Analysis of RNA-Seq Data with R/Bioconductor

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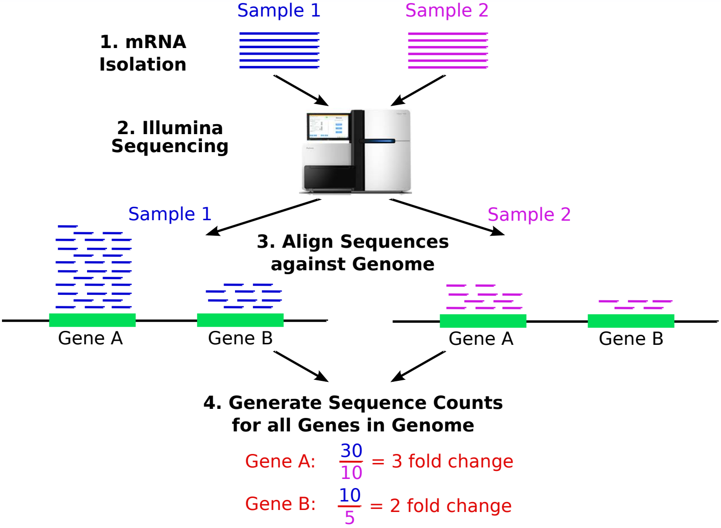
Outline

RNA-Seq Technology



Analysis Workflow of RNA-Seq Gene Expression Data

1. Alignment of RNA reads to reference
   - Reference can be genome or transcriptome.
2. Count reads overlapping with annotation features of interest
   - Most common: counts for exonic gene regions, but many viable alternatives exist here: counts per exons, genes, introns, etc.
3. Normalization
   - Main adjustment for sequencing depth and compositional bias.
4. Identification of Differentially Expressed Genes (DEGs)
   - Identification of genes with significant expression differences.
   - Identification of expressed genes possible for strongly expressed ones.
5. Specialty applications
   - Splice variant discovery (semi-quantitative), gene discovery, antisense expressions, etc.
6. Cluster Analysis
   - Identification of genes with similar expression profiles across many samples.
7. Enrichment Analysis of Functional Annotations
   - Gene ontology analysis of obtained gene sets from steps 5-6.

Important Aspects in RNA-Seq Analysis

- Alignment reference
  - Genome
  - Transcript models
  - Both
- How to quantify expression?
  - Read count per range
  - Coverage statistics per range
- What features?
  - Genes, transcript models, exons
- Alternative splicing
  - Often restricted to splice junction analysis
  - Objective: discovery vs. quantification

Important Considerations for NGS Alignments

- In NGS we usually want to find the origin of reads (NG sequences) in a reference genome or transcriptome. Thus, we are mostly interested in finding the best scoring or multiple best scoring locations for each read, but not lower scoring alternative solutions as in paralog/ortholog search applications.
- Ambiguous mappings should be removed, because there is no evidence for their origin. However, for certain applications one needs to include them, *e.g.* when mapping RNA-Seq reads against transcript sequences instead of genome.

Short Read Aligner for RNA-Seq

No special requirements for alignments with low number of variants

- ChIP-Seq
- RNA-Seq (if mapping against transcriptome or intron-less genome)
- Bis-Seq (with injected reference)
- ...

Variant tolerant aligners to account for mismatches and indels

- VAR-Seq
- Bis-Seq (without injected reference)
- ...

Splice tolerant aligner to account for introns

- RNA-Seq (if mapping against genome with introns)

Sequence Alignment/Map (SAM/BAM) Format

SAM is a tab-delimited alignment format consisting of a header section (lines starting with @) and an alignment section with 12 columns. BAM is the compressed, indexed and binary version of this format.

The below sample alignment contains the following features: (1) bases in lower cases are clipped from the alignment; (2) read r001/1 and r001/2 constitute a read pair; (3) r003 is a chimeric read; (4) r004 represents a split alignment.

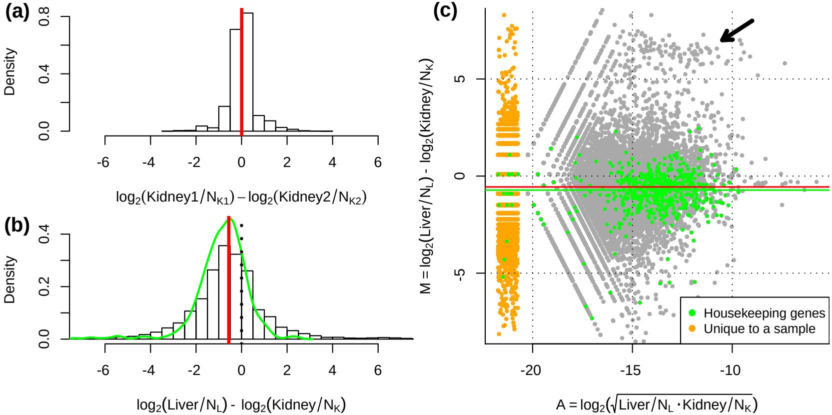
```
Coor     12345678901234  5678901234567890123456789012345
ref      AGCATGTTAGATAA**GATAGCTGTGCTAGTAGGCAGTCAGCGCCAT
+r001/1        TTAGATAAAGGATA*CTG
+r002           aaaAGATAA*GGATA
+r003         gcctaAGCTAA
+r004                       ATAGCT..............TCAGC
-r003                              ttagctTAGGC
-r001/2                                          CAGCGGCAT
```

⇓ SAM Format

```
r001   163 ref  7 30 8M2I4M1D3M = 37  39 TTAGATAAAGGATACTG   *
r002     0 ref  9 30 3S6M1P1I4M *  0   0 AAAAGATAAGGATA      *
r003     0 ref  9 30 5S6M       *  0   0 GCCTAAGCTAA         * SA:Z:ref,29,-,6H5M,17,0;
r004     0 ref 16 30 6M14N5M    *  0   0 ATAGCTTCAGC         *
r003  2064 ref 29 17 6H5M       *  0   0 TAGGC               * SA:Z:ref,9,+,5S6M,30,1;
r001    83 ref 37 30 9M         =  7 -39 CAGCGGCAT           * NM:i:1
```

For details see the SAM Format Specification Link

Normalization Required



Log ratio distributions (a and b) and MA plot (c) for two tissue samples (from Robinson and Oshlack, 2010).

Be Careful with RPKM/FPKM Values

RPKM Concept (FPKM is paired-end version of it)

- RPKM (FPKM): reads (fragments) per kp per million mapped reads
- The more we sequence, the more reads we expect from each gene. This is the most relevant correction of this method.
- Longer transcript are expected to generate more reads. The latter is only relevant for comparisons among different genes which we rarely perform!
- RPKM/FPKM are not suitable for statistical testing. Why? Consider the following example: in two libraries, each with one million reads, gene X may have 10 reads for treatment A and 5 reads for treatment B, while it is 100x as many after sequencing 100 millions reads from each library. In the latter case we can be much more confident that there is a true difference between the two treatments than in the first one. However, the RPKM values would be the same for both scenarios.
- Thus, RPKM/FPKM are useful for reporting expression values, but not for statistical testing!

TMM Method Corrects for RNA Composition Bias

Trimmed Mean of M Values (TMM) by Robinson and Oshlack (2010)

- Many normalization RNA-Seq normalization methods perform poorly on samples with extreme composition bias. For instance, in one sample a large number of reads comes from rRNAs while in another they have been removed more efficiently. Most scaling based methods, including RPKM and CPM, will underestimate the expression of weaker expressed genes in the presence of extremely abundant mRNAs (less sequencing real estate available for them). The TMM methods tries to correct this bias.
- Method implemented in edgeR library (Robinson et al., 2010).

Analysis of Differentially Expressed Genes (DEGs)

- Data is discrete, positively skewed
  - $\Rightarrow$ no (log-)normal model
- Small numbers of replicates
  - $\Rightarrow$ no rank based or permutation methods
- Sequencing depth (coverage) varies among samples
  - $\Rightarrow$ normalization

DEG Analysis Methods

Requirements

- One would like to perform a t-test or something similar for each gene.
- t-test assumes normal distribution and no mean-variance dependence. Both are not appropriate assumptions for RNA-Seq data.
- Variance estimation and rank-order statistics is difficult on small sample numbers.

Statistical Testing

- Poisson distribution (initially used but not very common anymore)
- Most statistical methods for RNA-Seq DEG analysis use negative binomial distribution along with modified statistical tests based on that.
- The mutiple testing issue is very similar as in microarray data analysis. Thus, most tools provide False Discovery Rates (FDRs), which are derived from p-values corrected for multiple testing using the Benjamini-Hochberg method.
- For variance estimation most methods borrow information across genes

Software for RNA-Seq DEG Analysis

- edgeR (Robinson et al., 2010)
- DESeq/DESeq2 (Anders and Huber, 2010)
- DEXSeq (Anders et al., 2012)
- limmaVoom
- Cuffdiff/Cuffdiff2 (Trapnell et al., 2013)
- PoissonSeq
- baySeq
- ...

Packages for RNA-Seq Analysis in R

- GenomicRanges Link: high-level infrastructure for range data
- Rsamtools Link: BAM support
- rtracklayer Link: Import/export of range and annotation data, interface to online genome browsers, etc.
- DESeq Link: RNA-Seq DEG analysis
- DESeq2 Link: RNA-Seq DEG analysis
- edgeR Link: RNA-Seq DEG analysis
- DEXSeq Link: RNA-Seq Exon analysis
- QuasR Link: RNA-Seq workflows

Outline

Data Sets and Experimental Variables

To make the following sample code work, please follow these instructions:

- Download and unpack the sample data Link for this practical.
- Direct your R session into the resulting `Rrnaseq` directory. It contains four slimmed down FASTQ files (SRA023501 Link) from *A. thaliana*, as well as the corresponding reference genome sequence (FASTA) and annotation (GFF) file.
- Start the analysis by opening in your R session the `Rrnaseq.R` script Link which contains the code shown in this slide show in pure text format.

The FASTQ files are organized in the provided `targets.txt` file. This is the only file in this analysis workflow that needs to be generated manually, *e.g.* in a spreadsheet program. To import `targets.txt`, we run the following commands from R:

```
> download.file("http://biocluster.ucr.edu/~tgirke/HTML_Presentations/Manuals/Wo

> targets <- read.delim("./data/targets.txt")
> targets

        FileName SampleName Factor Factor_long
1 SRR064154.fastq   AP3_fl4a    AP3     AP3_fl4
2 SRR064155.fastq   AP3_fl4b    AP3     AP3_fl4
3 SRR064166.fastq    Tl_fl4a    TRL      Tl_fl4
4 SRR064167.fastq    Tl_fl4b    TRL      Tl_fl4
```

Outline

Align Reads Option 1: *QuasR*

QuasR is an extremely versatile NGS mapping and postprocessing pipeline for RNA-Seq and many other application areas, such as BS-Seq, allele-specific RNA-Seq, etc. It uses *Rbowtie* for ungapped alignments and SpliceMap for spliced alignments.

(1) Evironment settings

```
> library(QuasR)
> targets <- read.delim("data/targets.txt")
> write.table(targets[,1:2], "data/QuasR_samples.txt", row.names=FALSE, quote=FALSE, sep="\t")
> sampleFile <- "./data/QuasR_samples.txt"
> genomeFile <- "./data/tair10chr.fasta"
> results <- "./results" # defines location where to write results
> cl <- makeCluster(1) # defines number of CPU cores to use
```

(2) Single command to index reference, align all samples and generate BAM files.

```
> proj <- qAlign(sampleFile, genome=genomeFile, maxHits=1, splicedAlignment=FALSE, alignmentsDir=results,
+                clObj=cl, cacheDir=results)
>                # Note: splicedAlignment should be set to TRUE when the reads are >=50nt long
> (alignstats <- alignmentStats(proj)) # Alignment summary report

                 seqlength  mapped unmapped
AP3_fl4a:genome     7e+05 1607234    26022
AP3_fl4b:genome     7e+05 1647774    21272
Tl_fl4a:genome      7e+05  206041     4366
Tl_fl4b:genome      7e+05  283742     5279
```

Align Reads Option 2: *Rsubread*

Rsubread is an R/Bioc package that implements an extremely fast aligner for RNA-Seq data. It is currently only available for OS X and Linux, but not for Windows.

(1) Index reference genome

```
> library(Rsubread); library(Rsamtools)
> dir.create("results") # Note: all output data will be written to directory 'results'
> buildindex(basename="./results/tair10chr.fasta", reference="./data/tair10chr.fasta") # Build indexed referenc
```

(2) Align all FASTQ files with *Rsubread* in loop. Includes generation of indexed BAM files.

```
> targets <- read.delim("./data/targets.txt") # Import experiment design information
> input <- paste("./data/", targets$FileName, sep="")
> output <- paste("./results/", targets$FileName, ".sam", sep="")
> reference <- "./results/tair10chr.fasta"
> for(i in seq(along=targets$FileName)) {
+         align(index=reference, readfile1=input[i], output_file=output[i], nthreads=8, indels=1, TH1=2)
+         asBam(file=output[i], destination=gsub(".sam", "", output[i]), overwrite=TRUE, indexDestination=TRUE)
+         unlink(output[i])
+ }
```

Align Reads Option 3: Bowtie2/Tophat2

Note: this step requires the command-line tools `tophat2/bowtie2` Link.

(1) Index reference genome

```
> library(modules) # Skip this and next line if you are not using IIGB's biocluster
> moduleload("bowtie2/2.1.0"); moduleload("tophat/2.0.8b") # loads bowtie2/tophat2 from module system
> system("bowtie2-build ./data/tair10chr.fasta ./data/tair10chr.fasta")
```

(2) Align all FASTQ files with Bowtie2/Tophat2 in loop. Includes generation of indexed BAM files.

```
> library(Rsamtools)
> dir.create("results") # Note: all output data will be written to directory 'results'
> input <- input <- paste("./data/", targets$FileName, sep="")
> output <- paste("./results/", targets$FileName, sep="")
> reference <- "./data/tair10chr.fasta"
> for(i in seq(along=input)) {
+         unlink(paste(output[i], ".tophat", sep=""), force=TRUE, recursive=TRUE)
+         tophat_command <- paste("tophat -p 4 -g 1 --segment-length 15 -i 30 -I 3000 -o ", output[i], ".tophat
+                 # -G: supply GFF with transcript model info (preferred!)
+                 # -g: ignore all alginments with >g matches
+                 # -p: number of threads to use for alignment step
+                 # -i/-I: min/max intron lengths
+                 # --segment-length: length of split reads (25 is default)
+         system(tophat_command)
+         sortBam(file=paste(output[i], ".tophat/accepted_hits.bam", sep=""), destination=paste(output[i], ".top
+         indexBam(paste(output[i], ".tophat/accepted_hits.bam", sep=""))
+ }
```

Alignment Summary

The following enumerates the number of reads in each FASTQ file and how many of them aligned to the reference. Note: the percentage of aligned reads is 100% in this particular example because only alignable reads were selected when generating the sample FASTQ files for this exercise. For *QuasR* this step can be omitted because the `qAlign` function generats this information automatically.

```
> library(ShortRead); library(Rsamtools)
> Nreads <- countLines(dirPath="./data", pattern=".fastq$")/4
> bfl <- BamFileList(paste0("./results/", targets$FileName, ".bam"), yieldSize=5000
> Nalign <- countBam(bfl)
> (read_statsDF <- data.frame(FileName=names(Nreads), Nreads=Nreads, Nalign=Nalign$r
+                              Perc_Aligned=Nalign$records/Nreads*100))
```

```
                     FileName  Nreads  Nalign Perc_Aligned
SRR064154.fastq SRR064154.fastq 1633256 1633256          100
SRR064155.fastq SRR064155.fastq 1669046 1669046          100
SRR064166.fastq SRR064166.fastq  210407  210407          100
SRR064167.fastq SRR064167.fastq  289021  289021          100
```

```
> write.table(read_statsDF, "results/read_statsDF.xls", row.names=FALSE, quote=FALSE
```

Quality Reports

The following shows how to create read quality reports with *QuasR's* `qQCReport` function or with the custom `seeFastq` function.

```
> qQCReport(proj, pdfFilename="results/qc_report.pdf")
> source("http://faculty.ucr.edu/~tgirke/Documents/R_BioCond/My_R_Scripts/fastqQuali
> myfiles <- paste0("data/", targets$FileName); names(myfiles) <- targets$SampleName
> fqlist <- seeFastq(fastq=myfiles, batchsize=50000, klength=8)
> pdf("results/fastqReport.pdf", height=18, width=4*length(myfiles)); seeFastqPlot(f
```

Outline

Import Annotation Data from GFF

Annotation data from GFF

```
> library(rtracklayer); library(GenomicRanges); library(Rsamtools)
> gff <- import.gff("./data/TAIR10_GFF3_trunc.gff", asRangedData=FALSE)
> seqlengths(gff) <- end(ranges(gff[which(elementMetadata(gff)[,"type"]=="chromosome
> subgene_index <- which(elementMetadata(gff)[,"type"] == "exon")
> gffsub <- gff[subgene_index,] # Returns only gene ranges
> gffsub[1:4, c(2,5)]

GRanges with 4 ranges and 2 metadata columns:
      seqnames         ranges strand |     type               group
         <Rle>      <IRanges>  <Rle> | <factor>            <factor>
  [1]     Chr1 [3631, 3913]      + |     exon Parent=AT1G01010.1
  [2]     Chr1 [3996, 4276]      + |     exon Parent=AT1G01010.1
  [3]     Chr1 [4486, 4605]      + |     exon Parent=AT1G01010.1
  [4]     Chr1 [4706, 5095]      + |     exon Parent=AT1G01010.1
  ---
  seqlengths:
     Chr1   Chr2   Chr3   Chr4   Chr5   ChrC   ChrM
   100000 100000 100000 100000 100000 100000 100000

> ids <- gsub("Parent=|\\..*", "", elementMetadata(gffsub)$group)
> gffsub <- split(gffsub, ids) # Coerce to GRangesList
```

More Robust: Store Annotations in TranscriptDb

Storing annotation ranges in *TranscriptDb* databases makes many operations more robust and convenient.

```
> library(GenomicFeatures)
> txdb <- makeTranscriptDbFromGFF(file="data/TAIR10_GFF3_trunc.gff",
+         format="gff3",
+         dataSource="TAIR",
+         species="Arabidopsis thaliana")
> saveDb(txdb, file="./data/TAIR10.sqlite")
> txdb <- loadDb("./data/TAIR10.sqlite")
> eByg <- exonsBy(txdb, by="gene")
```

Read Counting with countOverlaps

Number of reads overlapping gene ranges

```
> samples <- as.character(targets$FileName)
> samplespath <- paste("./results/", samples, ".bam", sep="")
> names(samplespath) <- samples
> countDF <- data.frame(row.names=names(eByg))
> for(i in samplespath) {
+         aligns <- readGAlignmentsFromBam(i) # Substitute next two lines with this
+         counts <- countOverlaps(eByg, aligns, ignore.strand=TRUE)
+         countDF <- cbind(countDF, counts)
+ }
> colnames(countDF) <- samples
> countDF[1:4,]

          SRR064154.fastq SRR064155.fastq SRR064166.fastq SRR064167.fastq
AT1G01010              52              26              60              75
AT1G01020             145              77              82              64
AT1G01030               5               1              13              14
AT1G01040             482             347             302             358

> write.table(countDF, "./results/countDF", quote=FALSE, sep="\t", col.names = NA)
> countDF <- read.table("./results/countDF")
```

Read Counting with `summarizeOverlaps`

The `summarizeOverlaps` function from the `GenomicRanges` package is easier to use, it provides more options and it is much more memory efficient. See here Link for details.

```
> library(GenomicRanges)
> bfl <- BamFileList(samplespath, yieldSize=50000, index=character())
> countDF2 <- summarizeOverlaps(eByg, bfl, mode="Union", ignore.strand=TRUE)
> countDF2 <- assays(countDF2)$counts
> colnames(countDF2) <- samples
> countDF2[1:4,]
```

```
          SRR064154.fastq SRR064155.fastq SRR064166.fastq SRR064167.fastq
AT1G01010              52              26              60              75
AT1G01020             145              77              82              64
AT1G01030               5               1              13              14
AT1G01040             482             346             285             339
```

Read Counting with qCount from QuasR

QuasR does everything in one command.

```
> countDF3 <- qCount(proj, txdb, reportLevel="gene", orientation="any")
> countDF3[1:4,]
```

	width	AP3_fl4a	AP3_fl4b	Tl_fl4a	Tl_fl4b
AT1G01010	1688	46	24	59	70
AT1G01020	1774	115	71	73	50
AT1G01030	1905	5	0	13	14
AT1G01040	6254	464	323	286	349

```
> write.table(countDF3, "results/countDFgene.xls", col.names=NA, quote=FALSE, sep="\
```

Simple RPKM Normalization

RPKM: reads per kilobase of exon model per million mapped reads

```
> returnRPKM <- function(counts, gffsub) {
+         geneLengthsInKB <- sum(width(reduce(gffsub)))/1000 # Length of exon union
+         millionsMapped <- sum(counts)/1e+06 # Factor for converting to million of
+         rpm <- counts/millionsMapped # RPK: reads per kilobase of exon model.
+         rpkm <- rpm/geneLengthsInKB # RPKM: reads per kilobase of exon model per m
+         return(rpkm)
+ }
> countDFrpkm <- apply(countDF, 2, function(x) returnRPKM(counts=x, gffsub=eByg))
> countDFrpkm[1:4,]
```

```
          SRR064154.fastq SRR064155.fastq SRR064166.fastq SRR064167.fastq
AT1G01010       231.83437      139.974951       1197.1649       1158.4825
AT1G01020       615.12206      394.445066       1556.8093        940.6477
AT1G01030        19.75249        4.770396        229.8389        191.6169
AT1G01040       580.01080      504.221101       1626.3883       1492.5394
```

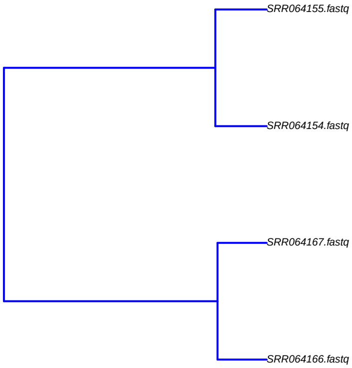
RPKM: for QuasR results

```
> rpkmDFgene <- t(t(countDF3[,-1]/countDF3[,1] * 1000)/colSums(countDF3[,-1]) *1e6)
```

Reproducibility Check by Sample-Wise Clustering

QC check of the sample reproducibility by computing a correlating matrix and plotting it as a tree. Note: the `plotMDS` function from *edgeR* is a more robust method for this task.

```
> library(ape)
> d <- cor(countDFrpkm, method="spearman")
> hc <- hclust(dist(1-d))
> plot.phylo(as.phylo(hc), type="p", edge.col=4, edge.width=3, show.node.label=TRUE, no.margin=TRUE)
```



Exercise 1: *QuasR* with Antisense Read Counting

Task 1 Align reads from all 4 samples.

Task 2 Count reads in sense and antisense. Discuss differences. Why is this analysis meaningless for the provided non-strand-specific RNA-Seq samples?

Task 3 Identify all genes where the antisense counts are $\geq$3-fold higher than the sense counts in at least 2 out of the 4 samples.

Task 4 Plot the result of the most pronounced antisense expression case with *ggbio*.

Outline

Identify DEGs with Simple Fold Change Method

Compute mean values for replicates

```
> source("http://faculty.ucr.edu/~tgirke/Documents/R_BioCond/My_R_Scripts/colAg.R")
> countDFrpkm_mean <- colAg(myMA=countDFrpkm, group=c(1,1,2,2), myfct=mean)
> countDFrpkm_mean[1:4,]
```

```
          SRR064154.fastq_SRR064155.fastq SRR064166.fastq_SRR064167.fastq
AT1G01010                       185.90466                       1177.8237
AT1G01020                       504.78356                       1248.7285
AT1G01030                        12.26145                        210.7279
AT1G01040                       542.11595                       1559.4639
```

Log2 fold changes

```
> countDFrpkm_mean <- cbind(countDFrpkm_mean, log2ratio=log2(countDFrpkm_mean[,2]/countDFrpkm_mean[,1]))
> countDFrpkm_mean <- countDFrpkm_mean[is.finite(countDFrpkm_mean[,3]), ]
> degs2fold <- countDFrpkm_mean[countDFrpkm_mean[,3] >= 1 | countDFrpkm_mean[,3] <= -1,]
> degs2fold[1:4,]
```

```
          SRR064154.fastq_SRR064155.fastq SRR064166.fastq_SRR064167.fastq log2ratio
AT1G01010                       185.90466                       1177.8237  2.663489
AT1G01020                       504.78356                       1248.7285  1.306723
AT1G01030                        12.26145                        210.7279  4.103180
AT1G01040                       542.11595                       1559.4639  1.524377
```

```
> write.table(degs2fold, "./results/degs2fold.xls", quote=FALSE, sep="\t", col.names = NA)
> degs2fold <- read.table("./results/degs2fold.xls")
```

Identify DEGs with DESeq Library

Raw count data are expected here!

```
> library(DESeq)
> countDF <- read.table("./results/countDF")
> conds <- targets$Factor
> cds <- newCountDataSet(countDF, conds) # Creates object of class CountDataSet derived from eSet class
> counts(cds)[1:4, ] # CountDataSet has similar accessor methods as eSet class.

          SRR064154.fastq SRR064155.fastq SRR064166.fastq SRR064167.fastq
AT1G01010              52              26              60              75
AT1G01020             145              77              82              64
AT1G01030               5               1              13              14
AT1G01040             482             347             302             358

> cds <- estimateSizeFactors(cds) # Estimates library size factors from count data. Alternatively, one can provi
> cds <- estimateDispersions(cds) # Estimates the variance within replicates
> res <- nbinomTest(cds, "AP3", "TRL") # Calls DEGs with nbinomTest
> res <- na.omit(res)
> res2fold <- res[res$log2FoldChange >= 1 | res$log2FoldChange <= -1,]
> res2foldpadj <- res2fold[res2fold$padj <= 0.05, ]
> res2foldpadj[1:4,1:8]

          id  baseMean  baseMeanA  baseMeanB foldChange log2FoldChange         pval         padj
5  AT1G01050 595.24510 275.126601 915.363593 3.32706322       1.734249 7.878492e-18 9.946596e-17
6  AT1G01060 299.40527 170.693390 428.117153 2.50810621       1.326598 7.141055e-08 4.507791e-07
7  AT1G01070  29.50693   5.717372  53.296498 9.32185294       3.220617 1.413061e-05 6.487233e-05
14 AT2G01008  20.01065  37.575725   2.445565 0.06508364      -3.941561 4.908712e-05 2.155565e-04
```

Identify DEGs with *edgeR's* Exact Method

DEG analysis with classical *edgeR* approach. Note: raw read count data are expected by all methods!

```
> library(edgeR)
> countDF <- read.table("./results/countDF")
> y <- DGEList(counts=countDF, group=conds) # Constructs DGEList object
> y <- estimateCommonDisp(y) # Estimates common dispersion
> y <- estimateTagwiseDisp(y) # Estimates tagwise dispersion
> et <- exactTest(y, pair=c("AP3", "TRL")) # Computes exact test for the negative binomial distribution.
> topTags(et, n=4)
```

```
Comparison of groups:  TRL-AP3
              logFC   logCPM        PValue           FDR
AT3G01120  3.189185 15.78303 3.500250e-128 4.060290e-126
AT1G01100  2.747447 17.07336 3.289500e-115 1.907910e-113
AT1G01050  3.539622 13.79932 6.577536e-115 2.543314e-113
ATMG00030 -4.415745 13.12701 2.338291e-107 6.781044e-106
```

```
> edge <- as.data.frame(topTags(et, n=50000))
> edge2fold <- edge[edge$logFC >= 1 | edge$logFC <= -1,]
> edge2foldpadj <- edge2fold[edge2fold$FDR <= 0.01, ]
```

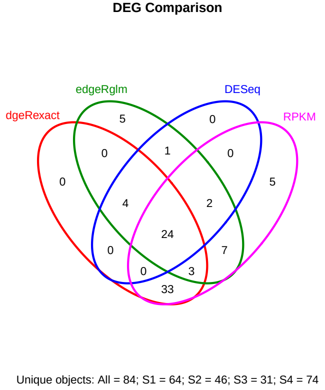
Identify DEGs with *edgeR's* GLM Approach

DEG analysis with *edgeR* using generalized linear models (glms)

```
> library(edgeR)
> countDF <- read.table("./results/countDF")
> y <- DGEList(counts=countDF, group=conds) # Constructs DGEList object
> ## Filtering and normalization
> keep <- rowSums(cpm(y)>1) >= 2; y <- y[keep, ]
> y <- calcNormFactors(y)
> design <- model.matrix(~0+group, data=y$samples); colnames(design) <- levels(y$samples$group) # Design matrix
> ## Estimate dispersion
> y <- estimateGLMCommonDisp(y, design, verbose=TRUE) # Estimates common dispersions

Disp = 0.01892 , BCV = 0.1375

> y <- estimateGLMTrendedDisp(y, design) # Estimates trended dispersions
> y <- estimateGLMTagwiseDisp(y, design) # Estimates tagwise dispersions
> ## Fit the negative binomial GLM for each tag
> fit <- glmFit(y, design) # Returns an object of class DGEGLM
> contrasts <- makeContrasts(contrasts="AP3-TRL", levels=design) # Contrast matrix is optional
> lrt <- glmLRT(fit, contrast=contrasts[,1]) # Takes DGEGLM object and carries out the likelihood ratio test.
> edgeglm <- as.data.frame(topTags(lrt, n=length(rownames(y))))
> ## Filter on fold change and FDR
> edgeglm2fold <- edgeglm[edgeglm$logFC >= 1 | edgeglm$logFC <= -1,]
> edgeglm2foldpadj <- edgeglm2fold[edgeglm2fold$FDR <= 0.01, ]
```

Comparison Among DEG Results

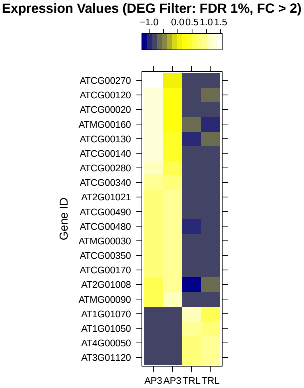
```
> source("http://faculty.ucr.edu/~tgirke/Documents/R_BioCond/My_R_Scripts/overLapper.R")
> setlist <- list(edgeRexact=rownames(edge2foldpadj), edgeRglm=rownames(edgeglm2foldpadj), DESeq=as.character(r
> OLlist <- overLapper(setlist=setlist, sep="_", type="vennsets")
> counts <- sapply(OLlist$Venn_List, length)
> vennPlot(counts=counts, mymain="DEG Comparison")
```



Heatmap of Top Ranking DEGs

Note: gene-wise clustering is not possible with a single sample pair. The following shows the scaled expression values (here RPKMs) in form of a heatmap.

```
> library(lattice); library(gplots)
> y <- countDFrpkm[rownames(edgeglm2foldpadj)[1:20],]
> colnames(y) <- targets$Factor
> y <- t(scale(t(as.matrix(y))))
> y <- y[order(y[,1]),]
> levelplot(t(y), height=0.2, col.regions=colorpanel(40, "darkblue", "yellow", "white"), main="Expression Values
```



Outline

Enrichment of GO Terms in DEG Sets

The following performs GO term enrichment analysis of one of the identified DEG sets using the *GOstats* Link package. Another package, among many others, to consider here is the *goseq* Link that considers gene length bias in RNA-Seq data.

```
> library(GOstats); library(GO.db); library(ath1121501.db)
> geneUniverse <- rownames(countDF)
> geneSample <- res2foldpadj[,1]
> params <- new("GOHyperGParams", geneIds = geneSample, universeGeneIds = geneUniverse,
+         annotation="ath1121501", ontology = "MF", pvalueCutoff = 0.5,
+         conditional = FALSE, testDirection = "over")
> hgOver <- hyperGTest(params)
> summary(hgOver)[1:4,]

      GOMFID      Pvalue OddsRatio ExpCount Count Size
1 GO:0008324 0.002673178        18 2.126582     6    7                         cation transmembrane transporter a
2 GO:0015075 0.002673178        18 2.126582     6    7                            ion transmembrane transporter a
3 GO:0015077 0.002673178        18 2.126582     6    7 monovalent inorganic cation transmembrane transporter a
4 GO:0015078 0.002673178        18 2.126582     6    7                   hydrogen ion transmembrane transporter a

> htmlReport(hgOver, file = "results/MyhyperGresult.html")
```

Outline

Inspect Results in IGV

View results in IGV

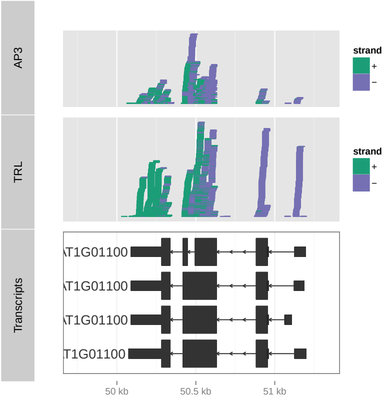
- Download and open IGV Link
- Select in menu in top left corner `A. thaliana (TAIR10)`
- Upload the following indexed/sorted Bam files with `File -> Load from URL...`

http://faculty.ucr.edu/~tgirke/HTML_Presentations/Manuals/Workshop_Dec_6_10_2012/Rrnaseq/results/SRR064154.fastq.ba
http://faculty.ucr.edu/~tgirke/HTML_Presentations/Manuals/Workshop_Dec_6_10_2012/Rrnaseq/results/SRR064155.fastq.ba
http://faculty.ucr.edu/~tgirke/HTML_Presentations/Manuals/Workshop_Dec_6_10_2012/Rrnaseq/results/SRR064166.fastq.ba
http://faculty.ucr.edu/~tgirke/HTML_Presentations/Manuals/Workshop_Dec_6_10_2012/Rrnaseq/results/SRR064167.fastq.ba

- To view area of interest, enter its coordinates `Chr1:49,457-51,457` in position menu on top.

Generate Similar View with *ggbio* Programmatically

```
> library(ggbio)
> AP3 <- readGAlignmentsFromBam("./results/SRR064154.fastq.bam", use.names=TRUE, param=ScanBamParam(which=GRange
> TRL <- readGAlignmentsFromBam("./results/SRR064166.fastq.bam", use.names=TRUE, param=ScanBamParam(which=GRange
> p1 <- autoplot(AP3, geom = "rect", aes(color = strand, fill = strand))
> p2 <- autoplot(TRL, geom = "rect", aes(color = strand, fill = strand))
> p3 <- autoplot(txdb, which=GRanges("Chr1", IRanges(49457, 51457)), names.expr = "gene_id")
> tracks(AP3=p1, TRL=p2, Transcripts=p3, heights = c(0.3, 0.3, 0.4)) + ylab("")
```



Exercise 2: Venn Diagram for Up/Down DEGs

Task 1 Store the identifiers of the upregulated genes from each of the four DEG methods in separate components of a list. Note: the definition of up and down is arbitrary and one needs to check how it is defined by the different DEG methods!

Task 2 Do the same for the downregulated genes.

Task 3 Compare the overlaps among the different up/down sets in a single 4-way venn diagram.

Outline

Analysis of Differential Exon Usage with DEXSeq

Number of reads overlapping gene ranges

```
> source("data/Fct/gffexonDEXSeq.R")
> gffexonDEXSeq <- exons2DEXSeq(gff=gff)
> ids <- as.character(elementMetadata(gffexonDEXSeq)[, "ids"])
> countDFdex <- data.frame(row.names=ids)
> for(i in samplespath) {
+         aligns <- readBamGappedAlignments(i) # Substitute next two lines with this one.
+         counts <- countOverlaps(gffexonDEXSeq, aligns)
+         countDFdex <- cbind(countDFdex, counts)
+ }
> colnames(countDFdex) <- samples
> countDFdex[1:4,1:2]
```

```
                                                          SRR064154.fastq SRR064155.fastq
Parent=AT1G01010:E001__Chr1_3631_3913_+_Parent=AT1G01010.1               2               4
Parent=AT1G01010:E002__Chr1_3996_4276_+_Parent=AT1G01010.1               2               1
Parent=AT1G01010:E003__Chr1_4486_4605_+_Parent=AT1G01010.1               3               3
Parent=AT1G01010:E004__Chr1_4706_5095_+_Parent=AT1G01010.1               6               1
```

```
> write.table(countDFdex, "./results/countDFdex", quote=FALSE, sep="\t", col.names = NA)
> countDFdex <- read.table("./results/countDFdex")
```

Analysis of Differential Exon Usage with DEXSeq

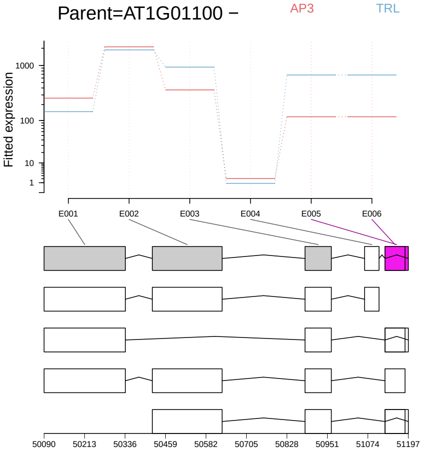
Identify genes with differential exon usage

```
> library(DEXSeq)
> samples <- as.character(targets$Factor); names(samples) <- targets$FileName
> countDFdex[is.na(countDFdex)] <- 0
> ## Construct ExonCountSet from scratch
> exset <- newExonCountSet2(countDF=countDFdex) # fData(exset)[1:4,]
> ## Performs normalization
> exset <- estimateSizeFactors(exset)
> ## Evaluate variance of the data by estimating dispersion using Cox-Reid (CR) likelihood estimation
> exset <- estimateDispersions(exset)

....
Done

> ## Fits dispersion-mean relation to the individual CR dispersion values
> exset <- fitDispersionFunction(exset)
> ## Performs Chi-squared test on each exon and Benjmini-Hochberg p-value adjustment for mutliple testing
> exset <- testForDEU(exset)
> ## Estimates fold changes of exons
> exset <- estimatelog2FoldChanges(exset)
> ## Obtain results in data frame
> deuDF <- DEUresultTable(exset)
> ## Count number of genes with differential exon usage
> table(tapply(deuDF$padjust < 0.01, geneIDs(exset), any))

FALSE  TRUE
   20     1
```

DEXSeq Plots

Sample plot showing fitted expression of exons

```
> plotDEXSeq(exset, "Parent=AT1G01100", displayTranscripts=TRUE, expression=TRUE, legend=TRUE)
> ## Generate many plots and write them to results directory
> mygeneIDs <- unique(as.character(na.omit(deuDF[deuDF$geneID %in% unique(deuDF$geneID),])[,"geneID"]))
> DEXSeqHTML(exset, geneIDs=mygeneIDs, path="results", file="DEU.html")
```



Session Information

```
> sessionInfo()

R version 3.0.2 (2013-09-25)
Platform: x86_64-unknown-linux-gnu (64-bit)
locale:
[1] C

attached base packages:
[1] parallel  stats     graphics  utils     datasets  grDevices methods   base

other attached packages:
 [1] DEXSeq_1.8.0           ggbio_1.10.7           ggplot2_0.9.3.1        xtable_1.7-1           ath11215
[10] GO.db_2.10.1           RSQLite_0.11.4         DBI_0.2-7              Matrix_1.1-0           gplots_2.
[19] ape_3.0-11             GenomicFeatures_1.14.0 AnnotationDbi_1.24.0   Biobase_2.22.0         rtrackla
[28] QuasR_1.2.2            Rbowtie_1.2.0          GenomicRanges_1.14.2   XVector_0.2.0          IRanges_

loaded via a namespace (and not attached):
 [1] AnnotationForge_1.4.2   BSgenome_1.30.0         BiocInstaller_1.12.0    GSEABase_1.24.0         Hmis
[10] RCurl_1.95-4.1          VariantAnnotation_1.8.5 XML_3.98-1.1            annotate_1.40.0         bioma
[19] colorspace_1.2-4        dichromat_2.0-0         digest_0.6.3            gdata_2.13.2            gene
[28] gtools_3.1.1            hwriter_1.3             labeling_0.2            latticeExtra_0.6-26     muns
[37] rpart_4.1-3             scales_0.2.3            splines_3.0.2           statmod_1.4.18          stat
```

Outline

References I

Anders, S., Huber, W., 2010. Differential expression analysis for sequence count data. Genome Biol 11 (10).
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URL http://www.hubmed.org/display.cgi?uids=23222703