RNA-Seq Data Analysis

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657.000 SS 2017

What is RNA-seq?

- An experimental protocol that uses next-generation sequencing technologies to sequence the RNA molecules within a biological sample in an effort to determine the primary sequence and relative abundance of each RNA
  - Martin JA, Wang Z (2011) Next-generation transcriptome assembly. Nat Rev Genet. 12(10):671-682

- Also known as "Whole Transcriptome Shotgun Sequencing" (WTSS)

Why RNA-seq?

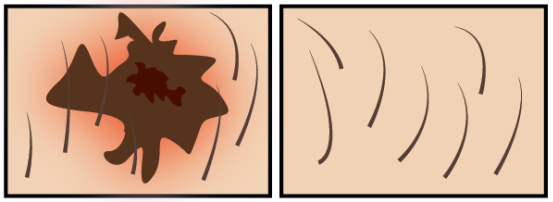
- Some studies are not possible with DNA sequences
- To study functions based on gene expression changes
  - Drug treatment vs. no treatment
  - Patients vs. healthy people
  - Wild type vs. knock-out
- Some features available only at RNA level
  - Alternative isoforms, RNA editing, transcript fusion

Goals of RNA-seq experiments

- Differential expression (DE) analysis
- Alternative expression analysis
- Novel transcript discovery
- Allele-specific expression analysis
  - SNPs, mutations
- Gene fusion detection

RNA sequencing

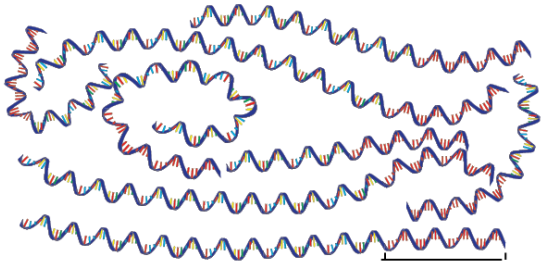
Samples of interest



Condition 1 (e.g. tumor)

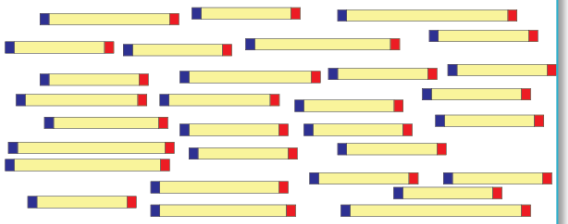
Condition 2 (e.g. normal)

Isolate RNAs

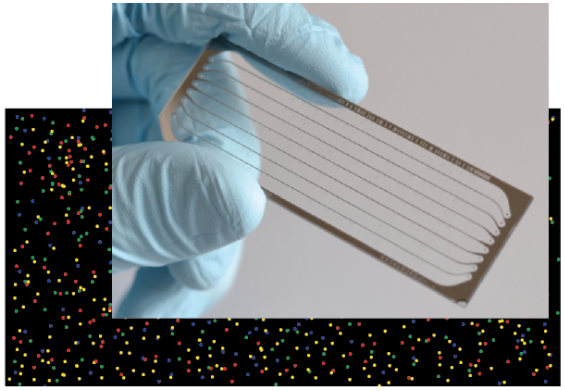


Poly(A) tail

Generate cDNA, Fragment, size select, add linkers



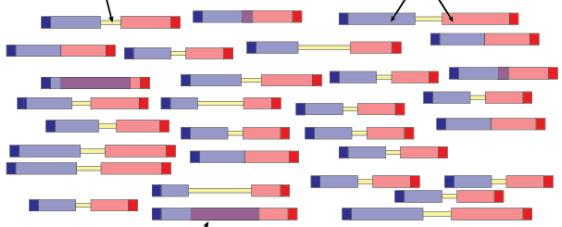
Sequence ends



100s of millions fo paired reads
10s of billions bases of sequence

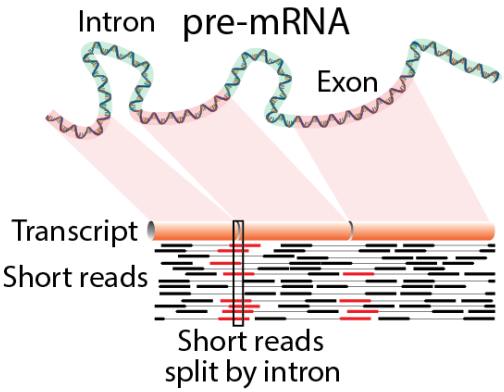
Unsequenced RNA

RNA reads



Short insert

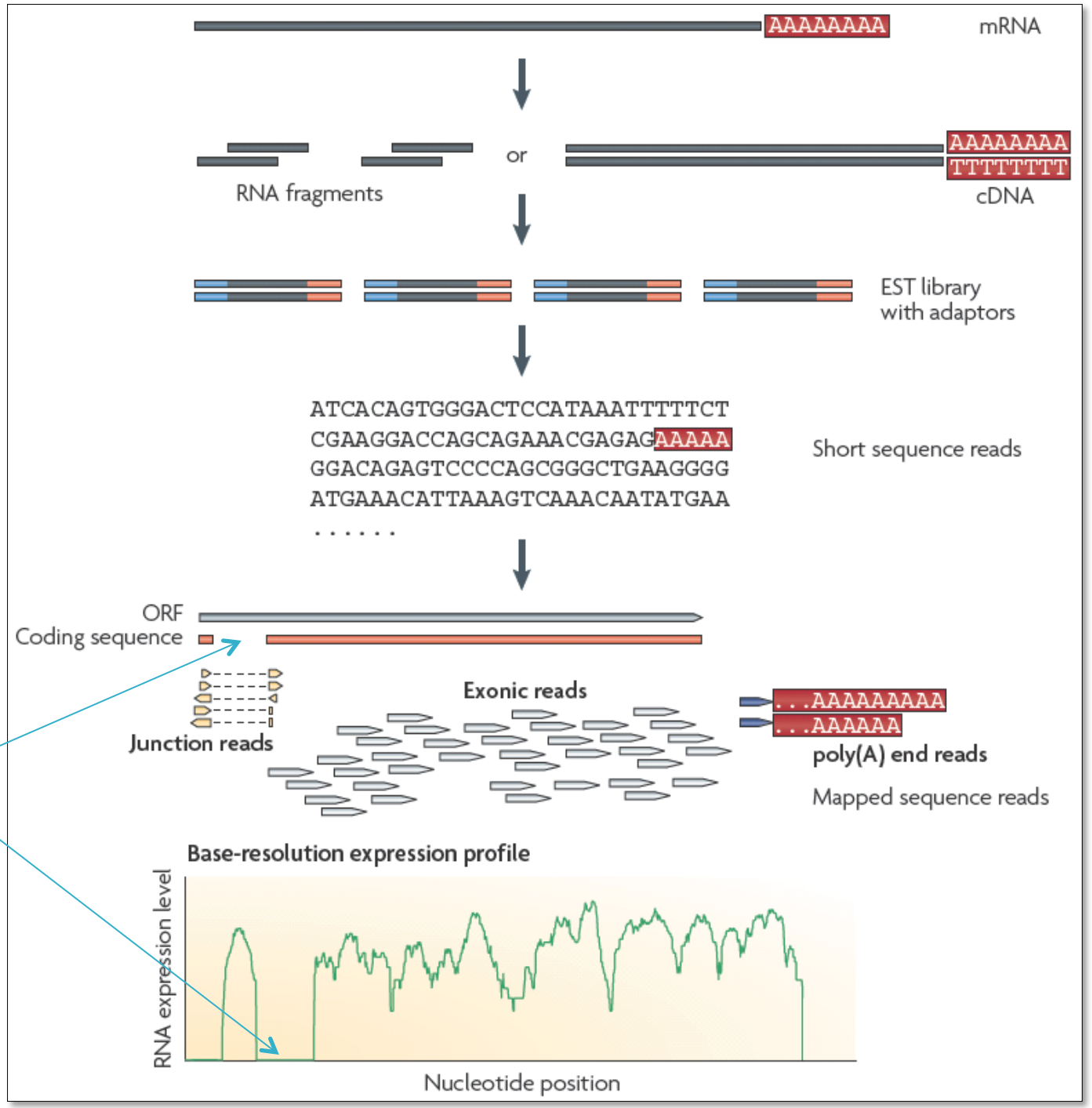
Map to genome, transcriptome, and predicted exon junctions



Short reads split by intron

Downstream analysis

RNA-Seq workflow



Wang Z, Gerstein M, Snyder M (2009) RNA-Seq: a revolutionary tool for transcriptomics. Nat Rev Genet. 10(1):57-63.

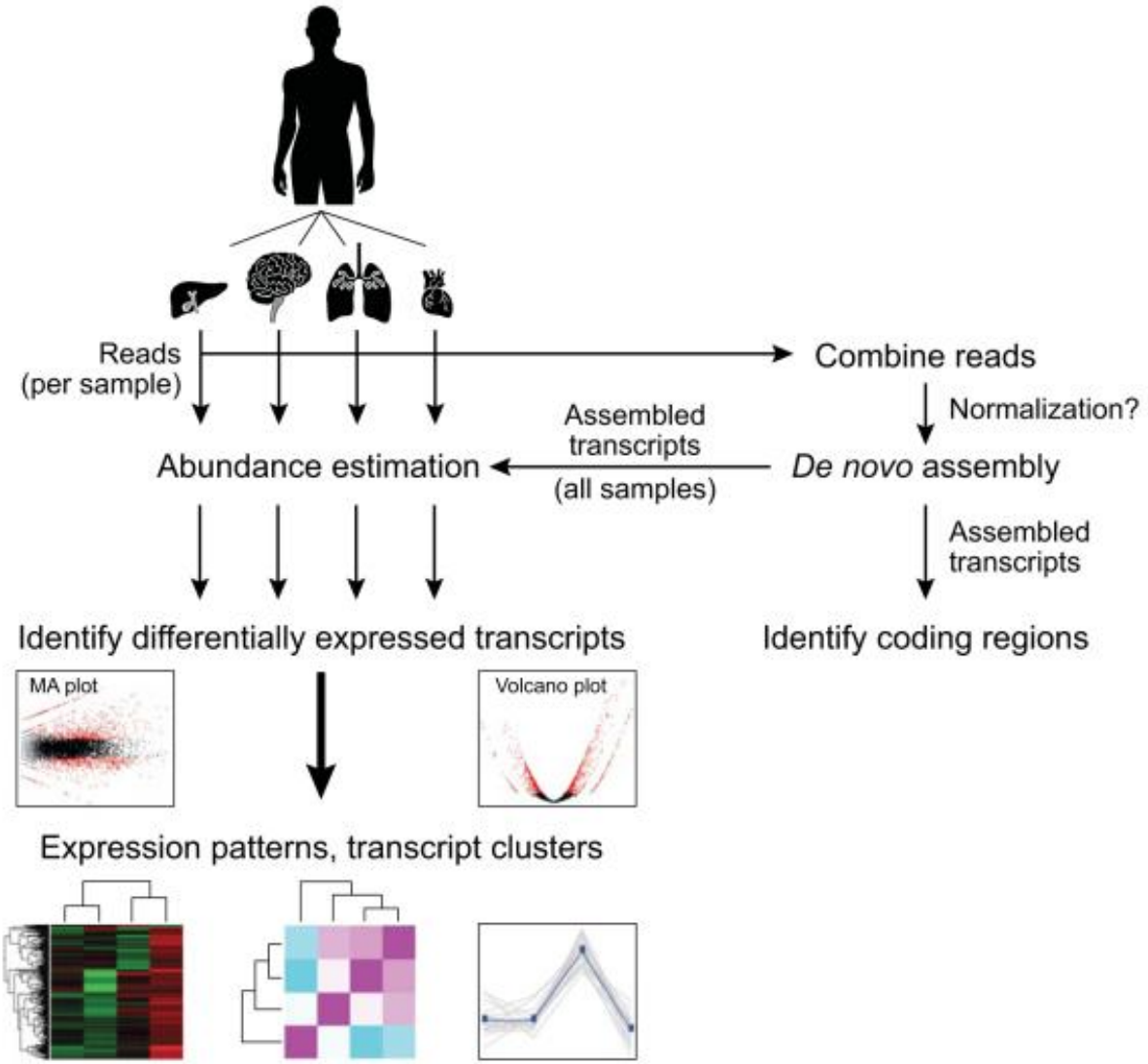
RNA-seq poses more challenges

- Difficulty with sampling
  - More fragile than DNA
  - Different sizes of RNA
- Relative abundance of RNAs hard to control
  - Can vary by orders of magnitude
  - Uneven coverage
    - Many reads from a small number of highly expressed genes
- Computational analysis challenges
  - Assembly, (spliced) alignment, quantification, normalization, visualization, …

RNA-Seq vs microarray

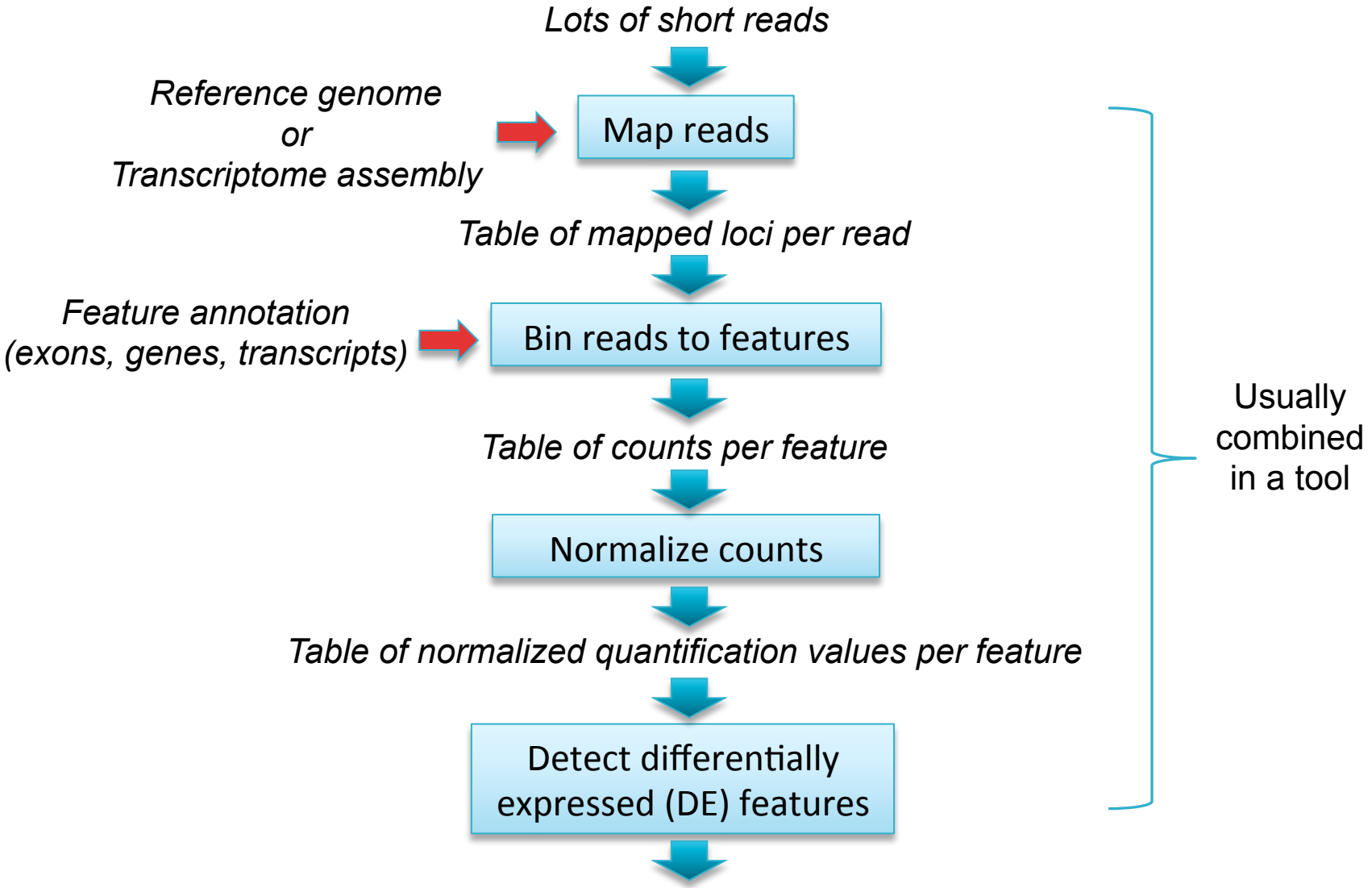
Characteristics	RNA-Seq	Microarray
Which transcripts?	All in a sample	Only those for which probes are designed
Transcript sequence generation	Yes	No
Low-abundance transcript detection	Yes	Limited
Abundance info source	Count (of the reads aligned to transcript)	Fluorescence level (of the probe spot for gene)
Resolution	Base	Probe sequence
Background noise	Low	High
Additional info	Alternative splicing, transcriptome-level variation	Limited

De novo transcriptome assembly and analysis



Haas, B. J., Papanicolaou, A., Yassour, M., Grabherr, M., Blood, P. D., Bowden, J., … Regev, A. (2013). *De novo* transcript sequence reconstruction from RNA-Seq: reference generation and analysis with Trinity. *Nature Protocols*, *8*(8)

RNA-Seq DE analysis data flow



Mapping reads

- Need a reference genome
  - Or *de novo* assembled transcriptome
- Issues
  - Reads spanning across exon junction
  - Alternative splicing
  - Reads mapping to multiple locations in the genome
  - Huge amounts of data
- Most common mapping results format
  - SAM: sequence alignment/map
  - BAM: binary format of SAM
- Many tools
  - Bowtie, SOAP, BWA, SHRiMP, mrFAST, mrsFAST, ZOOM, SSAHA2, Mosaik

SAM/BAM format

Example SAM/BAM header section (abbreviated)

```
mgriffit@linus270 ~> samtools view -H /gscmnt/gc13001/info/model_data/2891632684/build136494552/alignments/136080019.bam  | grep -P "SN\:22|HD|RG|PG"
@HD     VN:1.4  SO:coordinate
@SQ     SN:22   LN:51304566     UR:ftp://ftp.ncbi.nih.gov/genbank/genomes/Eukaryotes/vertebrates_mammals/Homo_sapiens/GRCh37/special_requests/GRCh37-lite.fa.gz AS:GRCh37-lite  M5:a718acaa6135fdca8357d5bfe9
4211dd  SP:Homo sapiens
@RG     ID:2888721359   PL:illumina     PU:D1BA4ACXX.3  LB:H_KA-452198-0817007-cDNA-3-lib1      PI:365  DS:paired end   DT:2012-10-03T19:00:00-0500     SM:H_KA-452198-0817007  CN:WUGSC
@PG     ID:2888721359   VN:2.0.8        CL:tophat --library-type fr-secondstrand --bowtie-version=2.1.0
@PG     ID:MarkDuplicates       PN:MarkDuplicates       PP:2888721359   VN:1.85(exported)       CL:net.sf.picard.sam.MarkDuplicates INPUT=[/gscmnt/gc13001/info/build_merged_alignments/merged-alignment-blad
e10-2-5.gsc.wustl.edu-jwalker-15434-136080019/scratch-ILg6Y/H_KA-452198-0817007-cDNA-3-lib1-2888360300.bam] OUTPUT=/gscmnt/gc13001/info/build_merged_alignments/merged-alignment-blade10-2-5.gsc.wustl.edu-jw
alker-15434-136080019/scratch-ILg6Y/H_KA-452198-0817007-cDNA-3-lib1-2888360300-post_dup.bam METRICS_FILE=/gscmnt/gc13001/info/build_merged_alignments/merged-alignment-blade10-2-5.gsc.wustl.edu-jwalker-1543
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ignments/merged-alignment-blade10-2-5.gsc.wustl.edu-jwalker-15434-136080019/scratch-ILg6Y] VALIDATION_STRINGENCY=SILENT MAX_RECORDS_IN_RAM=500000    PROGRAM_RECORD_ID=MarkDuplicates PROGRAM_GROUP_NAME=Mark
Duplicates MAX_SEQUENCES_FOR_DISK_READ_ENDS_MAP=50000 SORTING_COLLECTION_SIZE_RATIO=0.25 READ_NAME_REGEX=[a-zA-Z0-9]+:[0-9]:([0-9]+):([0-9]+):([0-9]+).* OPTICAL_DUPLICATE_PIXEL_DISTANCE=100 VERBOSITY=INFO
QUIET=false COMPRESSION_LEVEL=5 CREATE_INDEX=false CREATE_MD5_FILE=false
mgriffit@linus270 ~>
```

Example SAM/BAM alignment section (only 10 alignments shown)

```
mgriffit@linus270 ~> samtools view -f 3 -F 1804 /gscmnt/gc13001/info/model_data/2891632684/build136494552/alignments/136080019.bam | head
HWI-ST495_129147882:3:2114:15769:38646  99      1       11306   3       100M    =       11508   302     ACTGCGGGGCCCTCTTGCTTACTGTATAGTGGTGGCACGCCGCCTGCTGGCAGCTAGGGACATTGCAGGGTCCTCTTGCTCAAGGTGTAGTGGCAGCACGC
CCFFFFFHHHGHJJJJJJJIJJJHGIJJIJJHIIJJJJJJHFDDDDDDDDDDDDDCDDDDDDDDDDDDDD<CDDDDDDDDCCCC>@>CDDDDDDD?1<B     CC:Z:15 MD:Z:5A94       PG:Z:MarkDuplicates      RG:Z:2888721359 XG:i:0  NH:i:2  HI:i:0  NM:i:1  XM:i:
1       XN:i:0  XO:i:0  CP:i:102519765  AS:i:-5 XS:A:+  YT:Z:UU
HWI-ST495_129147882:3:2114:15769:38646  147     1       11508   3       100M    =       11306   -302    ACTCCTAAATATGGGATTCCTGGGTTTAAAAGTATAAAATAAATATGTTTAATTTGTGAACTGATTACCATCAGAATTGTACTGTTCTGTATCCCACCAG5
;5:CDCDCDECEFCD@9E=?7EEIIIIHCEGGIJJJJIIJJHF@?00IHHFFGG?*JJJIJGHGEIJJIJJJJJJJIHHCIEJJJHFHHGHFFEDFCCB     CC:Z:15 MD:Z:34A65      PG:Z:MarkDuplicates      RG:Z:2888721359 XG:i:0  NH:i:2  HI:i:0  NM:i:1  XM:i:
1       XN:i:0  XO:i:0  CP:i:102519563  AS:i:-6 XS:A:+  YT:Z:UU
HWI-ST495_129147882:3:1210:1257:16203   163     1       11810   3       100M    =       12055   345     CCTGCATGTAGTTTAAACGAGATTGCCAGCACCGGGTATCATTCACCATTTTTCTTTTCGTTAACTTGCCGTCAGCCTTTTCTTTGACCTCTTCTTTCTGC
CCFFFFFHFHAFGGIIIJJJEEHGIGGGIJIIJGI?@EHIGIJDGHIHIGGIJJJJJJJIJGHHHGHFFFCDDDDDDCDCCCCCA;>@>@AA@:AA>AA     CC:Z:15 MD:Z:100        PG:Z:MarkDuplicates      RG:Z:2888721359 XG:i:0  NH:i:2  HI:i:0  NM:i:0  XM:i:
0       XN:i:0  XO:i:0  CP:i:102519261  AS:i:0  XS:A:-  YT:Z:UU
HWI-ST495_129147882:3:1210:1257:16203   83      1       12055   3       100M    =       11810   -345    GAGCACTGGAGTGGAGTTTTCCTGTGGAGAGGAGCCATGCCTAGAGTGGGATGGGCCATTGTTCATCTTCTGGCCCCTGTTGTCTGCATGTAACTTAATAC
CC>4C>DCCCACACDCC?BDCEE@ECFFFFHHHHHIJJJIIJJIIIHHEHIIGJIJIJJIGHIIIJJJJJIIJJJJJIIJJIJIJJHGHHHDFEFFCCC     CC:Z:15 MD:Z:100        PG:Z:MarkDuplicates      RG:Z:2888721359 XG:i:0  NH:i:2  HI:i:0  NM:i:0  XM:i:
0       XN:i:0  XO:i:0  CP:i:102519016  AS:i:0  XS:A:+  YT:Z:UU
HWI-ST495_129147882:3:2111:3117:78828   163     1       12634   3       100M    =       12746   212     GCCCTTCCCCAGCATCAGGTCTCCAGAGCTGCAGAAGACGACGGCCGACTTGGATCACACTCTTGTGAGTGTCCCCAGTGTTGCACAGGTGAGAGGAGAG<
@@FFFFFDHHHH9FHGIIFGAFDHEGII>GHIIIIIIIIIIIIIIIFHDDFFEEECEECCCACCCCCC:AADCCBCC>CAC<CCCCCC:@CB@@BAB##     CC:Z:15 MD:Z:85G14      PG:Z:MarkDuplicates      RG:Z:2888721359 XG:i:0  NH:i:2  HI:i:0  NM:i:1  XM:i:
1       XN:i:0  XO:i:0  CP:i:102518437  AS:i:-5 XS:A:-  YT:Z:UU
HWI-ST495_129147882:3:2111:3117:78828   83      1       12746   3       100M    =       12634   -212    GGGAGTGGCGTCGCCCCTAGGGCTCTACGGGGCCGGCATCTCCTGTCTCCTGGAGAGGCTTCGATGCCCCTCCACACCCTCTTGATCTTCCCTGTGATGTD
DCABDBDDDDDDDDDDCDDDDBDB@BDDDB@;CCCCCDEFD@;.?<HIGGEIGEHIGJJJIIGIGIIHEGFEHFJIIIIIGJJJJHHHHHFFFFFC@@     CC:Z:15 MD:Z:37G62      PG:Z:MarkDuplicates      RG:Z:2888721359 XG:i:0  NH:i:2  HI:i:0  NM:i:1  XM:i:
1       XN:i:0  XO:i:0  CP:i:102518325  AS:i:-5 XS:A:-  YT:Z:UU
HWI-ST495_129147882:3:1102:4242:26638   99      1       13503   3       100M    =       13779   376     CGCTGTGCCCTTCCTTTGCTCTGCCCGCTGGAGACGGTGTTTGTCATGGGCCTGGTCTGCAGGGATCCTGCTACAAAGGTGAAACCCAGGAGAGTGTGGAC
CCFFFFFHHHHHJJJIJJJJJJJJJJJJJGIIIIJJFHGGIJGIJJJEGIJIJJHHIHHGHFFEFDEEEECCCAACDDACDCDDDDDB?8?<B>A@CDC     CC:Z:2  MD:Z:100        PG:Z:MarkDuplicates      RG:Z:2888721359 XG:i:0  NH:i:2  HI:i:0  NM:i:0  XM:i:
0       XN:i:0  XO:i:0  CP:i:114357414  AS:i:0  XS:A:+  YT:Z:UU
HWI-ST495_129147882:3:1309:15328:74082  99      1       13534   3       100M    =       13780   346     AGACGGTGTTTGTCATGGGCCTGGTCTGCAGGGATCCTGCTACAAAGGTGAAACCCAGGAGAGTGTGGAGTCCAGAGTGTTGCCAGGACCCAGGCACAGG@
CCFFFADHHHHFIJJJJJIJIJIHIJJJIHJJIJJIIIJJIJIJJJJJBFHIIJJJJJJIJHH=EEFFFFCEEECEDCDCDDDDDDDDDDDDDDDDBDCCD     CC:Z:2  MD:Z:100        PG:Z:MarkDuplicates      RG:Z:2888721359 XG:i:0  NH:i:2  HI:i:0  NM:i:0  XM:i:
0       XN:i:0  XO:i:0  CP:i:114357383  AS:i:0  XS:A:+  YT:Z:UU
HWI-ST495_129147882:3:1308:10126:19636  99      1       13779   3       100M    =       14027   348     CCTCTGCAGGAGGCTGCCATTTGTCCTGCCCACCTTCTTAGAAGCGAGACGGAGCAGACCCATCTGCTACTGCCCTTTCTATAATAACTAAAGTTAGCTGC
CCFFFFFHHGHHJJJJJJJJJJIJJJJJJIJJJJJJJJJIIJJJJJJJJJHJJJHGHHFFFFFFEEEEEEDDDDCDCDCDFADCACCACDDCCCCCCD     CC:Z:2  MD:Z:100        PG:Z:MarkDuplicates      RG:Z:2888721359 XG:i:0  NH:i:2  HI:i:0  NM:i:0  XM:i:
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HWI-ST495_129147882:3:1102:4242:26638   147     1       13779   3       100M    =       13503   -376    CCTCTGCAGGAGGCTGCCATTTGTCCTGCCCACCTTCTTAGAAGCGAGACGGAGCAGACCCATCTGCTACTGCCCTTTCTATAATAACTAAAGTTAGCTG#
##DCCDDDCCBBBABCCDDDCBDDBBDHC?=GIIJIIIIJIGIIIIJJHJJIJJIGCIIJJJJJIGHGJJIJIJJJJJIJIIIGGFGHHHHFFFFFCCC     CC:Z:2  MD:Z:100        PG:Z:MarkDuplicates      RG:Z:2888721359 XG:i:0  NH:i:2  HI:i:0  NM:i:0  XM:i:
0       XN:i:0  XO:i:0  CP:i:114357140  AS:i:0  XS:A:+  YT:Z:UU
mgriffit@linus270 ~>
```
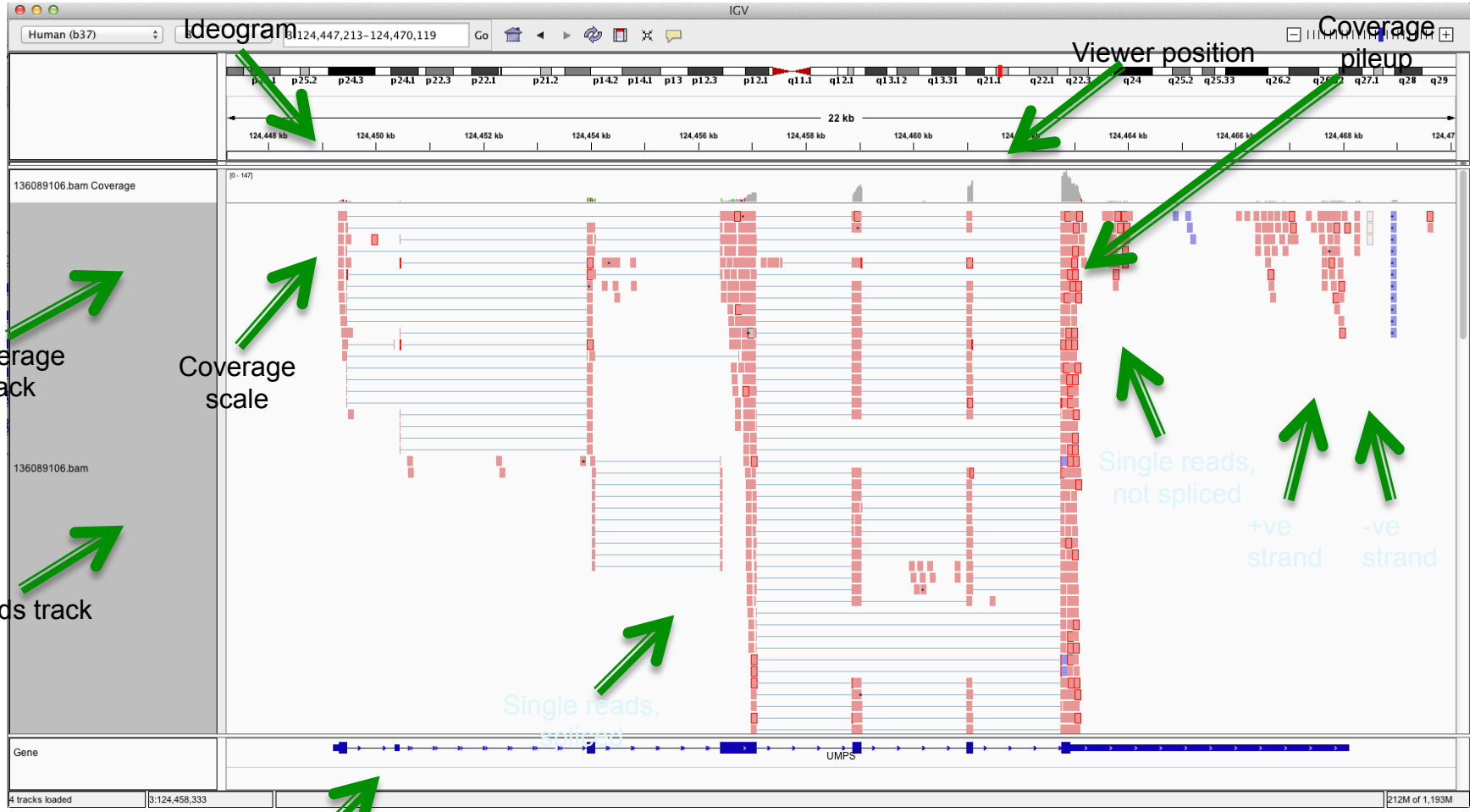
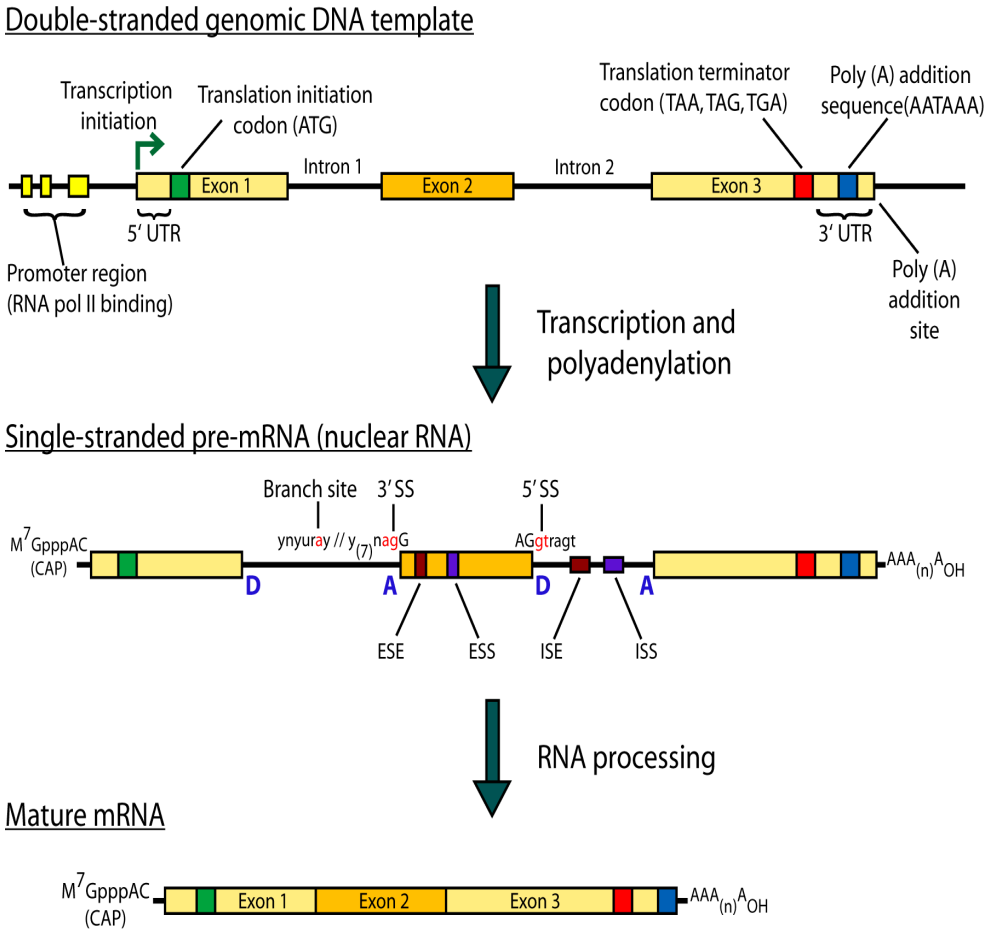

Alignment visualization with IGV



https://bioinformatics.ca/rnaseq2016module-3-ppt

Splice-aware alignment

- RNA-seq reads represent mRNA with introns removed
  - RNA-seq reads may span large introns
- But we usually align these reads back to reference genome
- Unless your reads are short (<50bp) you should use a splice-aware aligner
  - TopHat, STAR, MapSplice, etc.



https://bioinformatics.ca/rnaseq2016module-3-ppt

Downstream analysis

- Transcript abundance estimation
- Sample/replicate quality check
- Transcript functional annotation (*de novo* assembly)
- Differential expression analysis
- Coding region identification
- Alternative expression analysis

Transcript abundance estimation

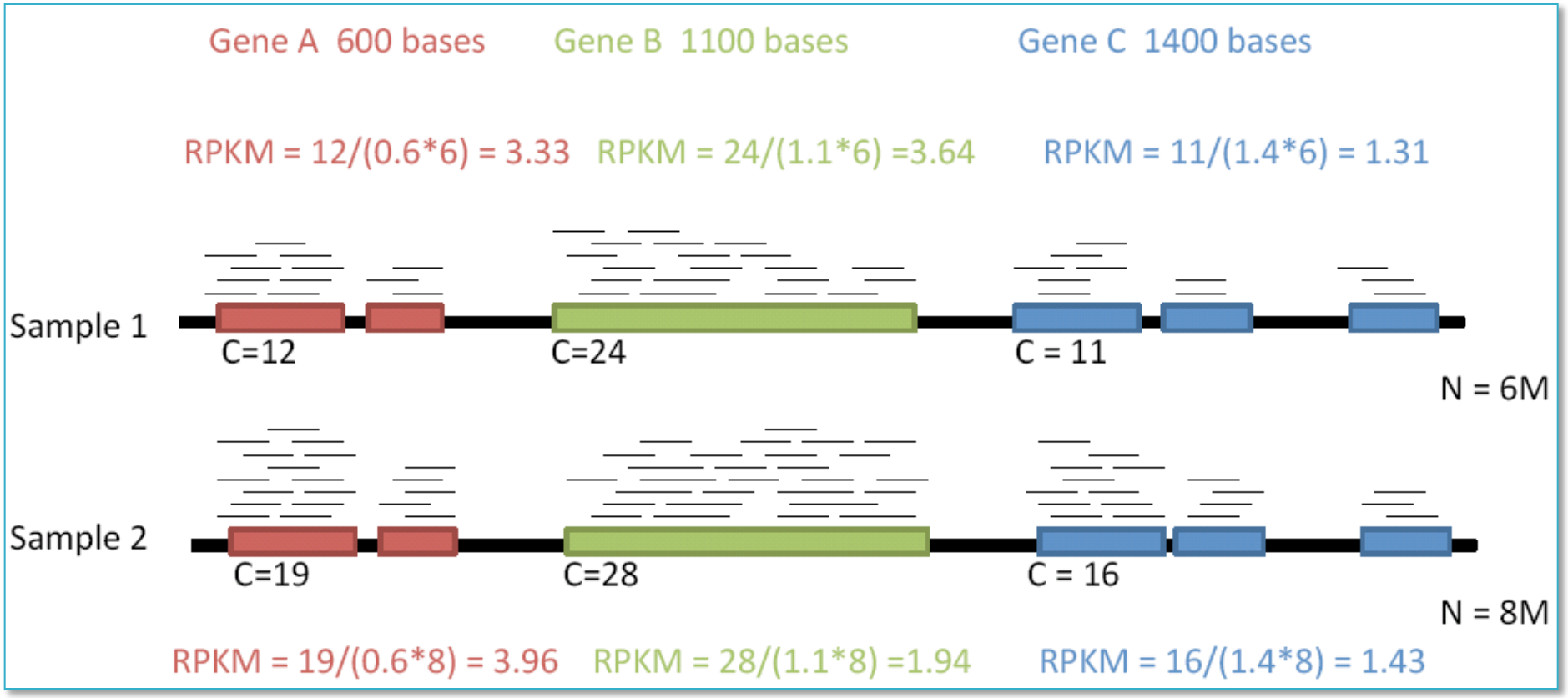
- For each transcript, count total number of reads mapped
  - Also called “binning” the reads
  - Counts are not directly comparable across features or samples (yet)
  - Followed by normalization into expression values

Normalizing counts

- Why normalize?
  - Longer features (naturally) can have more reads mapped
  - Deeper sequencing produces more reads

- RPKM (or FPKM) most commonly used
  - Reads (Fragments) per Kilobase per Million reads
  - Defined as $C/(LN)$
    - C = number of reads mapped to a feature
    - L = length of the feature (in kilobases)
    - N = total number of reads from the sample (in millions)

RPKM examples

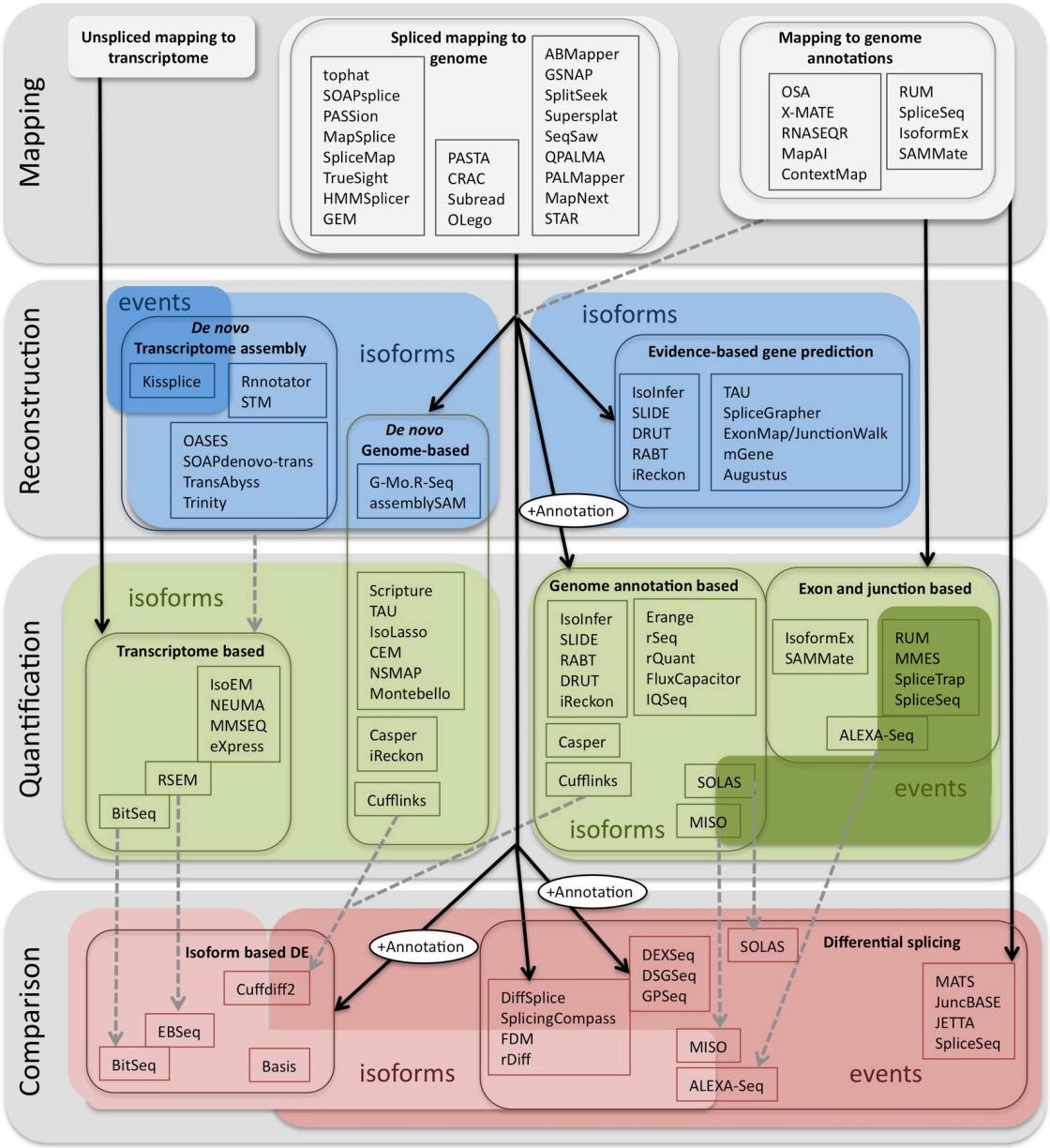


http://jura.wi.mit.edu/bio/education/hot_topics/RNAseq/RNA_Seq.pdf

Differential expression analysis

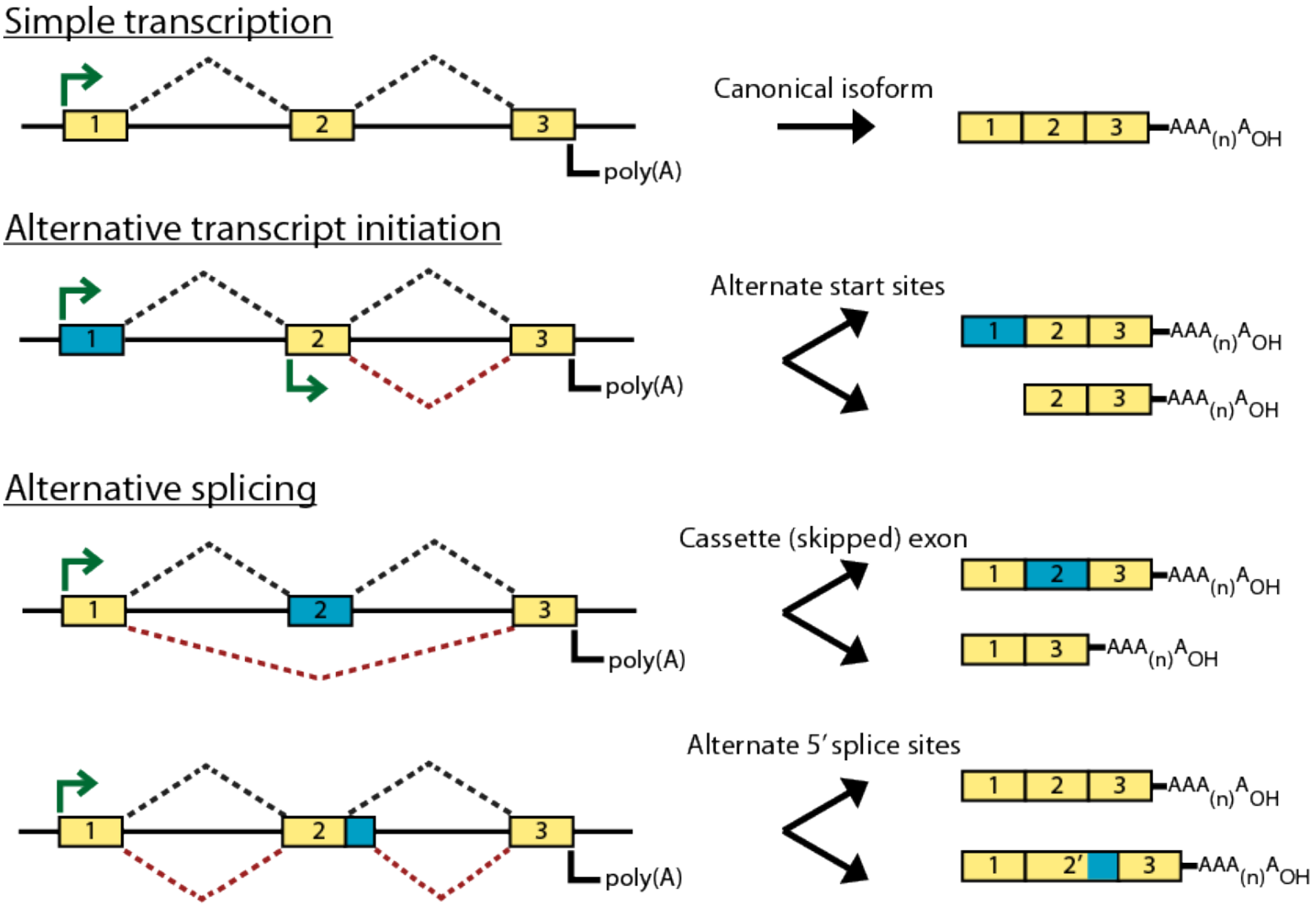
- Compare quantification values across samples or across features
  - The goal is to find differentially expressed (DE) features
- Many tools summarize/normalize counts and suggest DE features
  - Cufflinks/Cuffdiff, R packages (DESeq, edgeR, baySeq, TSPM), Samtools
- Determination of DE features depends on
  - Fold changes (FC)
  - Statistical significance of FC (FDR, p-value)

Studying splicing from RNA-seq

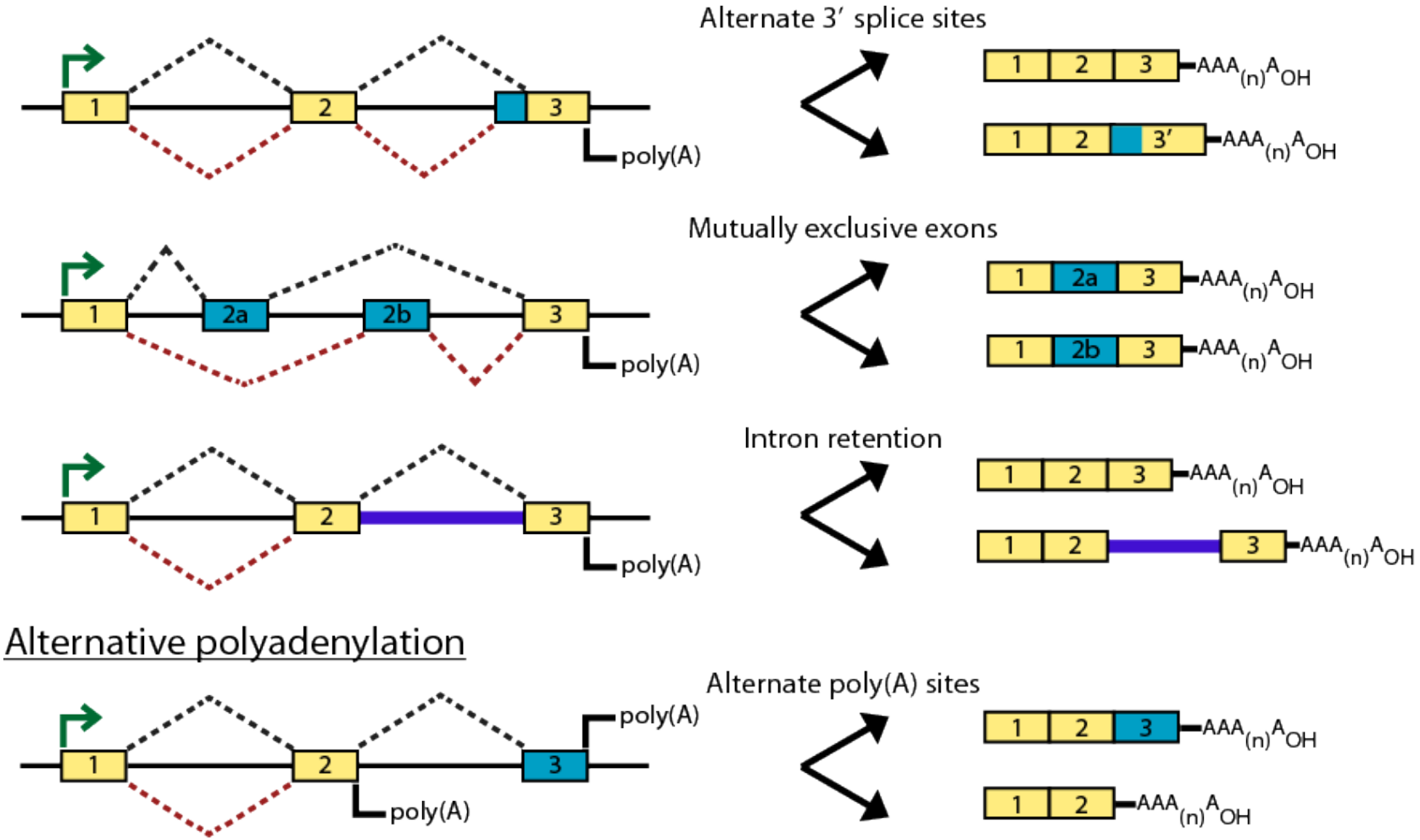


http://www.rna-seqblog.com/data-analysis/splicing-junction/methods-to-study-splicing-from-rna-seq/

Type of alternative expression



Types of alternative expression



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