University of Zurich[UZH]

URPP Evolution in Action

Next-Generation Sequencing 2 – Advanced Course

RNAseq

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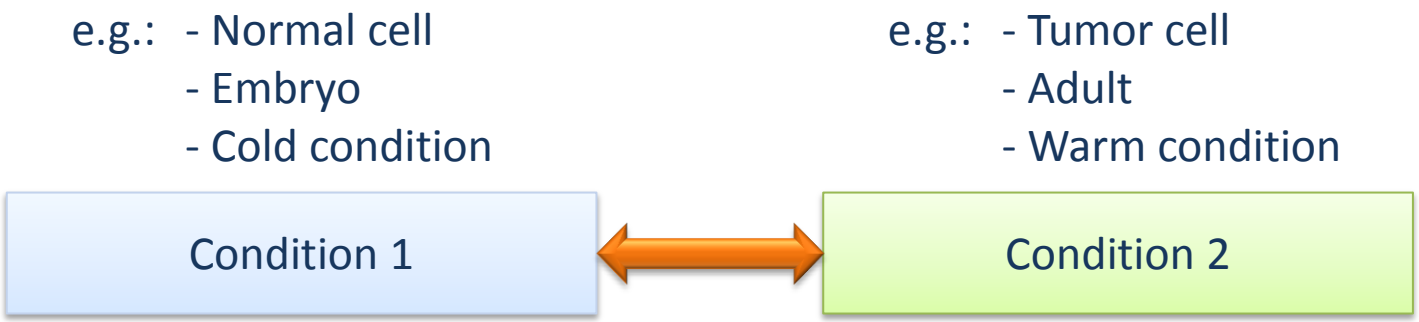
12 May, 2015

Why do organisms look like they look?

Why do cell types in an organism differ from each other, although they have the same genome?

Introduction

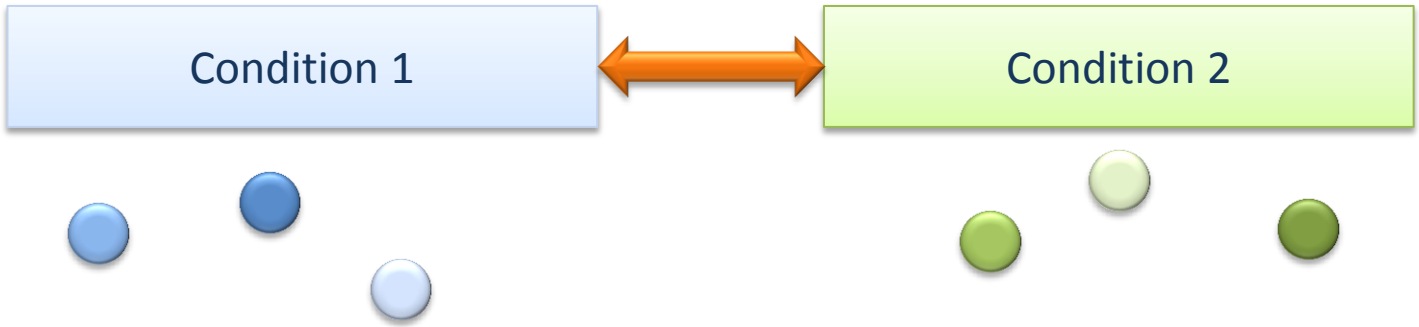
- Gene expression
  - cause phenotypic variations (e.g.: between sexes, along development)
  - allows to respond to spatial and temporal changes in environment
  - Some mutations have no effect on protein sequences, but on gene expression
  - essential for understanding the evolution of organisms



- What genes are turned on or off between these conditions?
- What about whole gene pathways?

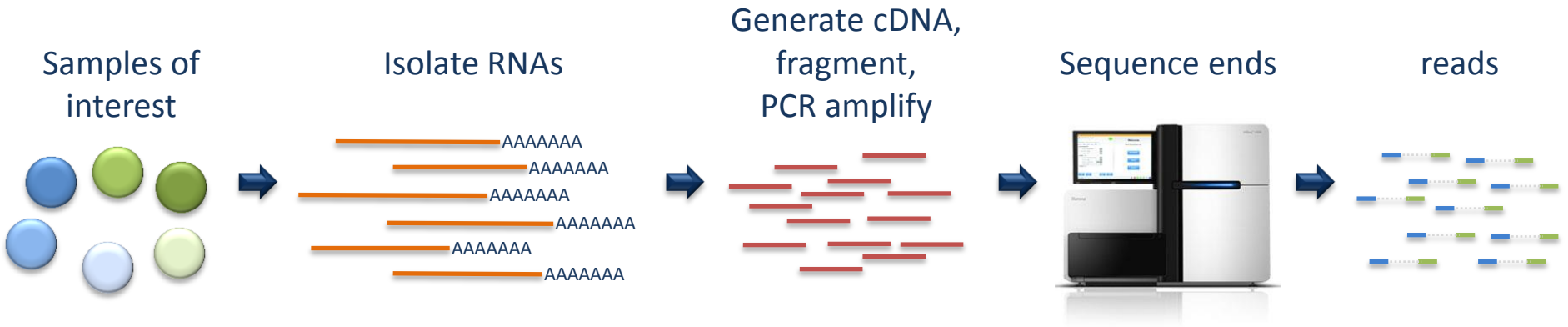
→ Change of expression of one gene may has effect on the expression of many genes

Design



- Multiple biological replicate per treatment group
  - Increase confidence that differentially expressed genes are due to treatment and not biological variance
  - does not account for technical variance
  - Biological variance > technical variance
    - →biological replicates are more useful than technical replicates
    - →attempt at least three replicates per condition

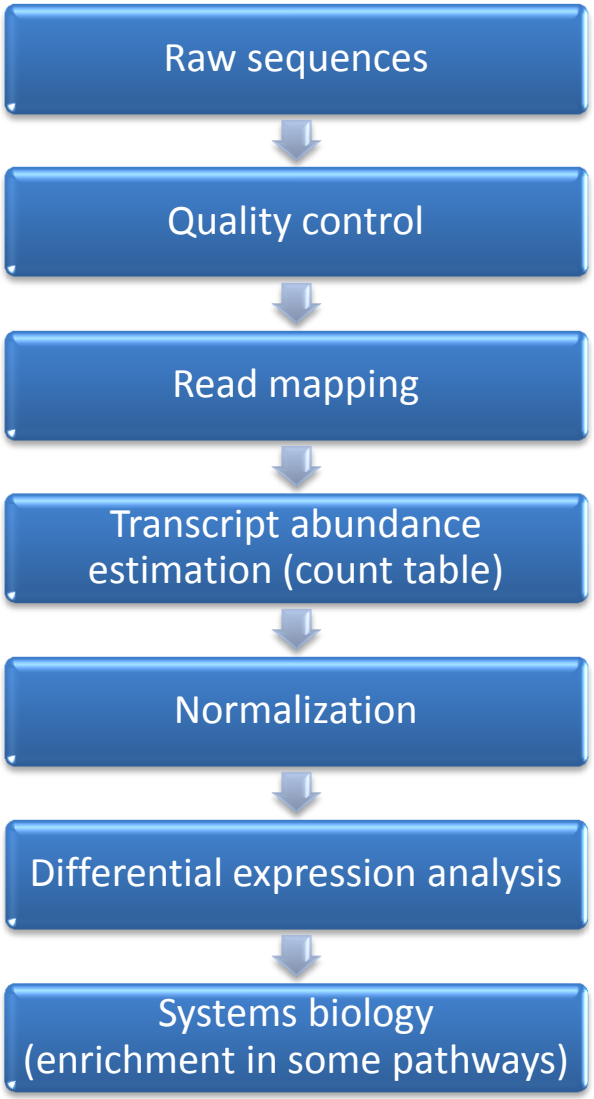
RNA-seq



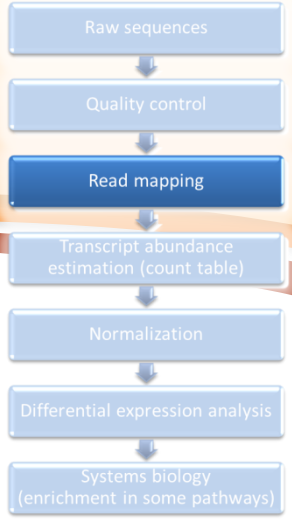
- RNA-Seq: next-generation sequencing of cDNA libraries
  - Measure gene expression in all transcripts (Microarrays: limited to array design)
  - Find new transcribed regions/genes
  - Detect low abundance transcripts
  - Study alternative splicing and allele specific expression

→ Possible for non-model organisms

RNAseq pipeline



Read mapping



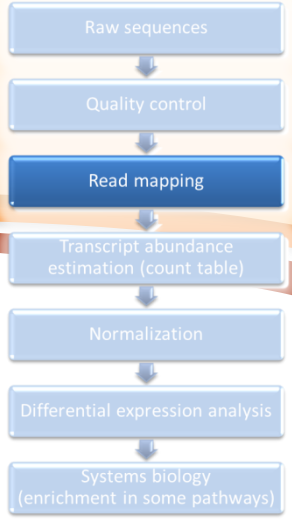
- Map against transcriptome (cDNA)

  - Use standard reference mapping tools (e.g.: BWA, Bowtie2)
  - Transcript level expression
  - Problem: shared exons → reads map to several positions
    - Gene level expression: map them randomly
    - Transcript level expression: map them proportional

- Map against genome

  - Need splice junction reference mapping tools (e.g.: TopHat, rna-star)
  - Problem: transcript level expression more difficult to estimate

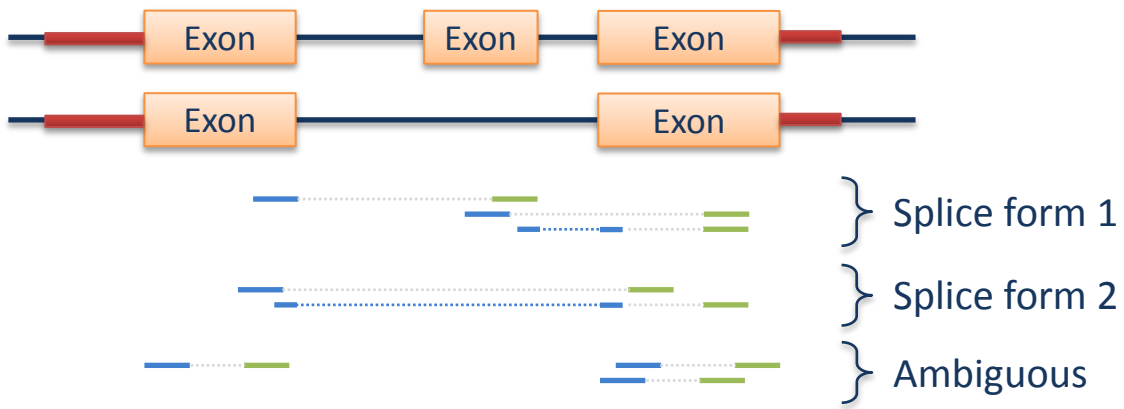
TopHat



- Automatically detects splice junctions
- Can provide annotation file (GFF/GTF)
  - Map reads first against transcriptome
  - Unassembled reads are then mapped against whole reference genome
- Requires Bowtie2
- Outputs BAM files

http://tophat.cbcb.umd.edu/manual.shtml

Get count data

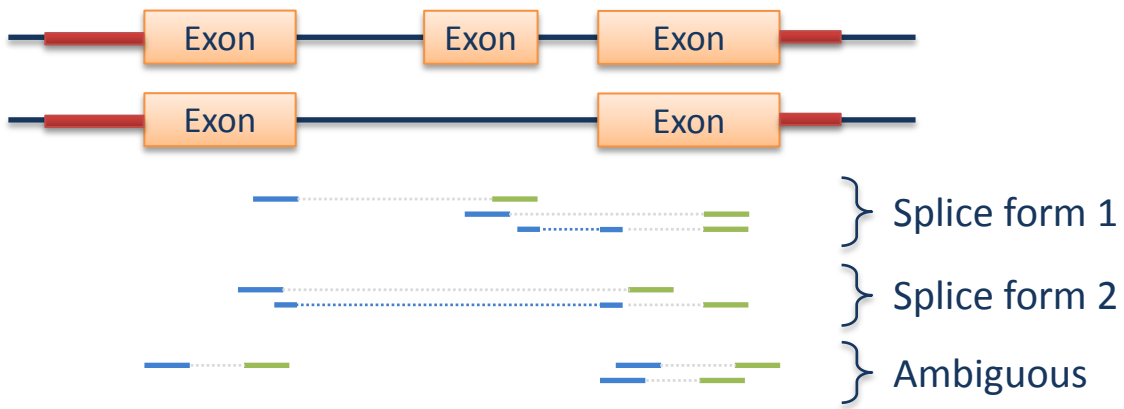


- Gene level (ignore splice variants):
  - Number of reads per gene (sum up reads from different splice forms)
  - Simple
  - Powerful
  - Inaccurate in some cases

→ BEDtools (multicov)
→ HTSeq (htseq-count)

} SAM/BAM, GFF

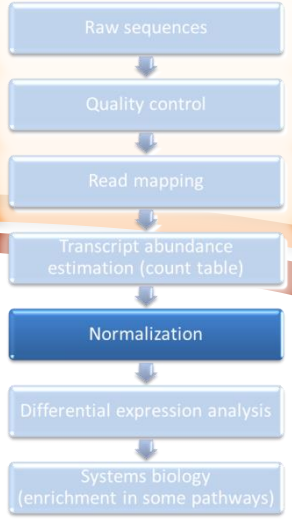
Get count data



- Transcript level:
  - Get number of reads per splice form
  - Cleaner
  - More powerful signal
  - Some degree of uncertainty: Ambiguous reads are assigned proportional to unique ones (maximum likelihood approach)

→ Cufflinks } BAM, GFF/GTF

Normalization

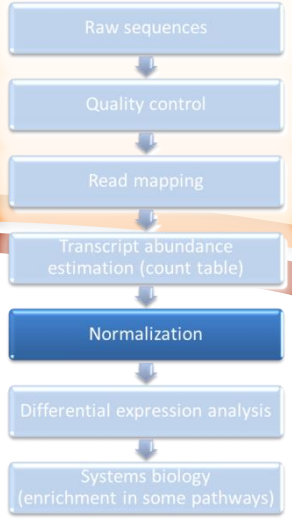


- Why do we need to normalize the count data?
  - Suppose cDNA from treatment 1 was sequenced deeper as cDNA from treatment 2
    → sequenced on different lanes
    → differences in DNA concentration
  - Everything in treatment 1 will appear as up regulated

- RPM (reads per million reads)
  - Correct for differences in coverage
  - Allows comparisons between treatments/samples

- RPKM (reads per kilobase per million reads) / FPKM (paired-end)
  - Correct for differences in coverage
  - Correct for gene length
  - Allows comparisons between treatments/samples and genes

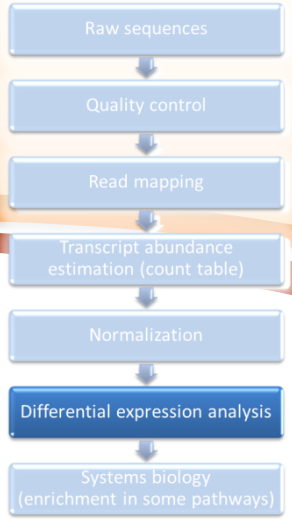
Normalization



- Problems of RPM/RPKM/FPKM
  - Small changes in highly expressed genes
    - → cause global shifts in all values
    - → as highly expressed genes consume substantial proportion of total number of reads
- EdgeR:
  - Estimates a scaling factor
  - Uses a trimmed mean of M-values (TMM) (Robinson and Oshlack, 2010)
  - Highly expressed genes have not a large influence on scaling factor
- DESeq:
  - Calculates a size factors for each sample
  - For each gene: counts of the samples are divided by the geometric means over all samples
  - Size factor: median of all gene ratios

→ Do not correct for gene length

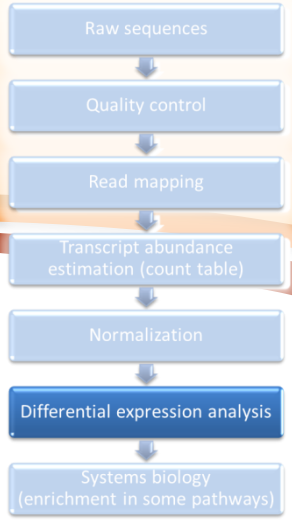
Differential expression analysis



- Test if the expression strength of a gene between two treatments is larger as compared to the variation within each treatment

- Estimate gene variance
  - Assume variance is similar for similarly expressed transcripts
  - Model variance as a function of expression
  - Use model to estimate variance for a transcript given its mean count

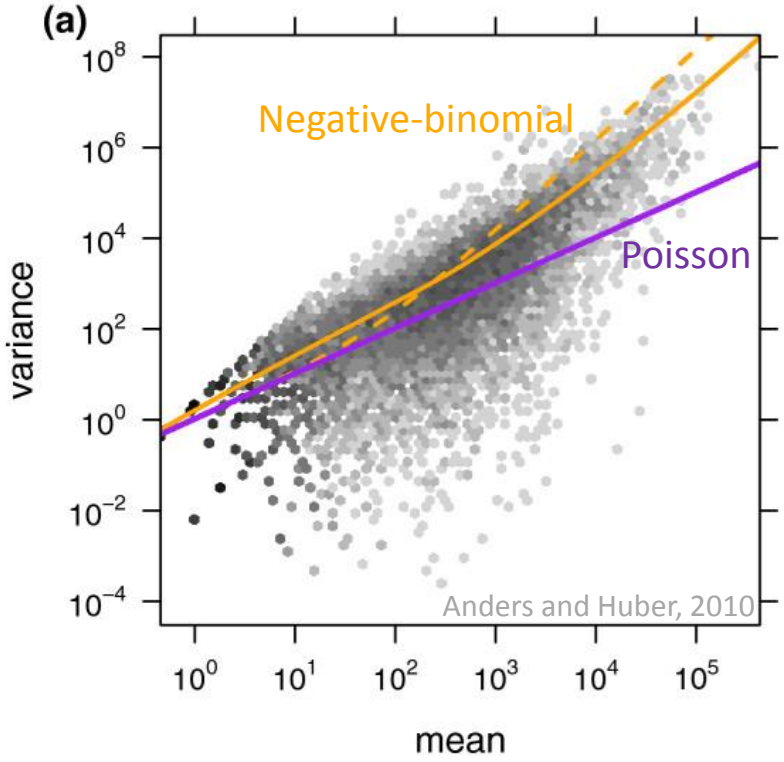
Differential expression analysis



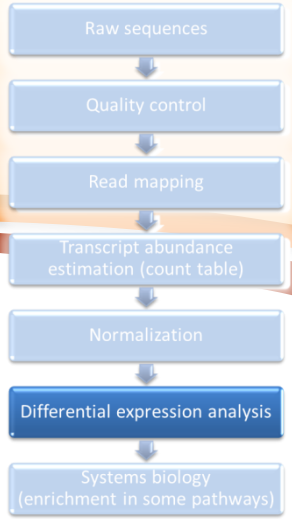
- Microarray data follow Poisson distribution
- RNAseq
  - Genes with high mean counts (longer or highly expressed) tend to show more variance
  - → Fit negative-binomial distribution better

Bioconductor packages (R) estimate means and variances of read counts under a

- Poisson distribution:
  - DEGSeq (Wang, Wang, 2009)
- negative-binomial distribution:
  - DESeq2 (Love, Anders, Huber, 2014)
  - edgeR (Robinson, Mcarthy, Smyth, 2010)
  - BaySeq (Hardcastle, 2012)



Differential expression analysis



- **Model:**
  - The count for a given gene in sample j come from negative binomial distributions with the mean $s_j \mu_\rho$ and variance $s_j \mu_\rho + s_j^2 \, v(\mu_\rho)$

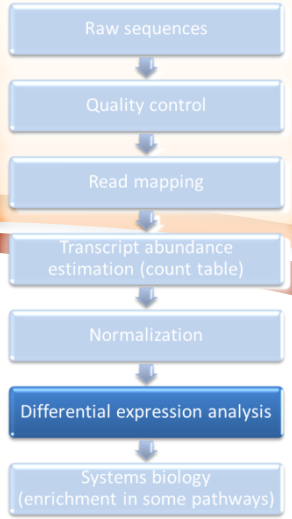
Relative size of library j — Mean value for condition ρ — fitted variance for mean μ_ρ

- **Null hypothesis:**
  - The experimental condition r has no influence on the expression of the gene under consideration
    → all samples have the same: μ_j

- **Alternative hypothesis:**
  - Mean is the same only within groups:
    $log\ \mu_j = \beta_0 + x_j \beta_T$

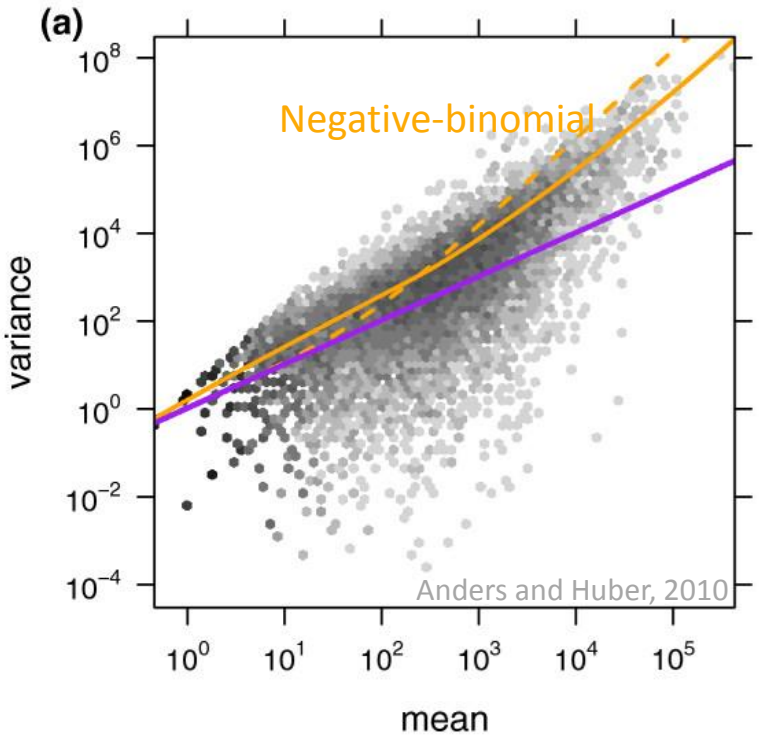
$x_j = 0$ if j is condition 1 sample
$x_j = 1$ if j is condition 2 sample

Differential expression analysis

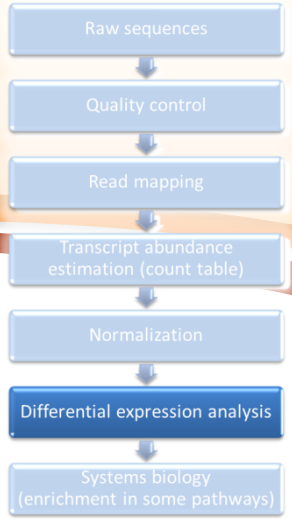


- Model fitting
  - Estimate the variance from replicates
  - Fit a negative-binomial line to get the variance-mean dependence

- Test for differential expression
  - Use a generalized linear model
    $log\ \mu_j = \beta_0 + x_j\ \beta_T$
  - Calculate the coefficients β that fit best the observed data
    → is the value for β_T significant different from null?
    → reject null hypothesis



Multiple testing



- Multiple testing
  - We test for differential expression simultaneously for n number of genes
  - Suppose we have 10,000 genes, just by chance we expect that 10,000*0.05 = 500 genes have a p-value < 0.05

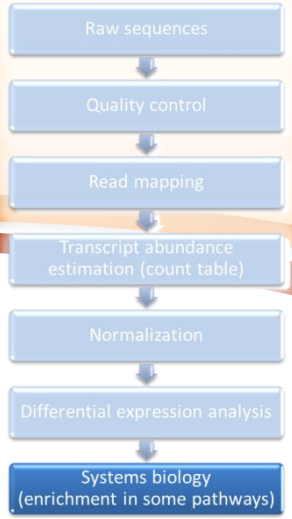
  → p-values for each gene no longer correspond to significant findings

- Bonferroni Correction:
  - p-value' = p-value/n
  - Problem: very conservative.

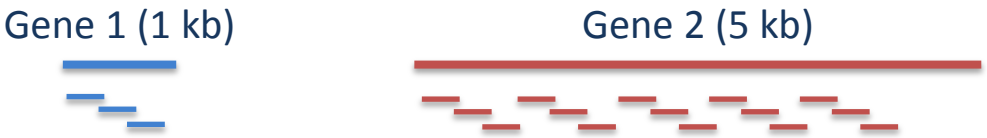
- False Discovery Rate (FDR) (Benjamini and Hochberg, 1995)
  - order p-values in increasing order and assign a rank (smallest: rank 1, second smallest: rank 2…)
  - FDR = p-value*n/rank
  - expected proportion of Type I errors among the rejected hypotheses

    →If we find 40 genes significant differential expressed at a 5% FDR, we expect 2 false discoveries

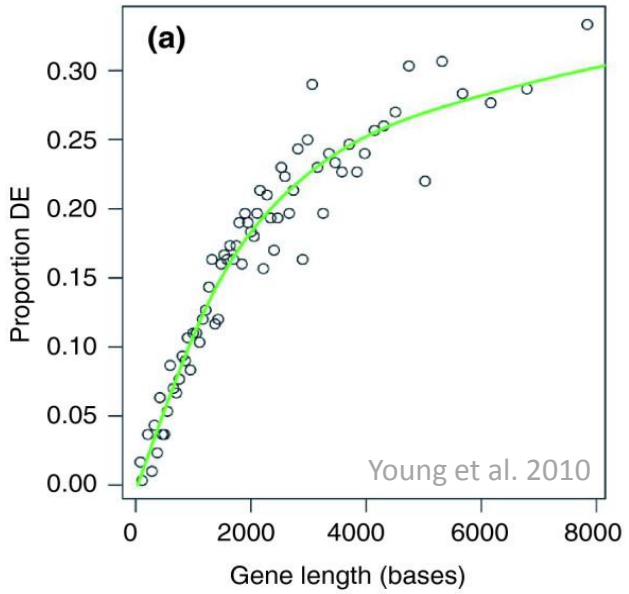
GO enrichment analysis



- Gene Ontology categories are tested for over representation amongst differentially expressed genes



- Problem: length bias
  - genes with same expression level → longer genes will have more reads
  - More information for longer transcripts
  - Longer genes have higher power to detect differential expression



- GOSeq (Young et al. 2010)
  - Correct length bias (probability weighting function) in null distribution
  - Random samples of genes are created by selecting a subset of genes from the experiment → null distribution