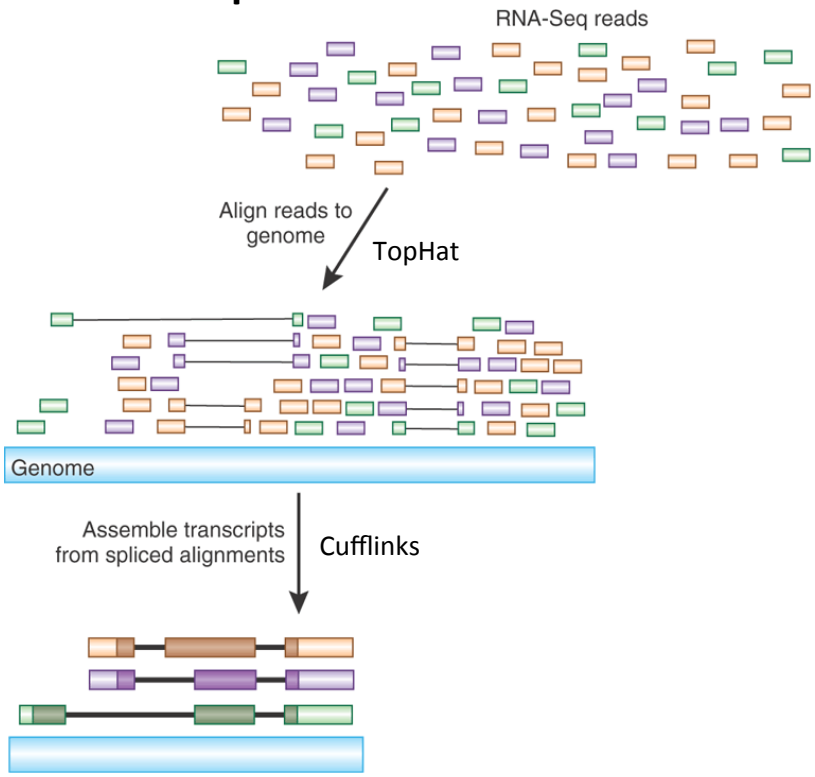
Advancing RNA-Seq analysis

Brian J Haas & Michael C Zody — Nature Biotech, 2010

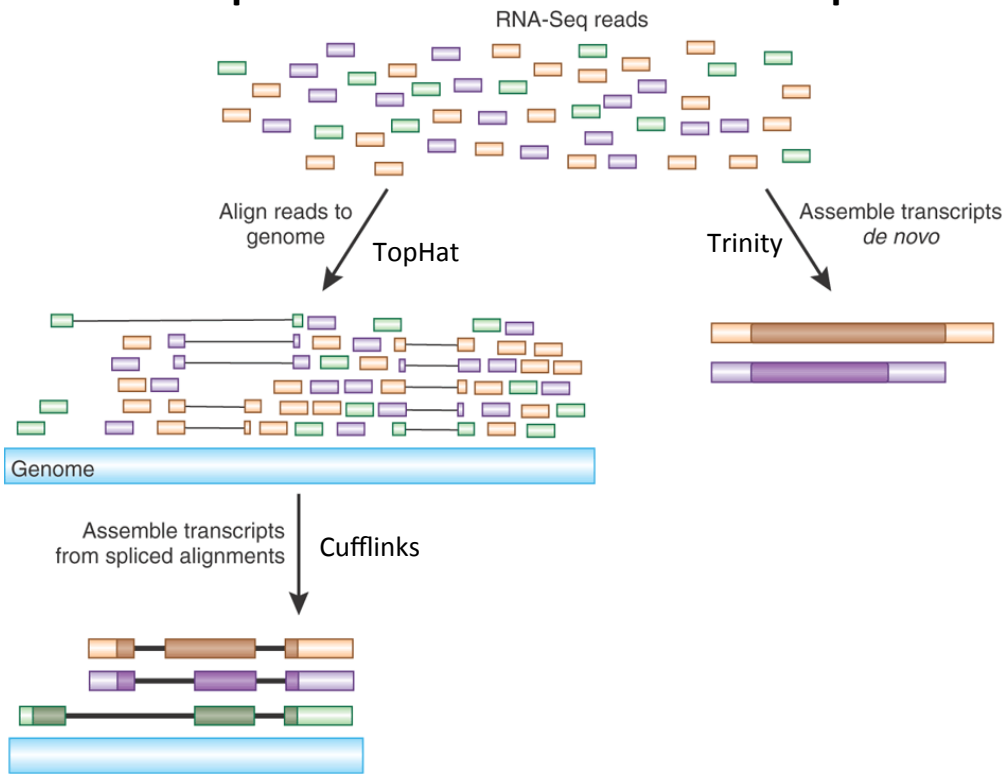
New methods for analyzing RNA-Seq data enable *de novo* reconstruction of the transcriptome.

Transcript Reconstruction from RNA-Seq Reads

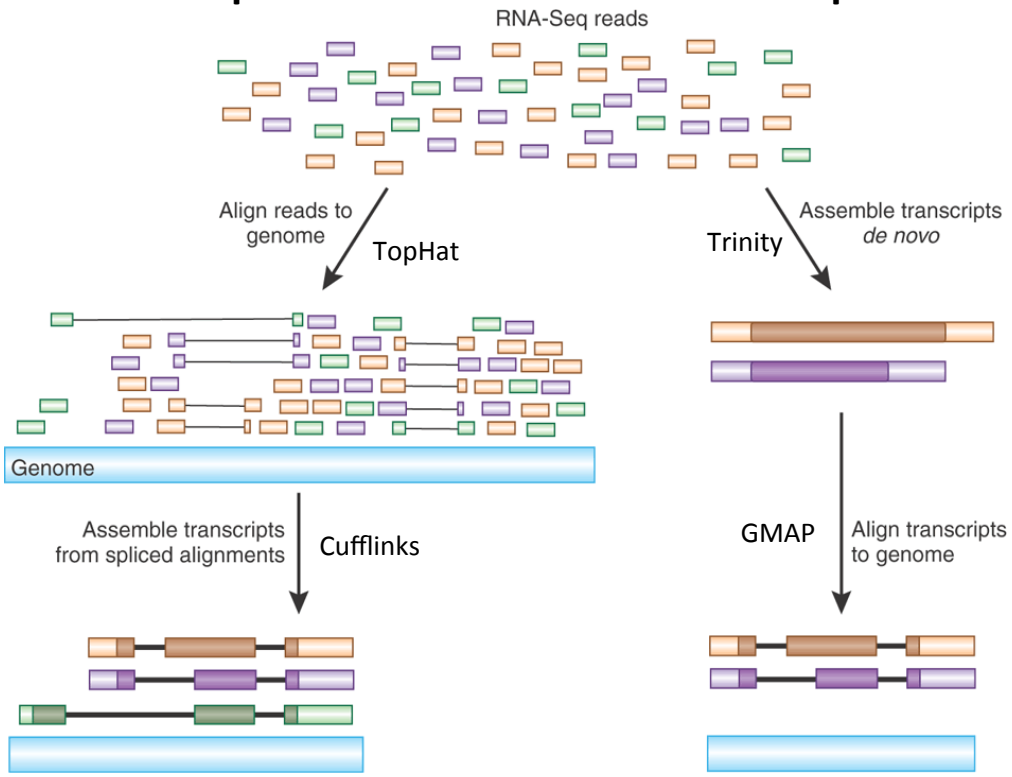
Transcript Reconstruction from RNA-Seq Reads



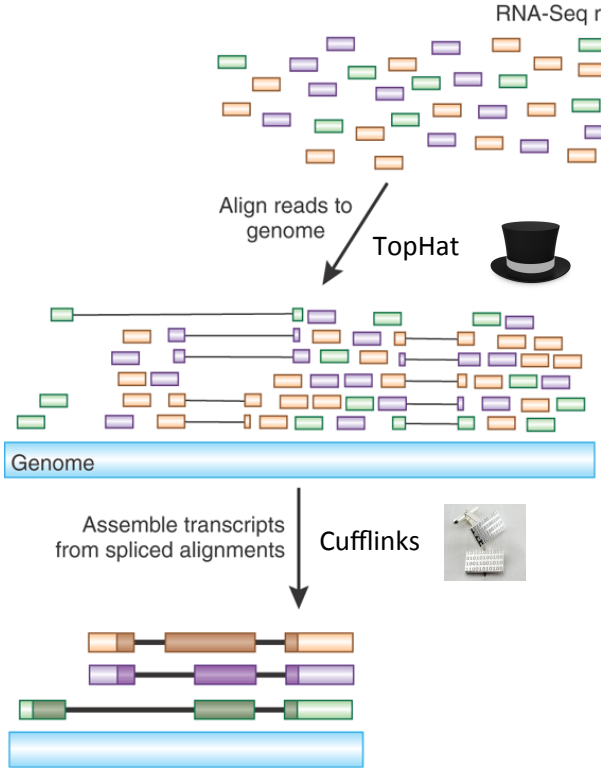
Transcript Reconstruction from RNA-Seq Reads



Transcript Reconstruction from RNA-Seq Reads



Transcript Reconstruction from RNA-Seq Reads



The Tuxedo Suite:

End-to-end Genome-based RNA-Seq Analysis Software Package

NATURE PROTOCOLS | PROTOCOL

Differential gene and transcript expression analysis of RNA-seq experiments with TopHat and Cufflinks

Cole Trapnell, Adam Roberts, Loyal Goff, Geo Pertea, Daehwan Kim, David R Kelley, Harold Pimentel, Steven L Salzberg, John L Rinn & Lior Pachter

Affiliations | Contributions | Corresponding author

Nature Protocols **7**, 562–578 (2012) | doi:10.1038/nprot.2012.016
Published online 01 March 2012

Overview of the Tuxedo Software Suite

Bowtie (fast short-read alignment)

TopHat (spliced short-read alignment)

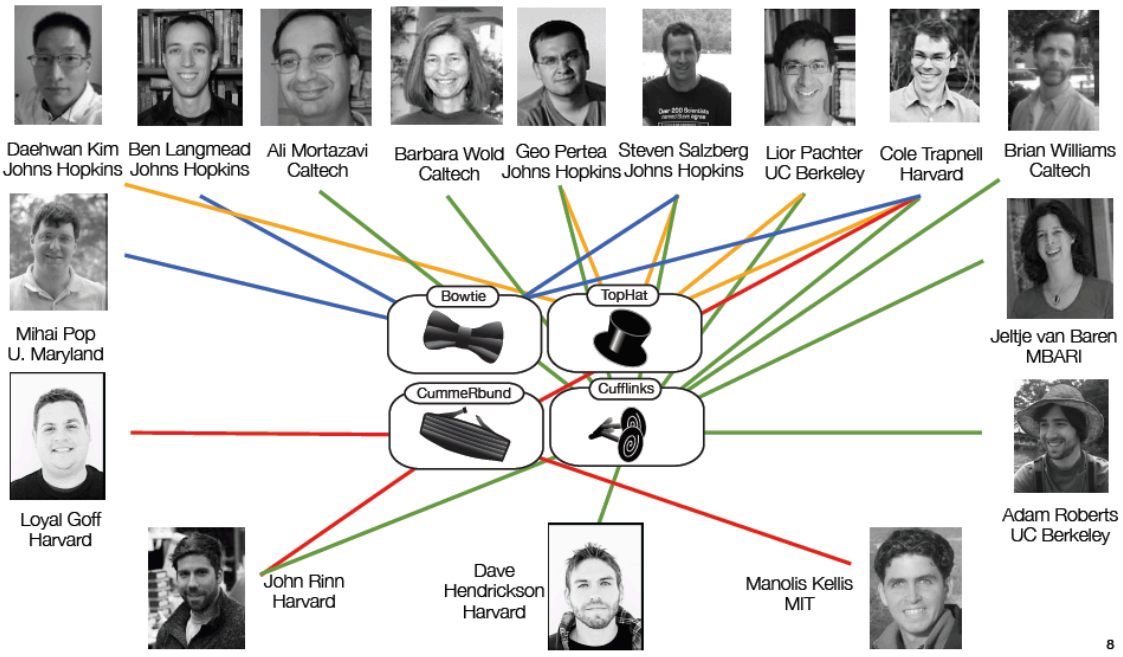
Cufflinks (transcript reconstruction from alignments)

Cuffdiff (differential expression analysis)

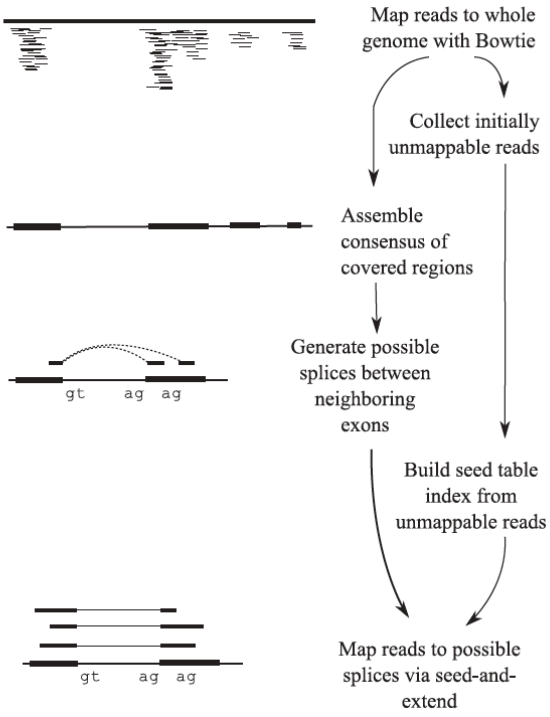
CummeRbund (visualization & analysis)

Slide courtesy of Cole Trapnell

Tuxedo development team



The TopHat Pipeline



From Trapnell, Pachter, & Salzberg. Bioinformatics. 2009

Alignments are reported in a compact representation: SAM format

```
0       61G9EAAXX100520:5:100:10095:16477
1       83
2       chr1
3       51986
4       38
5       46M
6       =
7       51789
8       -264
9       CCCAAACAAGCCGAACTAGCTGATTTGGCTCGTAAAGACCCGGAAA
10      ###CB?=ADDBCBCDEEFFDEFFFDEFFGDBEFGEDGCFGFGGGGG
11      MD:Z:67
12      NH:i:1
13      HI:i:1
14      NM:i:0
15      SM:i:38
16      XQ:i:40
17      X2:i:0
```

Link to SAM format description

Alignments are reported in a compact representation: SAM format

```
0       61G9EAAXX100520:5:100:10095:16477  (read name)
1       83  (FLAGS stored as bit fields; 83 = 00001010011 )
2       chr1  (alignment target)
3       51986 (position alignment starts)
4       38
5       46M  (Compact description of the alignment in CIGAR format)
6       =
7       51789
8       -264
9       CCCAAACAAGCCGAACTAGCTGATTTGGCTCGTAAAGACCCGGAAA   (read sequence, oriented according to the forward alignment)
10      ###CB?=ADDBCBCDEEFFDEFFFDEFFGDBEFGEDGCFGFGGGGG   (base quality values)
11      MD:Z:67
12      NH:i:1
13      HI:i:1
14      NM:i:0
15      SM:i:38          (Metadata)
16      XQ:i:40
17      X2:i:0
```

Link to SAM format description

Alignments are reported in a compact representation: SAM format


```
0       61G9EAAXX100520:5:100:10095:16477  (read name)
1       83  (FLAGS stored as bit fields; 83 = 00001010011 )
2       chr1  (alignment target)
```

Still not compact enough…
Millions to billions of reads takes up a lot of space!!

Convert SAM to binary – BAM format.


```
15      SM:i:38
16      XQ:i:40
17      X2:i:0
```

Link to SAM format description

Samtools

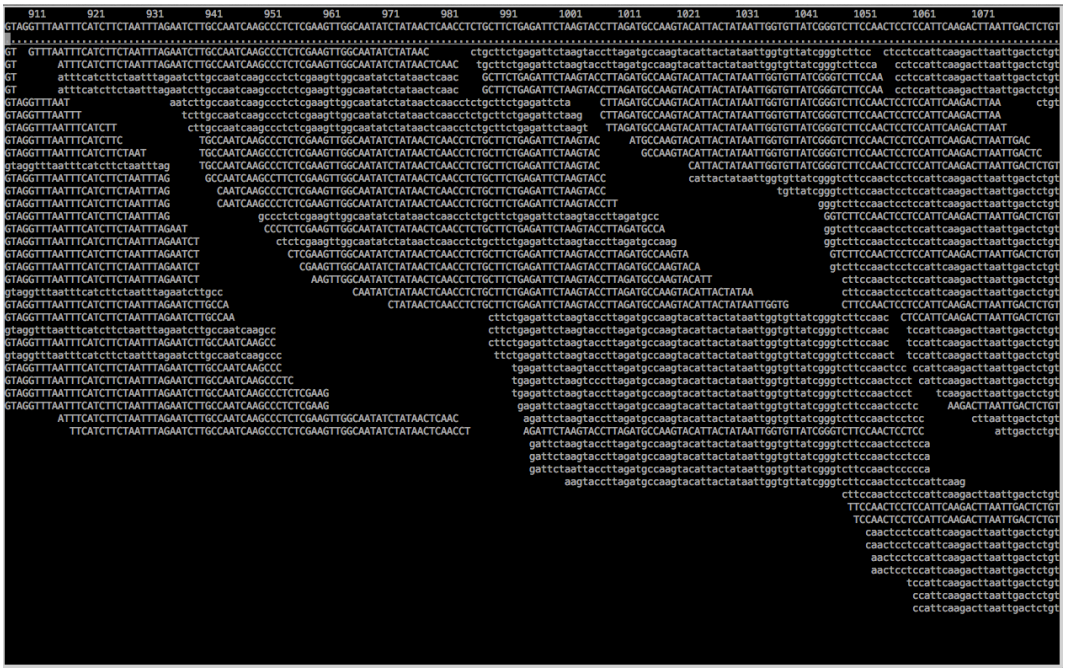
- Tools for
  - converting SAM <-> BAM
  - Viewing BAM files (eg. samtools view file.bam | less)
  - Sorting BAM files, and lots more:

```
Program: samtools (Tools for alignments in the SAM format)
Version: 0.1.18 (r982:295)

Usage:   samtools <command> [options]

Command: view        SAM<->BAM conversion
         sort        sort alignment file
         mpileup     multi-way pileup
         depth       compute the depth
         faidx       index/extract FASTA
         tview       text alignment viewer
         index       index alignment
         idxstats    BAM index stats (r595 or later)
         fixmate     fix mate information
         flagstat    simple stats
         calmd       recalculate MD/NM tags and '=' bases
         merge       merge sorted alignments
         rmdup       remove PCR duplicates
         reheader    replace BAM header
         cat         concatenate BAMs
         targetcut   cut fosmid regions (for fosmid pool only)
         phase       phase heterozygotes
```

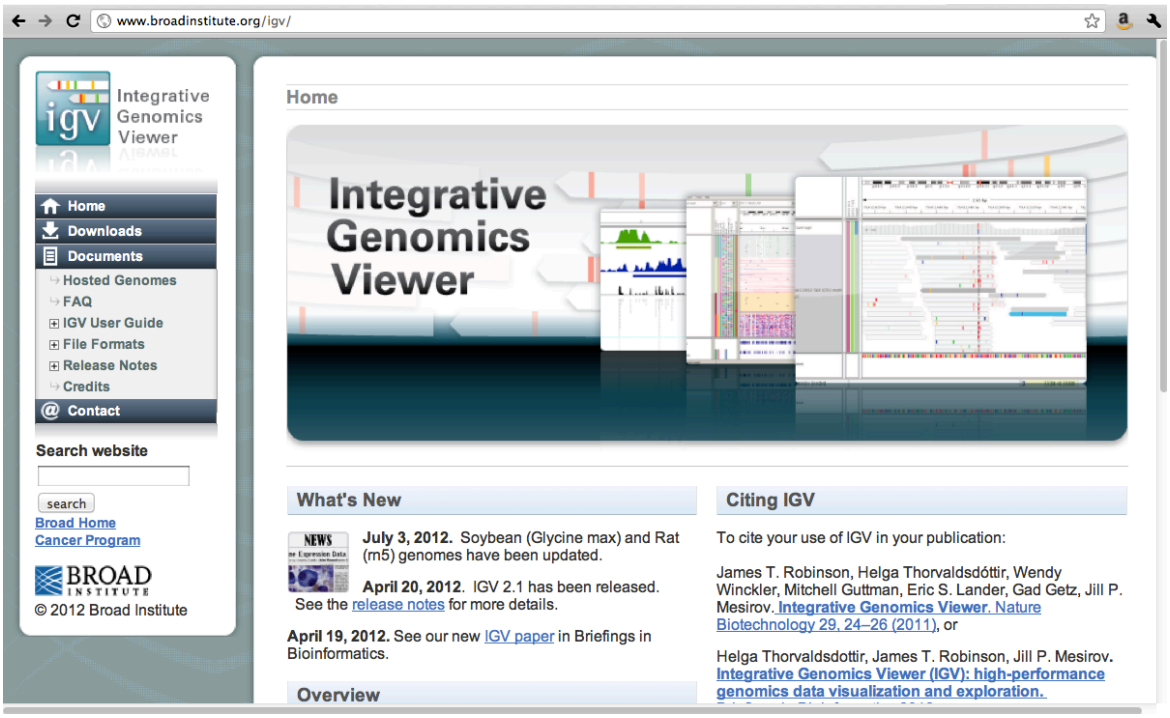
Visualizing Alignments of RNA-Seq reads

Text-based Alignment Viewer

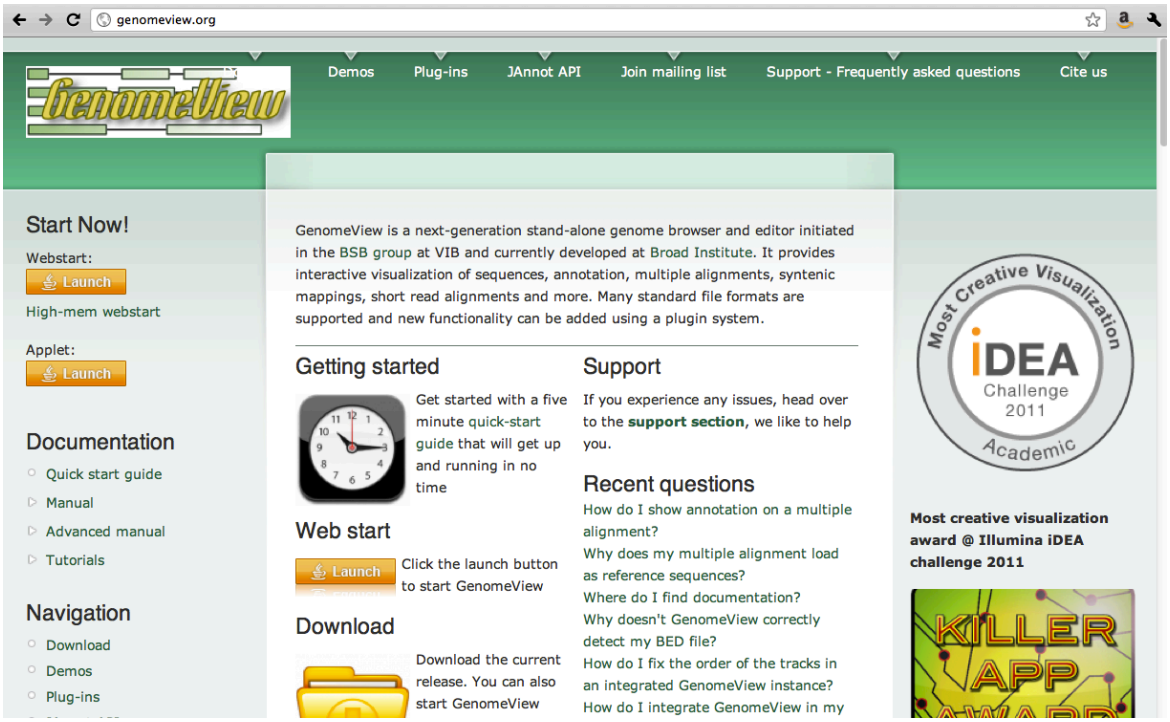
% samtools tview alignments.bam target.fasta



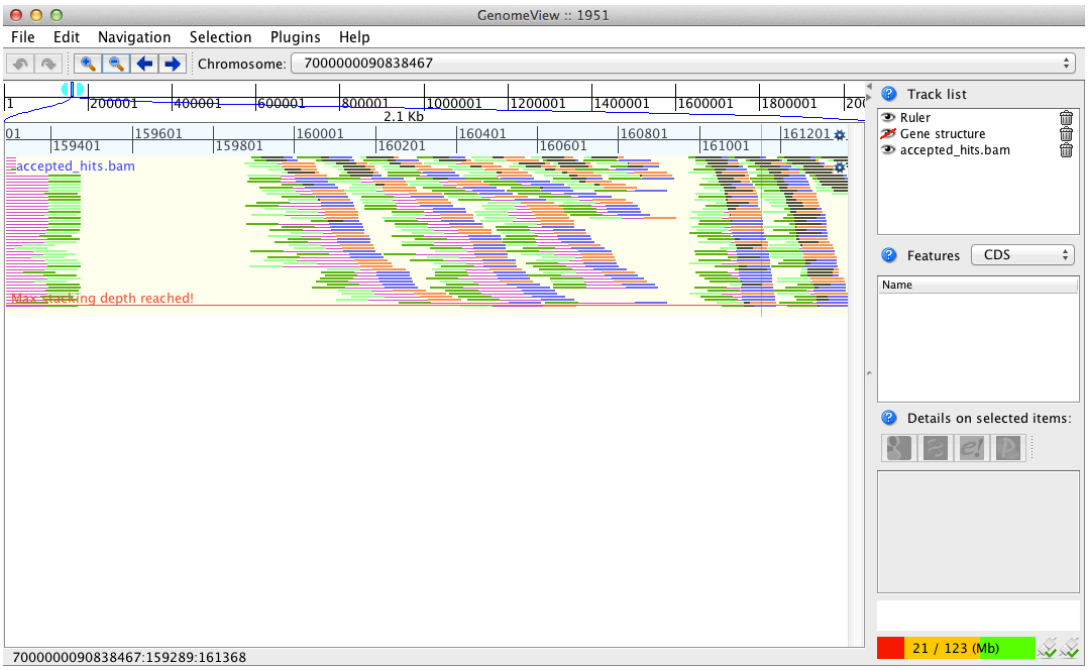
IGV



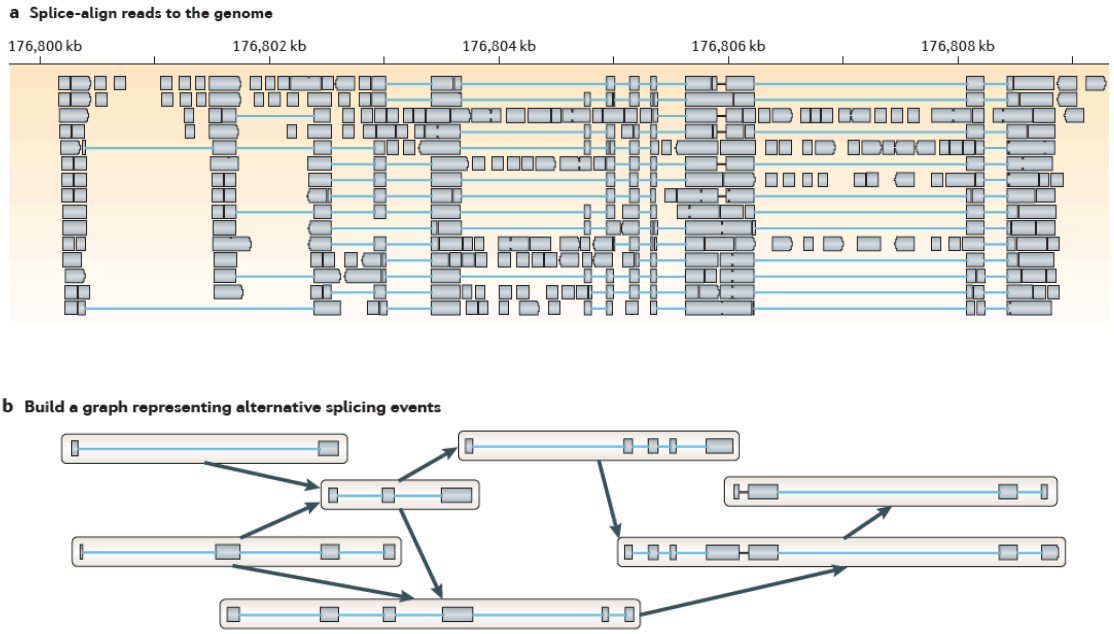
GenomeView



GenomeView: viewing TopHat alignments

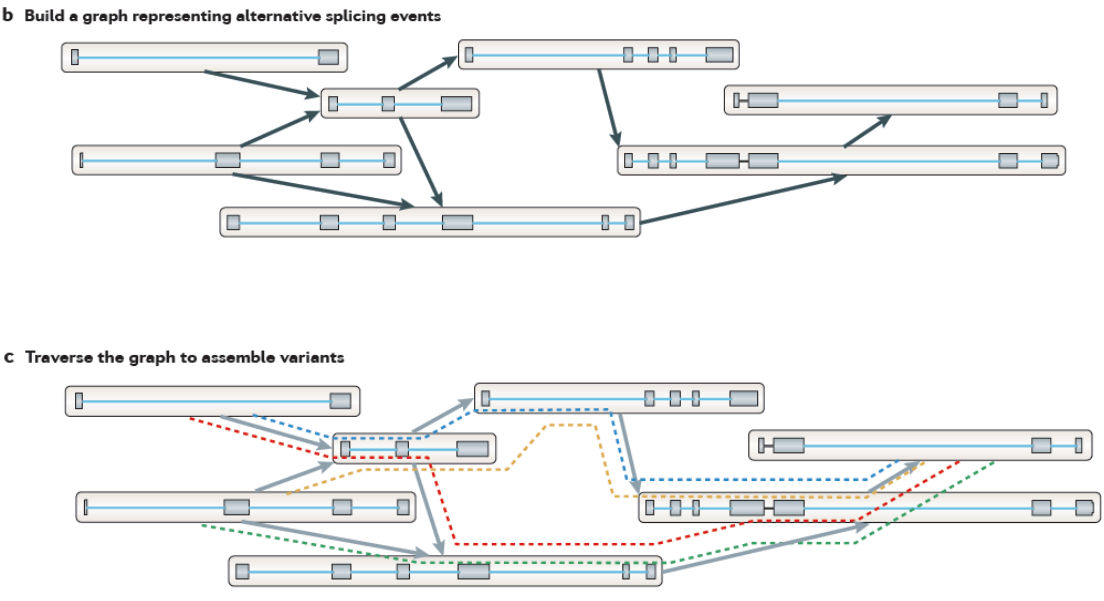


Transcript Reconstruction Using Cufflinks



From Martin & Wang. Nature Reviews in Genetics. 2011

Transcript Reconstruction Using Cufflinks



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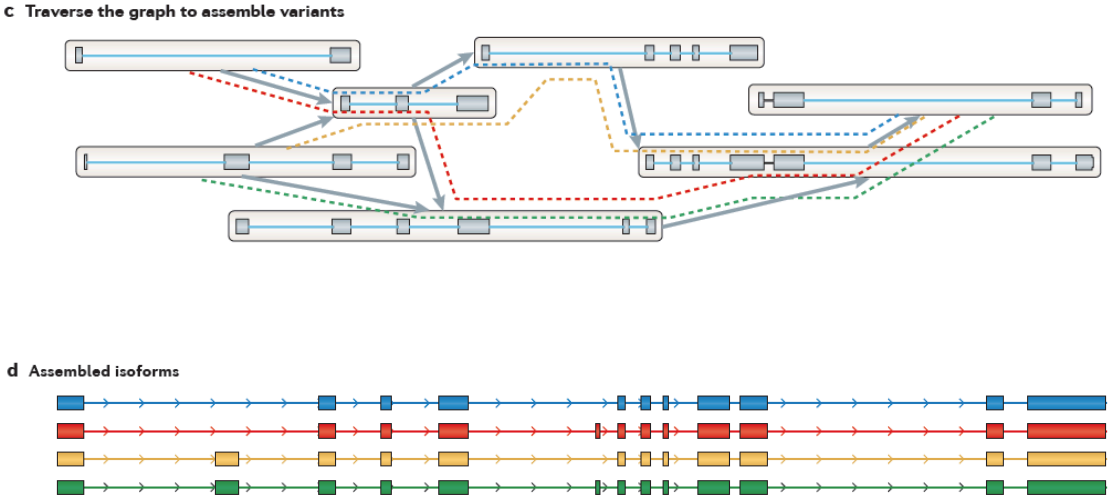
Transcript Reconstruction Using Cufflinks



From Martin & Wang. Nature Reviews in Genetics. 2011

Transcript Structures in **GTF** Format

(tab-delimited fields per line shown transposed to a column format here)

0	7000000090838467
1	Cufflinks
2	transcript
3	101
4	5716
5	1000
6	.
7	.
8	gene_id "CUFF.1"; transcript_id "CUFF.1.1"; FPKM "378.0239937260“

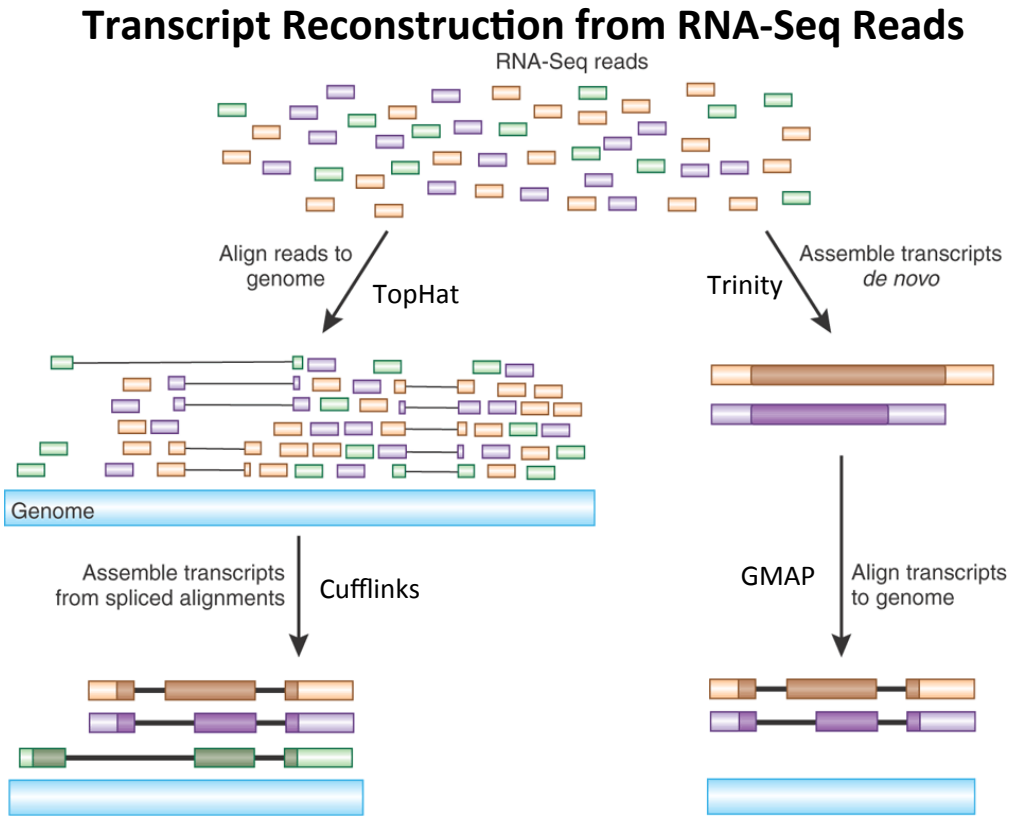
0	7000000090838467
1	Cufflinks
2	exon
3	101
4	5716
5	1000
6	.
7	.
8	gene_id "CUFF.1"; transcript_id "CUFF.1.1"; exon_number "1"; FPKM "378.0239937260“

De novo transcriptome assembly

No genome required

Empower studies of non-model organisms

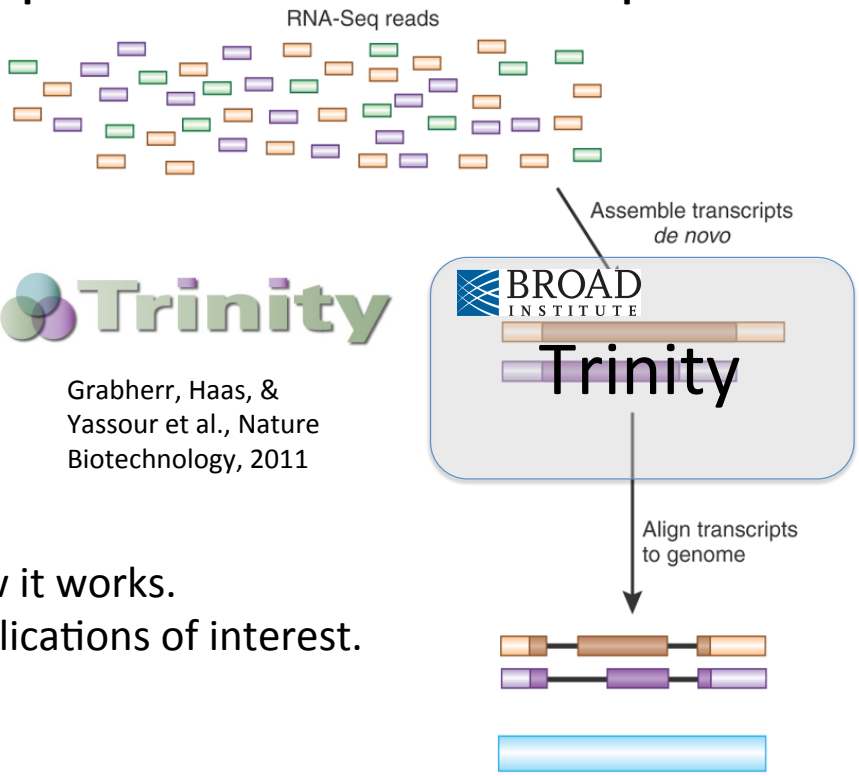
- expressed gene content
- transcript abundance
- differential expression



Transcript Reconstruction from RNA-Seq Reads

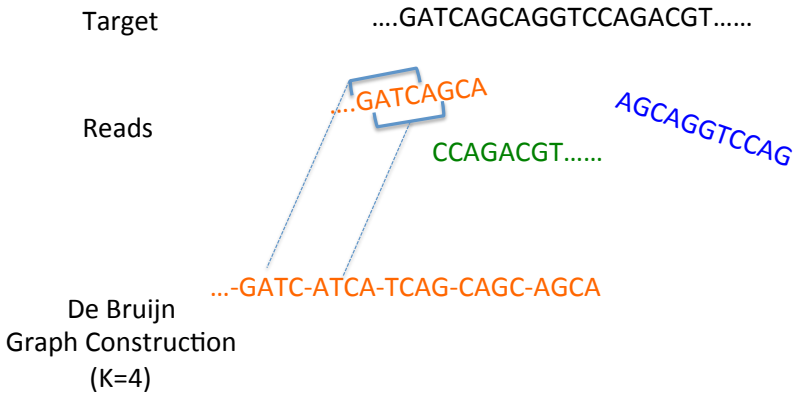
Trinity

Grabherr, Haas, &
Yassour et al., Nature
Biotechnology, 2011



- How it works.
- Applications of interest.

Short Read Assembly Using de Bruijn Graphs

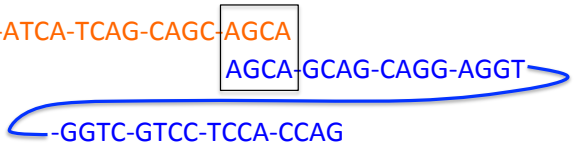


Short Read Assembly Using de Bruijn Graphs

Target ….GATCAGCAGGTCCAGACGT……

Reads ….GATCAGCA AGCAGGTCCAG CCAGACGT……

De Bruijn Graph Construction (K=4)



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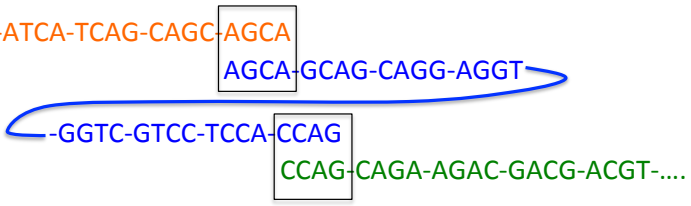
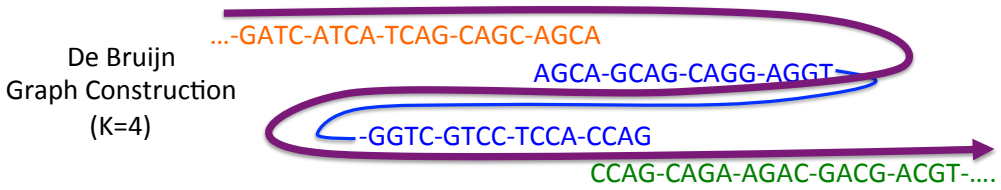
De Bruijn Graph Construction (K=4)



Short Read Assembly Using de Bruijn Graphs

Target ....GATCAGCAGGTCCAGACGT.....

Reads ....GATCAGCA AGCAGGTCCAG CCAGACGT.....



Sequence Reconstruction
By Path Traversal **....GATCAGCAGGTCCAGACGT.....**

Contrasting Genome and Transcriptome Assembly

Genome Assembly

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

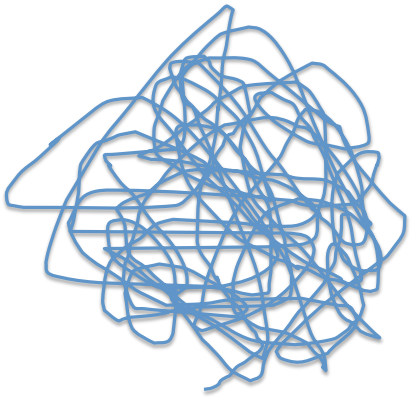
Transcriptome Assembly

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

Trinity

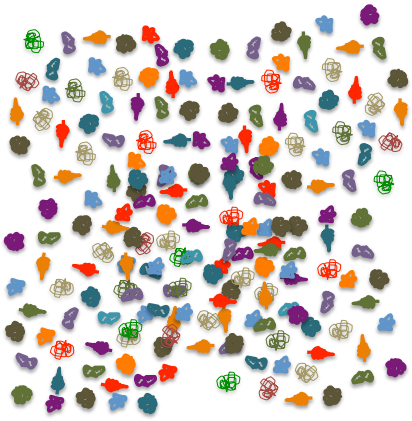
Trinity Aggregates Isolated Transcript Graphs

Genome Assembly
Single Massive Graph



Entire chromosomes represented.

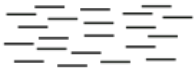
Trinity Transcriptome Assembly
Many Thousands of Small Graphs

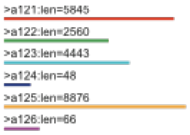


Ideally, one graph per expressed gene.

Trinity

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





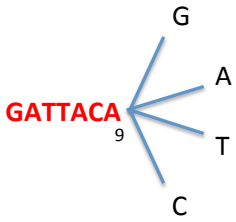
...CTTCGCAA...TGATCGGAT...
...ATTCGCAA...TCATCGGAT...

Inchworm Algorithm

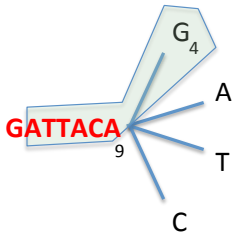
Decompose all reads into overlapping Kmers (25-mers)

Identify seed kmer as most abundant Kmer, ignoring low-complexity kmers.

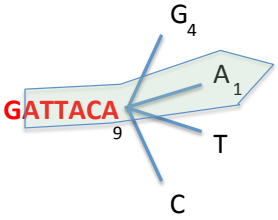
Extend kmer at 3' end, guided by coverage.



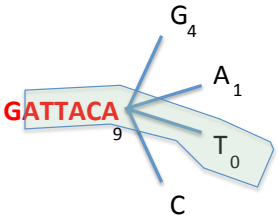
Inchworm Algorithm



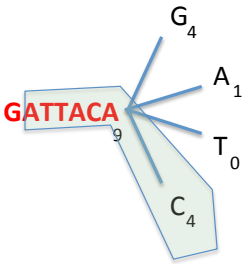
Inchworm Algorithm



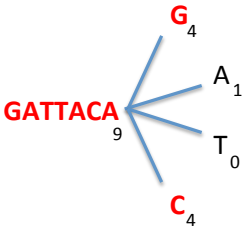
Inchworm Algorithm



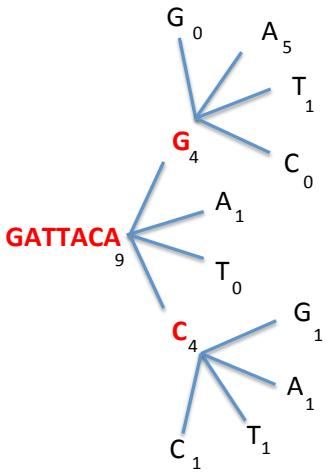
Inchworm Algorithm



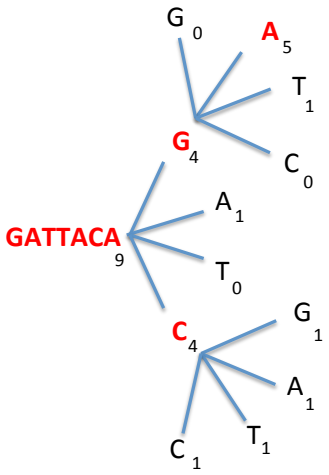
Inchworm Algorithm



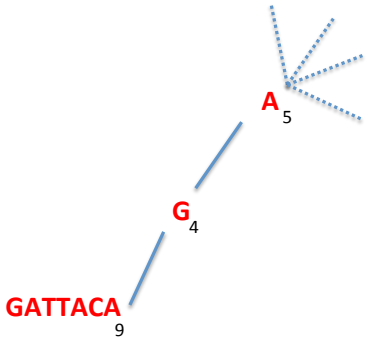
Inchworm Algorithm



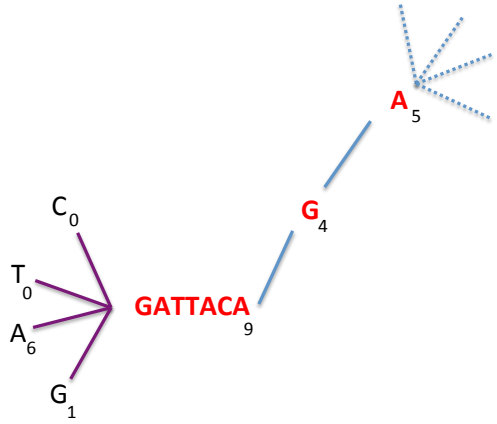
Inchworm Algorithm



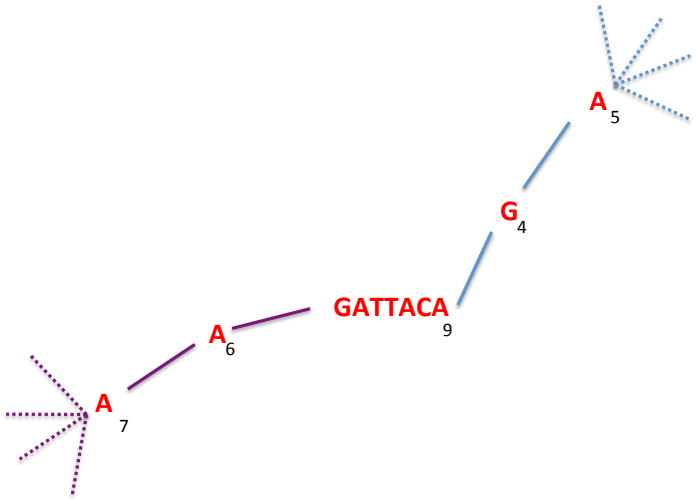
Inchworm Algorithm



Inchworm Algorithm



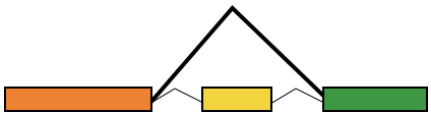
Inchworm Algorithm



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

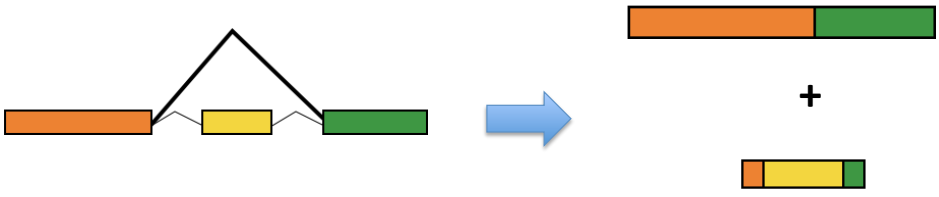
Remove assembled kmers from catalog, then repeat the entire process.

Inchworm Contigs from Alt-Spliced Transcripts

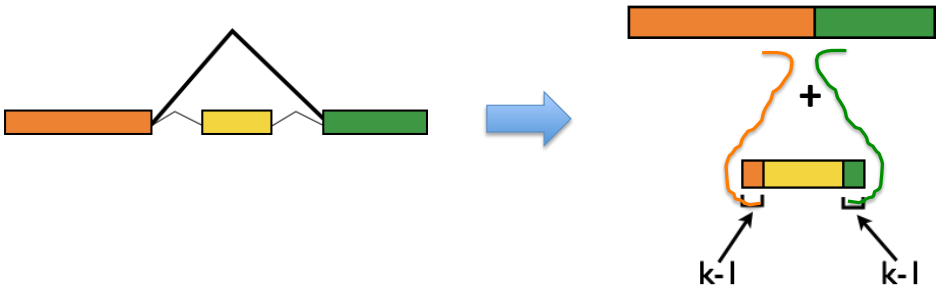


Inchworm Contigs from Alt-Spliced Transcripts

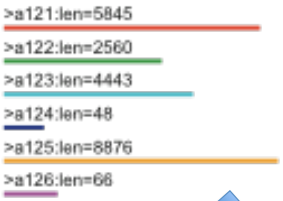
Inchworm Contigs from Alt-Spliced Transcripts



Inchworm Contigs from Alt-Spliced Transcripts

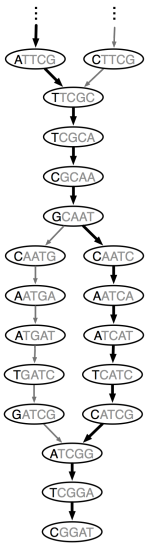


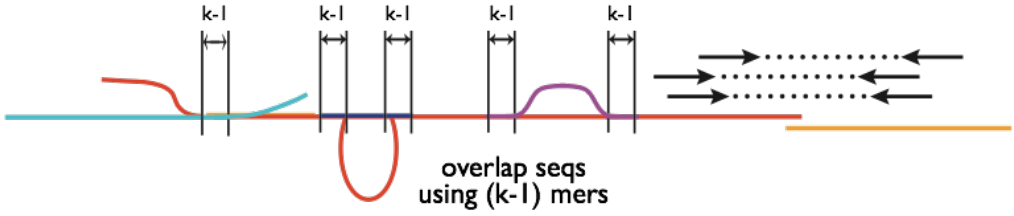
Chrysalis



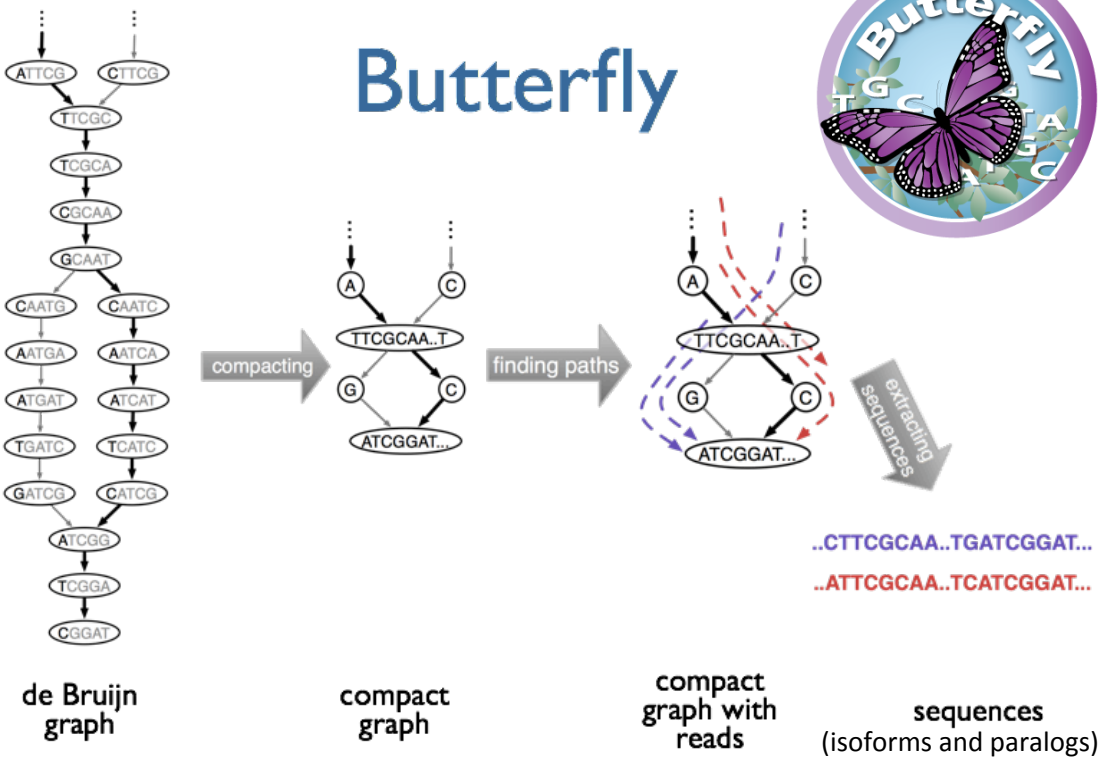
Integrate isoforms
via k-1 overlaps

Build de Bruijn Graphs
(ideally, one per gene)





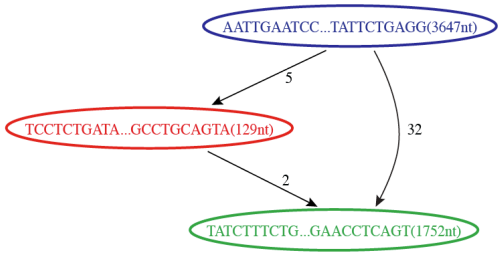
Butterfly



de Bruijn graph | compact graph | compact graph with reads | sequences (isoforms and paralogs)

Reconstruction of Alternatively Spliced Transcripts

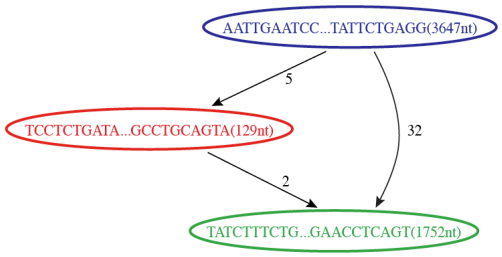
Butterfly's Compacted Sequence Graph



Reconstructed Transcripts

Reconstruction of Alternatively Spliced Transcripts

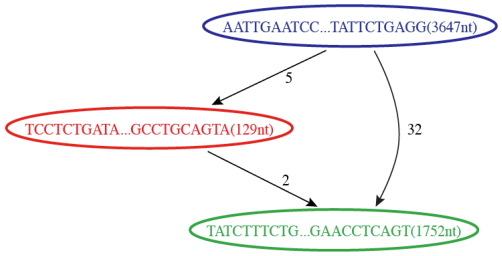
Butterfly's Compacted Sequence Graph



Reconstructed Transcripts

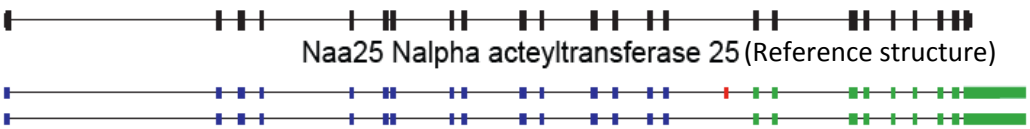
Reconstruction of Alternatively Spliced Transcripts

Butterfly's Compacted Sequence Graph



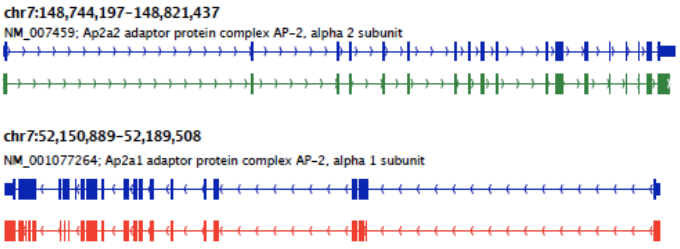
Reconstructed Transcripts

Aligned to Mouse Genome



Teasing Apart Transcripts of Paralogous Genes

Teasing Apart Transcripts of Paralogous Genes

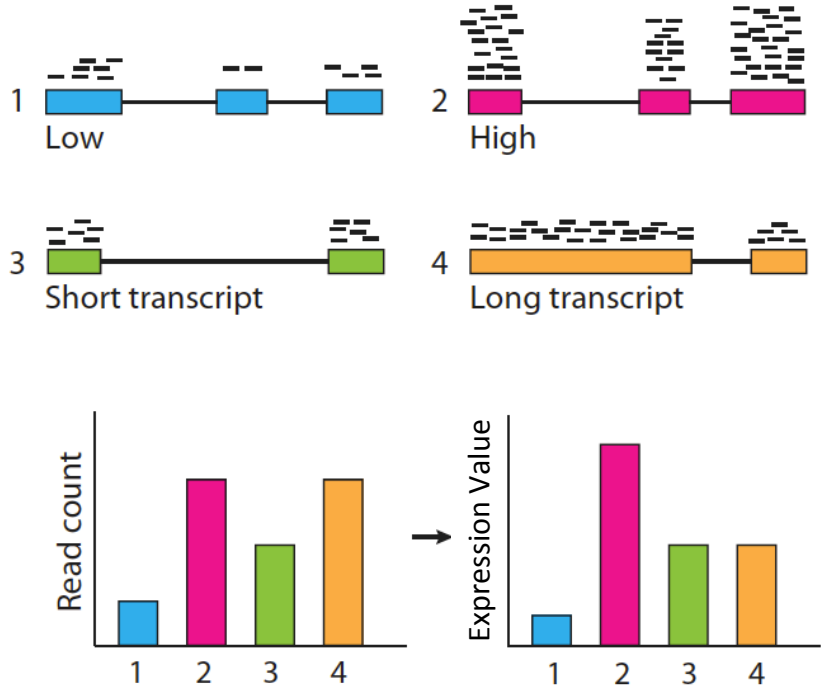


Trinity output: A multi-fasta file

Abundance Estimation

(Aka. Computing Expression Values)

Calculating expression of genes and transcripts



Slide courtesy of Cole Trapnell

Normalized Expression Values

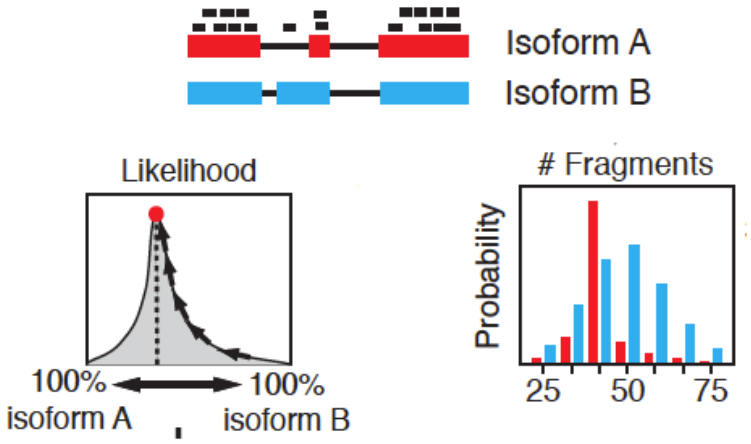
- Normalized for both length of the transcript and total depth of sequencing.

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

FPKM

Note, **R**PKM : **R**eads per … instead of Fragments is often used with single-end reads.

Sophisticated computations are required to estimate isoform expression where there is read mapping ambiguity.



Model considers unique and ambiguously mapping reads and the length of transcripts.

Illustrations courtesy of Cole Trapnell

Tools that perform abundance estimation

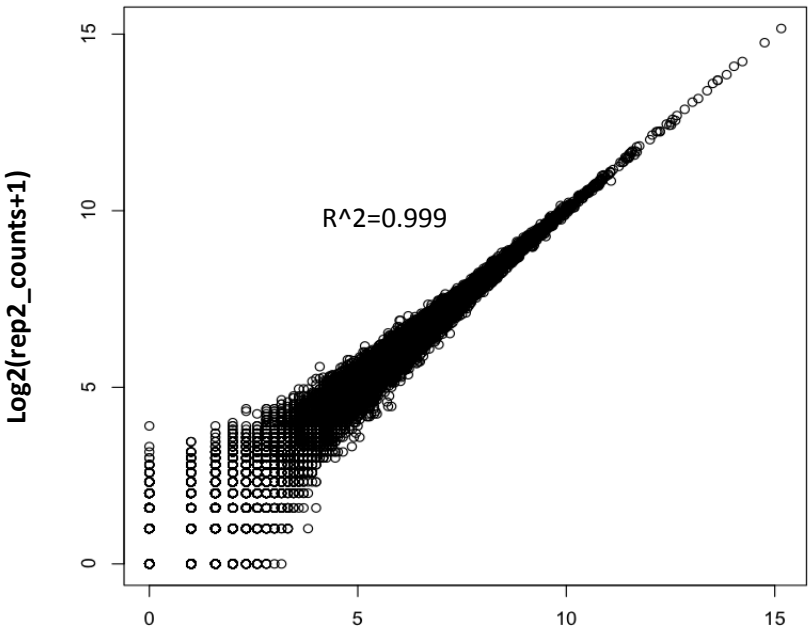
Cuffdiff

0	tracking_id	XLOC_000001
1	class_code	-
2	nearest_ref_id	-
3	gene_id	XLOC_000001
4	gene_short_name	-
5	tss_id	TSS1
6	locus	Chr1:180422-180902
7	length	-
8	coverage	-
9	condA_FPKM	10042.1
10	condA_conf_lo	0
11	condA_conf_hi	20319.6
12	condA_status	OK

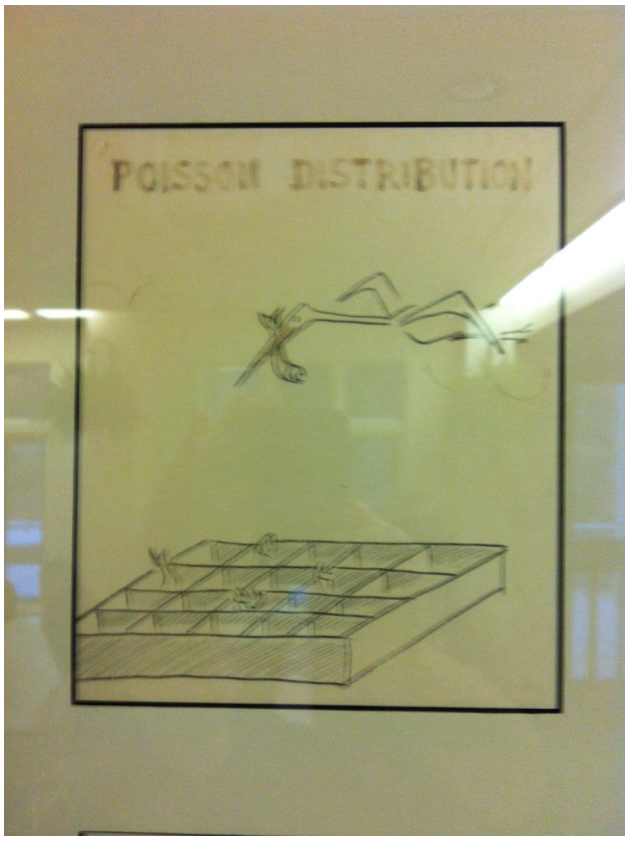
RSEM

0	transcript_id	comp100_c0_seq1
1	gene_id	comp100_c0
2	length	727
3	effective_length	534.74
4	expected_count	14.00
5	TPM	328.11
6	FPKM	532.77
7	IsoPct	100.00

Technical Replicates

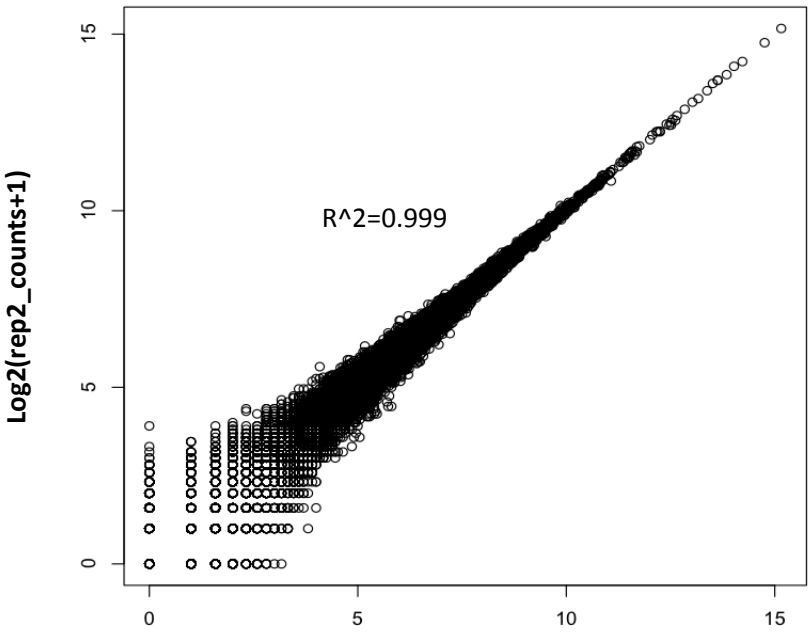


Kidney rna-seq samples
Data from Marioni et al. [2008]



See above the toasters in Blackford Hall, CSHL

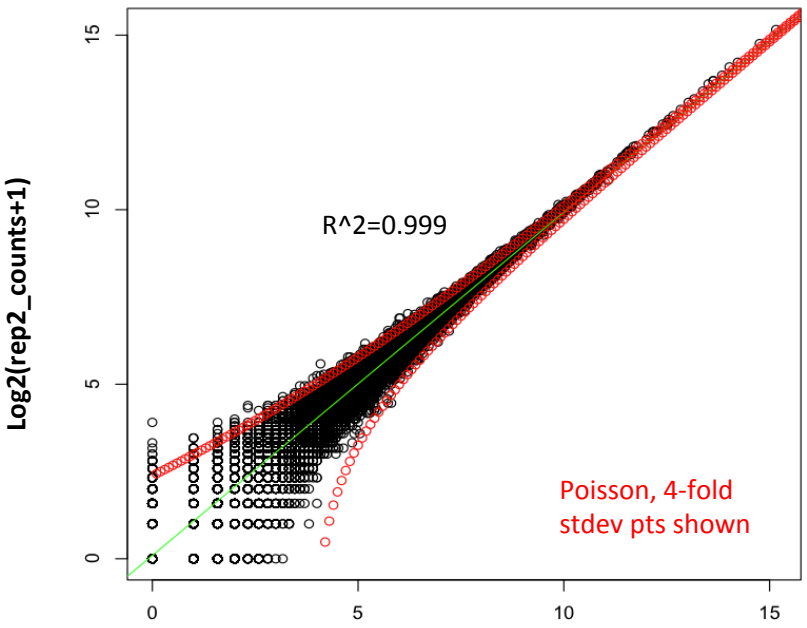
Technical Replicates



Kidney rna-seq samples
Data from Marioni et al. [2008]

Technical Replicates

Variation observed matches expectations due to random sampling (Poisson distribution)

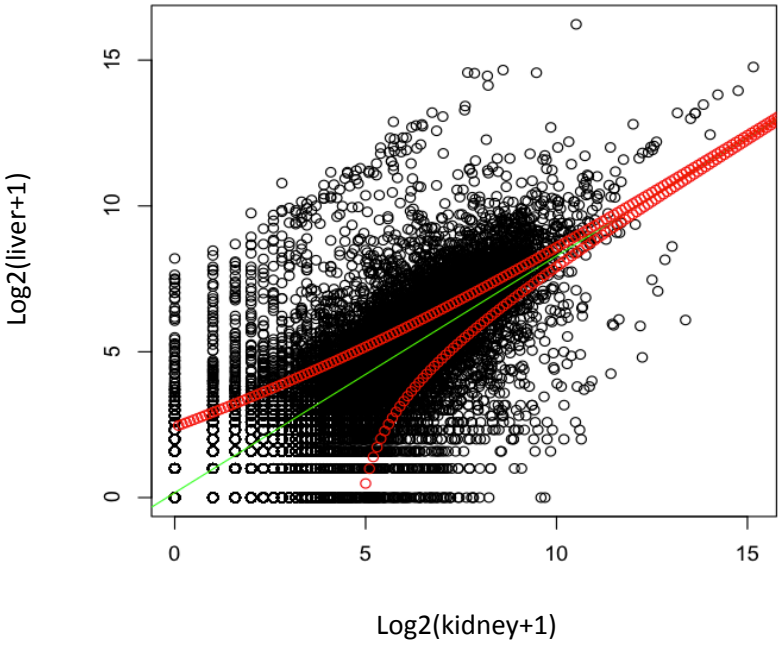


Kidney rna-seq samples
Data from Marioni et al. [2008]

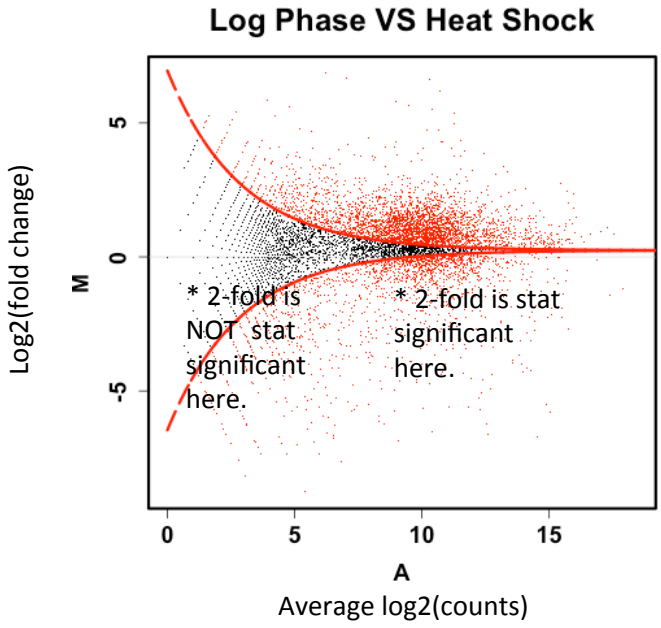
- Poisson well-describes variation observed in technical replicates.
- Negative binomial (overly dispersed poisson) better models biological replicates.

Comparing Samples and Identifying Differentially Expressed Transcripts

Kidney vs. Liver



Increased Power for Identifying Differentially Expressed Transcripts With Deeper Sequencing



Normalization Required
Otherwise, housekeeping genes look diff expressed due to sample composition differences

Subset of genes highly expressed in liver

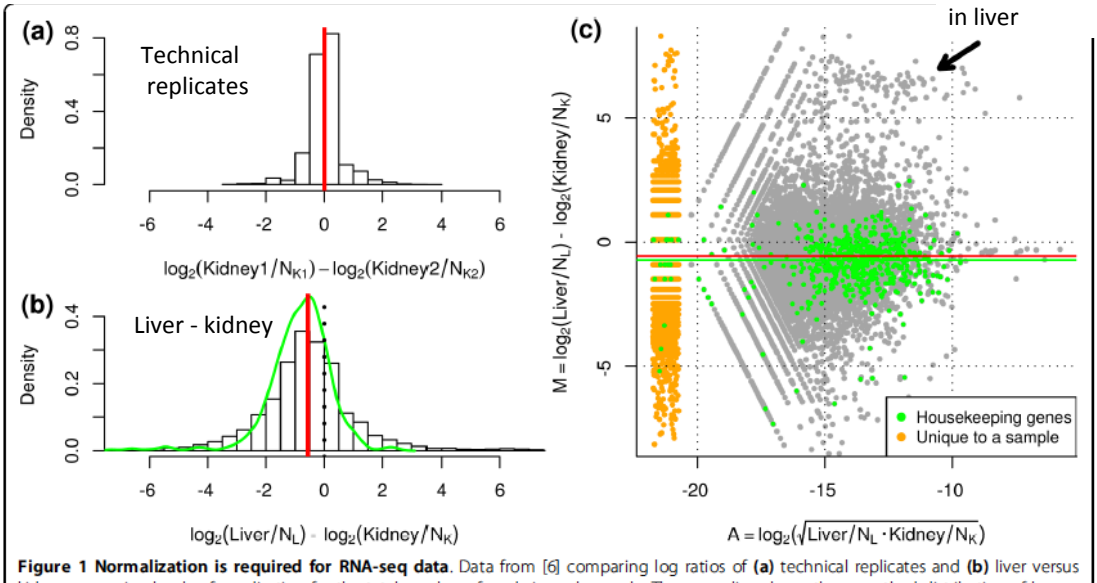


Figure 1 Normalization is required for RNA-seq data. Data from [6] comparing log ratios of **(a)** technical replicates and **(b)** liver versus kidney expression levels, after adjusting for the total number of reads in each sample. The green line shows the smoothed distribution of log-fold-changes of the housekeeping genes. **(c)** An M versus A plot comparing liver and kidney shows a clear offset from zero. Green points indicate 545 housekeeping genes, while the green line signifies the median log-ratio of the housekeeping genes. The red line shows the estimated TMM normalization factor. The smear of orange points highlights the genes that were observed in only one of the liver or kidney largely attributable for the overall bias in log-fold-changes.

Robinson and Oshlack, Genome Biology, 2010

Identifying Differentially Expressed Transcripts

- Statistical tests performed on fragment counts (not FPKM values).
- Given observed read counts for a transcript in each of two samples, what's the probability they were derived from the same distribution (null hypothesis)? (ex. Fishers exact test)

  If (P <= 0.05), significantly different
- Don't forget to adjust P-values due to false discovery rate (FDR) resulting from running many (thousands of) statistical tests. (ex. use Q-values)

Experimental Design

- Forego technical replicates
- Ideally, have at least 3 biological replicates
- Without biological replicates, can still model variation based on parametric distributions (eg. Negative binomial), but expect lower accuracy.

Statistical Analysis Software for Identifying Differentially Expressed Transcripts

- Bioconductor
  - EdgeR
  - DEGseq
  - DESeq
  - And others…
- Tuxedo suite
  - Cuffdiff
  - (analysis enabled with CummeRbund/Bioconductor)

Examples of Results

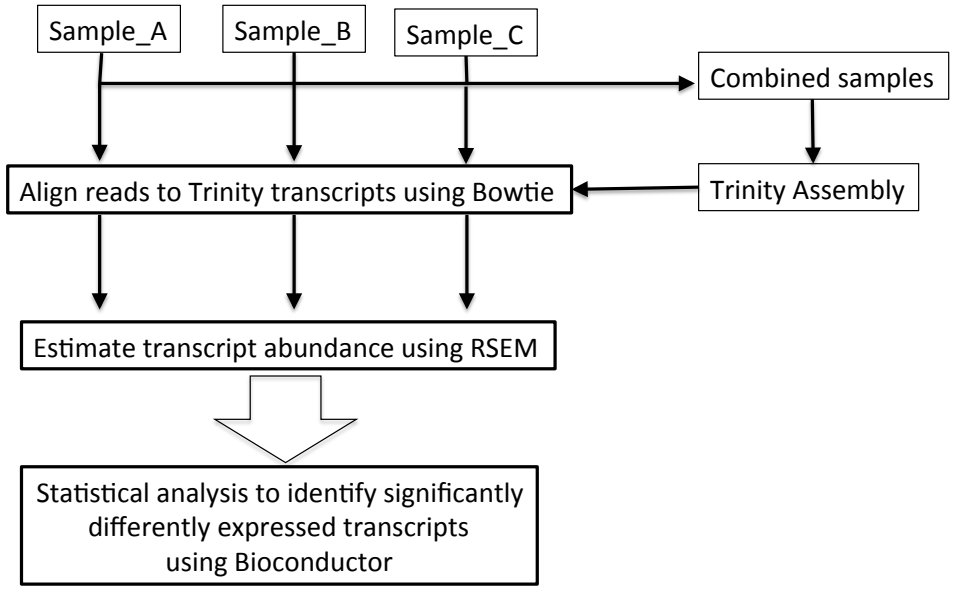
(Cuffdiff)

0	test_id	XLOC_000024
1	gene_id	XLOC_000024
2	gene	-
3	locus	7000000090838467:1335927-1338056
4	sample_1	condA
5	sample_2	condB
6	status	OK
7	value_1	680.167
8	value_2	68932
9	log2(fold_change)	6.66314
10	test_stat	-2.91993
11	p_value	0.00350111
12	q_value	0.0424377
13	significant	yes

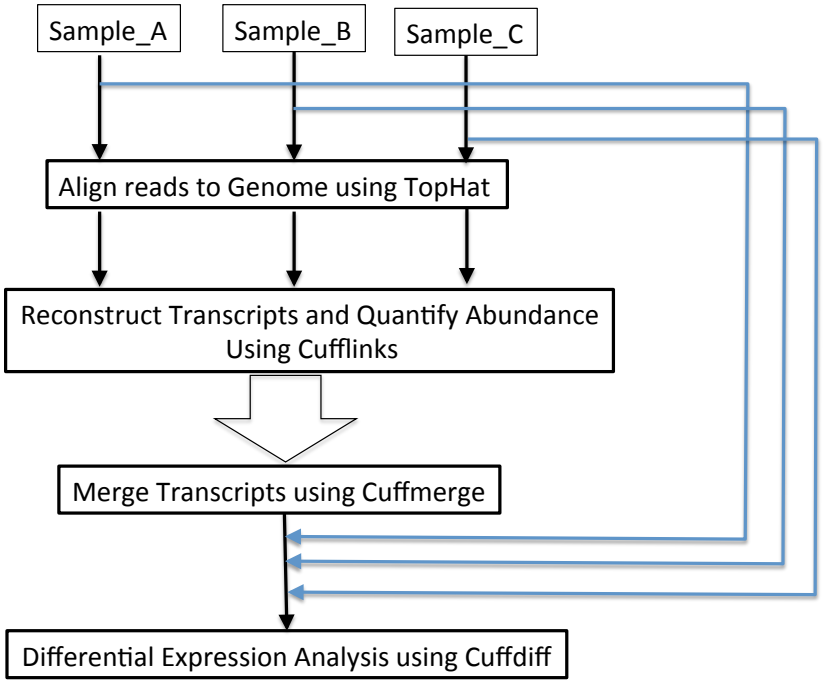
Examples of Results (example edgeR)

		comp217_c0_seq1
0	logFC	6.69056684523186
1	logCPM	16.1146897543805
2	PValue	2.06844466442231e-15
3	FDR	9.01969581996253e-13

Trinity Differential Expression Workflow

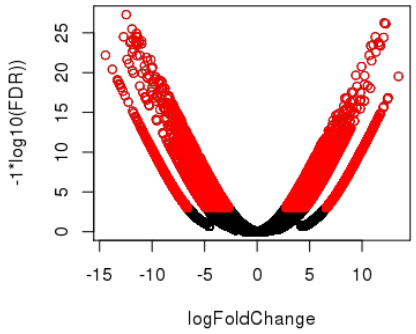


Tuxedo Differential Expression Workflow

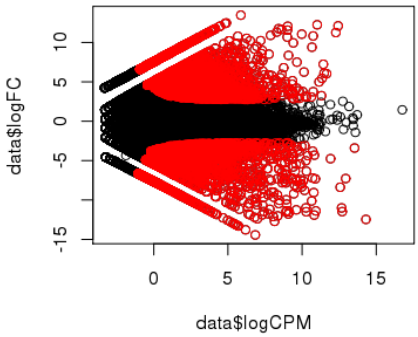


Plotting Pairwise Differential Expression Data

Volcano plot
(fold change vs. significance)



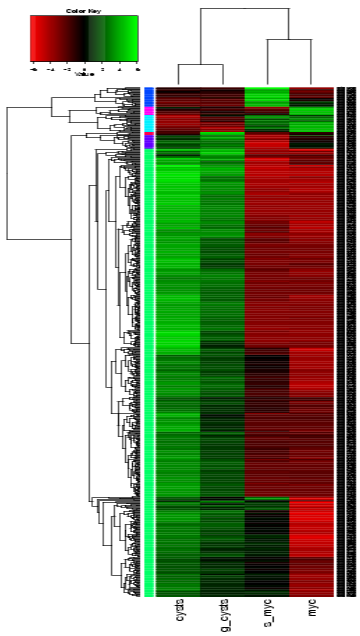
MA plot
(abundance vs. fold change)



Significantly differently expressed transcripts have FDR <= 0.001
(shown in red)

No replicates available, so modeled by edgeR using the
Negative Binomial with dispersion manually set to 0.1

Comparing Multiple Samples



Heatmaps provide an effective tool
for navigating differential expression across
multiple samples.

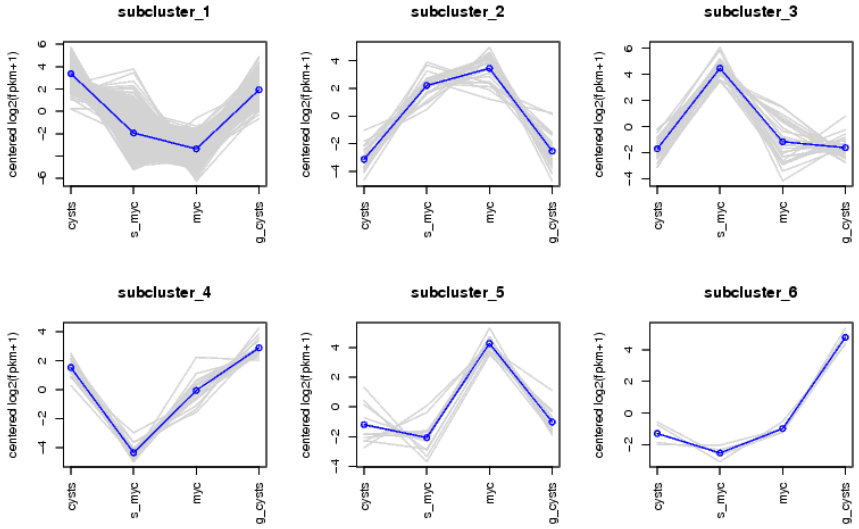
Clustering can be performed across both axes:

-cluster transcripts with similar expression patters.

-cluster samples according to similar expression values among transcripts.

Examining Patterns of Expression Across Samples

Can extract clusters of transcripts and examine them separately.



Hands-on Tutorials

- Tuxedo
  - Tophat alignment
  - Cufflinks transcript reconstructions
  - GenomeView for navigating the alignments
  - Cuffdiff for differential expression analysis
  - cummeRbund for exploring diff. express. results.
- Trinity
  - De novo assembly using Trinity
  - Bowtie and RSEM for abundance estimation
  - edgeR for differential expression analysis