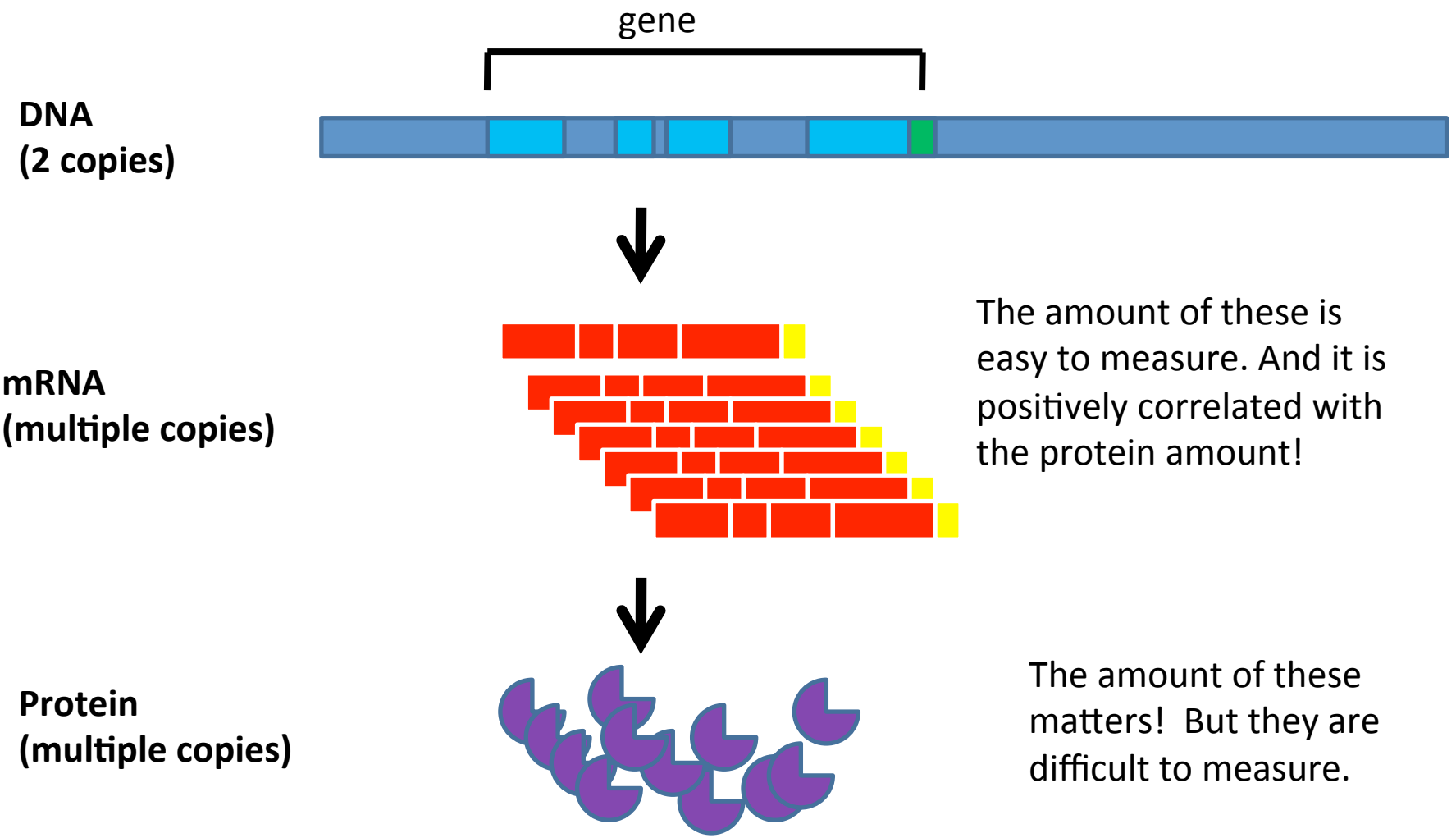
Introduction to RNA-seq data analyses

Outline

- Biological motivations and experimental procedures.
- Analyses of RNA-seq data: methods and useful software tools and Bioconductor packages.
  - Data summarization and normalization.
  - Differential expression.
  - Other issues: alternative splicing and isoform expression.

Review: Gene expression levels are measured through their mRNA abundance.

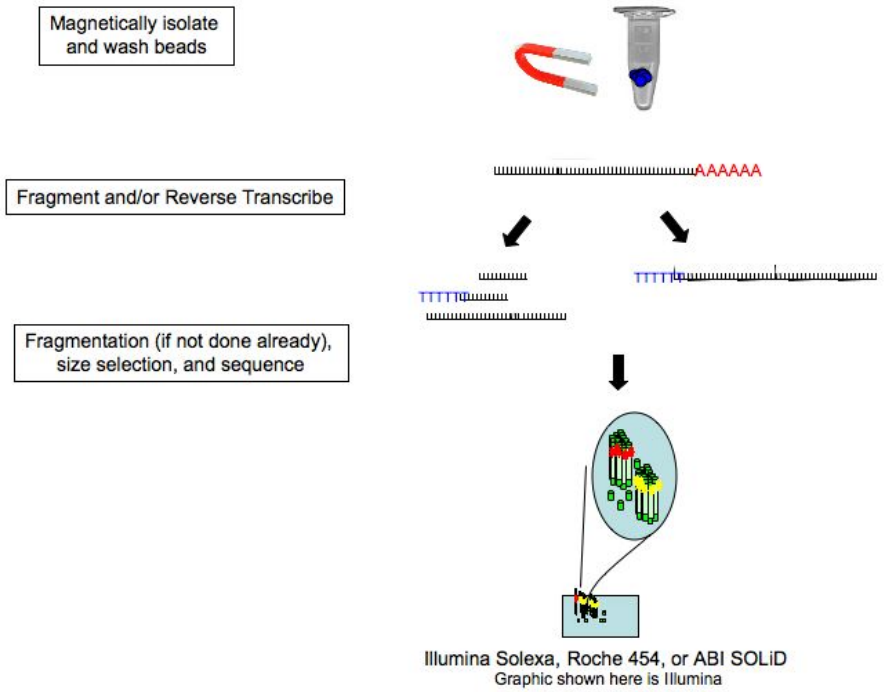


Measuring mRNA abundance

- Using microarray:
  - Probes are designed to target genes.
  - mRNA are converted to cDNA, labeled by dyes, hybridized to microarray (cDNA are attracted to probes with complementary sequences).
  - High gene expression -> more cDNA -> corresponding probes have higher intensities.
- Using RNA-seq:
  - Sequence the cDNA, then align all reads.
  - High gene expression -> more cDNA -> more reads aligned to the genes.
  - Different from microarrays: hybridization is replaced by sequencing.

RNA-seq experiment

1. Extract RNA from samples.
2. Generate cDNA.
3. Fragmentation (cut cDNA into small pieces), then select the fragments with certain lengths.
4. Sequence the fragmented cDNA.

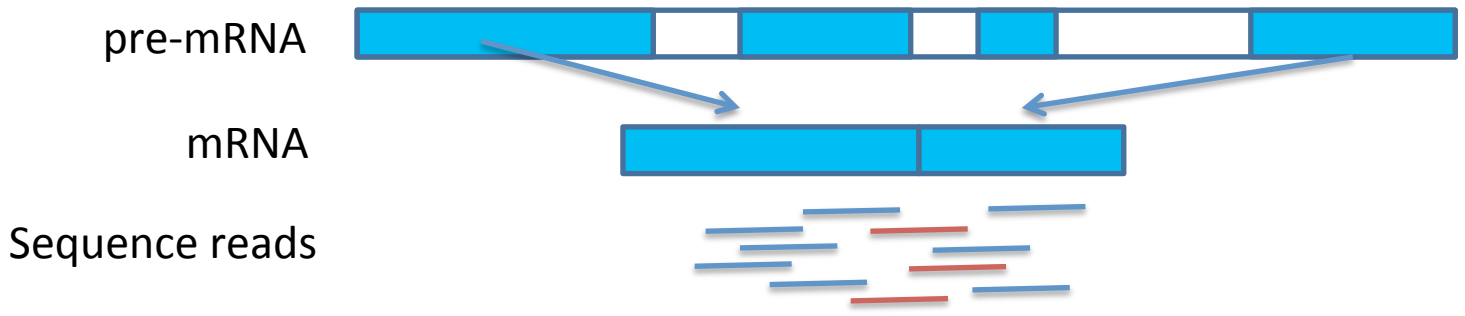


Beyond gene expressions

- RNA-seq provide much more information than gene expression microarrays. In additional to gene expressions, it provides information for:
  - alternative splicing and splicing efficiency.
  - structural changes of genes: gene fusion.
  - new genes/exons.

Alternative splicing

- Definition: the same pre-mRNA produces different mRNA products, through joining different exons.
  - Locations where two exons join is called "junction".
  - Can be detected and quantified using exon arrays, which probes are designed to target the junction regions.
  - From RNA-seq: look at "junction reads", which are reads overlap two exons.



Structural modification of transcriptome

- Example: gene fusion:
  - Two or more separate genes are “fused” together to form a new gene.
  - Often associated with cancer.
- Cannot be detected from microarray.
- Reads from “paired-end” RNA-seq provide information on it.
  - If a pair of reads are very far apart on the reference genome, it suggests gene fusion. Because the DNA segments are selected based on the sizes, and there shouldn’t be long cDNA segments.

Other information from RNA-seq

- Finding new genes/exons:
  - Reads aligned to genomes with no gene/exon annotation are possibly from new gene/exon.
- mRNA processing efficiency:
  - The percentage of reads mapped to introns represents the efficiency of mRNA processing (like splicing).
- RDD: RNA-DNA difference (Li *et al.* 2011, *Science*):
  - The transcription process (DNA -> RNA) is not perfect, based on different SNPs detected from DNA and RNA-seq data. This is very controversial!!

RNA-seq data analyses

- RNA-seq data: sequence reads.
- First step: alignment to the reference genome.
- Information for different tasks:
  - expression: read counts in genes/exons.
  - alternative splicing: junction reads.
  - gene fusion: distances between paired reads.
- We will focus on expression analysis in this class.

Gene expression analysis

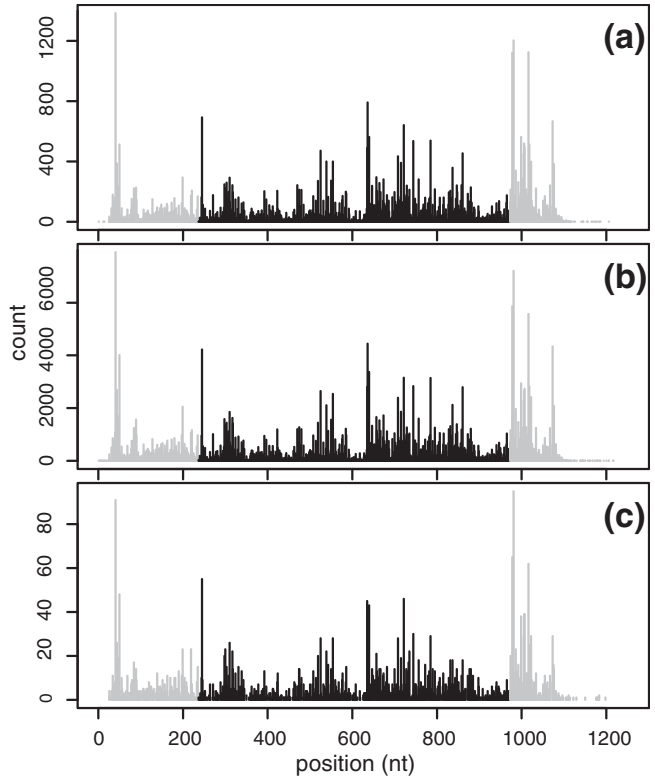
- Biological motivation: compare the expression of genes between different samples.
- Steps:
  - summarization: get a number for each gene to represent its expression level.
  - normalization: remove technical artifacts so that data from different samples are comparable.
  - differential expression detection: gene by gene statistical test.

Summarization of read counts

- From RNA-seq, the alignment result gives the chromosome/ position of each aligned read.
- For a gene, there are reads aligned to the gene body. How to summarized them into a number for the expression?
- Easiest: simply count the number, then normalized by gene lengths and total number of reads in the experiment – RPKM (reads per kilo-bp per million reads). Mortazavi *et al.* (2008), *Nature Method*.

Artifacts in the reads distribution

- The reads are NOT uniformly distributed within gene bodies. It affects by many things such as the sequence composition, chromatin structure, etc.



Li et al. (2010) *Genome Biology*

Weighted sum (Hensen et al. 2010 *NAR*)

- Discovered that reads from Illumina has a 7-bp motif at beginning: there are more reads started with certain 7-bp due to technical artifacts (the random priming bias).
- Down-weight the reads started with the motif.

$$w(h) = \frac{\frac{1}{6}\sum_{i=24}^{29} \hat{p}_{hep:i}(h)}{\frac{1}{2}(\hat{p}_{hep:1}(h)+\hat{p}_{hep:2}(h))}$$

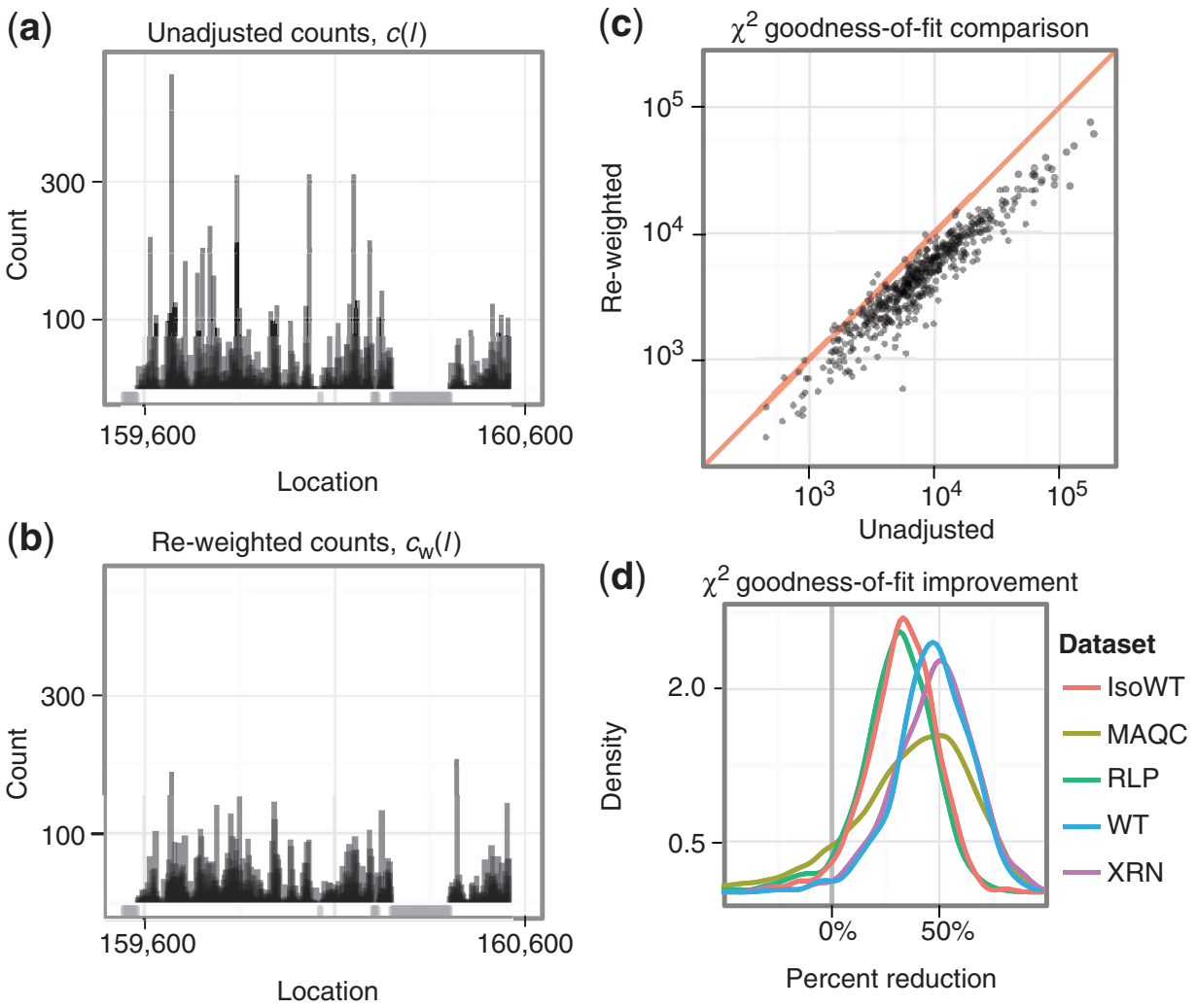
  - *w(h)*: weights for reads starting with heptamer *h*.
  - $\hat{p}_{hep:i}$: observed distribution of heptamers starting at position *i*.

Table 1. Data from a small genomic region in the sense strand of the YOL086C gene in *S. cerevisiae*

Strand	Location l	Heptamer $h(l)$	Count $c(l)$	Weight $w(h(l))$	Reweighted count $c_w(l)$
...					
−1	159792	TTGGTCG	17	1.39	23.6
−1	159793	TTTGGTC	17	0.25	4.3
−1	159794	TTTTGGT	65	0.31	20.4
−1	159795	GTTTTGG	72	0.32	23.3
−1	159796	CGTTTTG	10	1.66	16.6
...					

$c(l)$ denotes the number of mapped reads starting at a particular (stranded) location l and $h(l)$ is the unique heptamer associated with this location. $w(h(l))$ are weights such as in Equation (1) and $c_w(l) = c(l)w(h(l))$ are the location-specific reweighted counts. For this particular small genomic region, reweighting makes the counts more comparable between different locations. Data from the WT experiment.

Results: reweighting increase uniformity of read distribution within gene body



Model the read counts as a function of base compositions (Li et al. 2010 *GB*)

- Log-linear model: for nucleotide j of gene i,
  - n_{ij}: number of reads starting at this position.
  - μ_i: true expression of the gene.
  - ω_{ij}: sequence biases at this position.
  - let $\mu_{ij}=\mu_i*\omega_{ij}$, assume $n_{ij}/\mu_{ij} \sim Poisson(\mu_{ij})$, and

$$\log(\mu_{ij}) = \nu_i + \alpha + \sum_{k=1}^{K} \sum_{h\in\{A,C,G\}} \beta_{kh} I(b_{ijk} = h)$$

- Non-linear model: MART (multiple additive regression trees).

Results

- Results from the (linear or non-linear) model are estimated gene expression.
- Comparing the correlation with microarray data, MART model is slightly better than using sum:

Table 4: Spearman's rank correlation coefficients in mouse embryoid bodies

Fold change bin	SCC by uniform model	SCC by our MART model	Relative improvement
(1.00, 1.09)	0.465	0.466	0.1%
(1.09, 1.19)	0.437	0.444	1.4%
(1.19, 1.33)	0.413	0.434	5.1%
(1.33, 1.53)	0.481	0.520	8.2%
(1.53, 4.82)	0.389	0.490	26.0%

SCC: Spearman's rank correlation coefficient.

More complicated likelihood approach

- Roberts *et al.* (2011) *GB:*
  - Denote the transcript abundance by ρ.
  - Focus on relative expressions: $\sum_{t \in T} \rho_t = 1$
  - Whole data likelihood:

$$L(\rho|F) = \left(\prod_{g \in G} \beta_g^{X_g} \right) \left(\prod_{g \in G} \left(\prod_{f \in F: f \in g} \sum_{t \in g} \gamma_t \cdot D(t,f) \cdot \frac{b(t, e_{5'}(t,f), e_{3'}(t,f))}{B(t, I_t(f))} \right) \right)$$

  - Maximize the likelihood using iterative methods and obtain the estimates of relative expression.

Summary

- Sequence reads are not uniformly distributed within gene body.
- The distribution is highly dependent on sequence compositions.
- Read count summarization is still an open problem:
  - Proposed methods didn't provide convincing performance improvements.

Data normalization

- Data from different samples need to be normalized so that they are comparable.
- Most important – sequencing depth: sample with more total counts will have more counts in each gene on average.
- Easiest method: divided by the total number of counts – RPKM.

Data model for one sample

- The gene read counts from RNA-seq is a sampling process.
- for gene *i*, *i=1, …, G,* let
  - the true expression (number of cDNA fragments) be μ_i.
  - gene length be L_i.
- The probability of a read starting from gene *i* is: $p_i = \mu_i L_i / \sum_{i=1}^{G} \mu_i L_i$
- If the total number of reads is *N*, the count for gene *i*, denoted by Y_i, can be modeled as a Poisson random variable. Let $\lambda_i = Np_i$, $Y_i \mid \lambda_i \sim Poisson(\lambda_i)$
- Downstream DE test between sample 1 and 2 is: $H_0 : \mu_{1i} = \mu_{2i}$ which is NOT equivalent to $H_0 : \lambda_{1i} = \lambda_{2i}$ without proper normalization.

Concerns in RNA-seq data normalization

- When comparing two samples, if the distributions of p_i are approximate the same, normalizing by N will be sufficient – this is what RPKM does.
- However if that's not true we will be in trouble.
  - A toy example: if there are only two genes in the genome, their read counts are 10 and 20 in one sample, and 10 and 100 in another one. We don't know how to compare!
- The normalization procedure is to choose a proper "baseline" for different samples, then normalize data to the baseline so that the counts are comparable.

Single factor normalization methods – One normalization factor per sample

- Total or median counts.
- Bullard *et al. (*2010), *BMC Bioinformatics:*
  - use counts from house keeping genes.
  - use a certain quantile (75^{th}) for all counts.
- Anders et al. (2010), *Genome Biology*:
  - median of the ratios of observed counts.
- Robinson et al. (2010), *Genome Biology*: TMM (trimmed mean of M values).
  1. compute M (log fold changes) and A (log total counts) for all genes.
  2. Discard genes with extreme M and A values, and compute a weighted mean of M's for the rest of genes. The weights as the inverse of the approximate asymptotic variances.
  3. Underlying assumption is that most genes are not DE.

Gene-specific normalization – each gene has a different normalization factor

- Hansen et al. (*Biostatistics*):
  - The gene-specific biases (from GC content, gene length, etc.) need to be considered.

$$Y_{g,i} \mid \mu_{g,i} \sim \text{Poisson}(\mu_{g,i})$$

$$\mu_{g,i} = \exp\left\{ h_i(\theta_{g,i}) + \sum_{j=1}^{p} f_{i,j}(X_{g,j}) \right\}$$

true expression

biases, e.g., GC content

- A conditional quantile normalization (cqn) procedure is designed to estimate *h* and *f*, and then ϑ.

Summary

- RNA-seq normalization is difficult!
- Still an open statistical problem.
- The goal is to find a proper “baseline” to normalize data to.
- Single factor methods provide comparable results.
- Gene-specific normalization is promising, but be careful of over-fitting.

Differential expression (DE) analysis

- Goal: find genes that are expressed differently between (among) conditions.
  - Assign a score for each gene to represent its statistical significance of being different.
  - Rank the genes according to the score.
  - Find a proper threshold for the score for calling DE.
- Microarray methods are not directly applicable: continuous vs. count data, but ideas can be borrowed.

Simple ideas for DE

- Transform data into continuous scale (e.g., by logarithm) then use microarray methods:
  - Troublesome for genes with low counts.
- For each gene, perform two group Poisson or NB test for equal means. Use p-value<0.05 as threshold to call DE. But:
  - Number of replicates are usually small (e.g., 3 vs. 3). Asymptotic theories don't apply so the results (p-values) are not reliable.
  - Use 0.05 as threshold of p-values to call DE - multiple comparison problem: Tests are performed for 20,000 genes. Even if all are null (not DE), 1,000 will have p-value less than 0.05.

Variance shrinkage ideas

- Microarray DE analysis faces the same small sample size problem: limited number of replicates leads to unstable estimates of variances.
  - By chance some genes have very small variance, which will result in large t-statistics and tiny p-values even when the difference is small.
- Solution: "shrinkage estimator" for variances.
  - For a gene, instead of computing sample variances, borrow information from other genes to estimate variances.
  - Examples include SAM and limma.
  - Estimation procedure is often based on Bayesian hierarchical model.
- Similar technique can be used for RNA-seq data, but with caution since the variances and means are dependent.

Data generative model for replicated RNA-seq

- For a sample with M replicates, the counts for gene *i* replicate *j* is often modeled by following hierarchical model: $Y_{ij} \mid \lambda_i \sim Poisson(\lambda_i), \lambda_i \sim Gamma(\alpha, \beta)$
- Marginally, the Gamma-Poisson compound distribution is Negative binomial. So the counts for a gene from multiple replicates is often modeled as Negative binomial: $Y_{ij} \sim NB(\alpha, \beta)$.

A little more about the NB distribution

- NB is over-dispersed Poisson:
  - Poisson: $var = \mu$
  - NB: $var = \mu + \mu^2\phi$
- Dispersion parameter ϕ approximates the squared coefficient of variation: $\phi = \frac{var-\mu}{\mu^2} \approx \frac{var}{\mu^2}$
- Dispersion ϕ represents the biological variance, so shrinkage should be done for ϕ.
- NB distribution can be parameterized by mean and dispersion, but there's no conjugate prior for ϕ.

DEseq (Anders *et al.* 2010, *GB*)

- Counts are assumed to following NB, parameterized by mean and variance: $K_{ij} \sim \mathrm{NB}(\mu_{ij}, \sigma_{ij}^2)$,
- The variance is the sum of shot noise and raw variance:

$$\sigma_{ij}^2 = \underbrace{\mu_{ij}}_{\text{shot noise}} + \underbrace{s_j^2 v_{i,\rho(j)}}_{\text{raw variance}}.$$

- The raw variance is a smooth function of the mean: assumes that genes with same means will have the same variances.
- Hypothesis testing using exact test:

$$p_i = \frac{\sum_{\substack{a+b=k_{i\mathrm{S}} \\ p(a,b) \le p(k_{i\mathrm{A}}, k_{i\mathrm{B}})}} p(a,b)}{\sum_{a+b=k_{i\mathrm{S}}} p(a,b)}.$$

Bioconductor package `DEseq`

- Inputs are:
  - integer matrix for gene counts, rows for genes and columns for samples.
  - experimental design: samples for the columns.

```
library(DESeq)
conds=c(0,0,0,1,1,1)
cds=newCountDataSet(data, conds )
cds=estimateSizeFactors( cds )
cds=estimateVarianceFunctions( cds )
fit=nbinomTest( cds, 0, 1)
pval.DEseq=fit.DEseq$pval
```

edgeR

- From a series of papers by Robinson et al.(the same group developed limma): 2007 *Bioinformatics*, 2008 *Biostatistics*, 2010 *Bioinformatics*.
- Empirical Bayes ideas to "shrink" gene-specific estimations and get better estimates for variances.
- The parameter to shrink is over-dispersion (Φ) in NB, which controls the within group variances.
- There is no conjugate prior so a shrinkage is not straightforward.
- Used a conditional weighted likelihood approach to establish an approximate EB estimator for Φ.

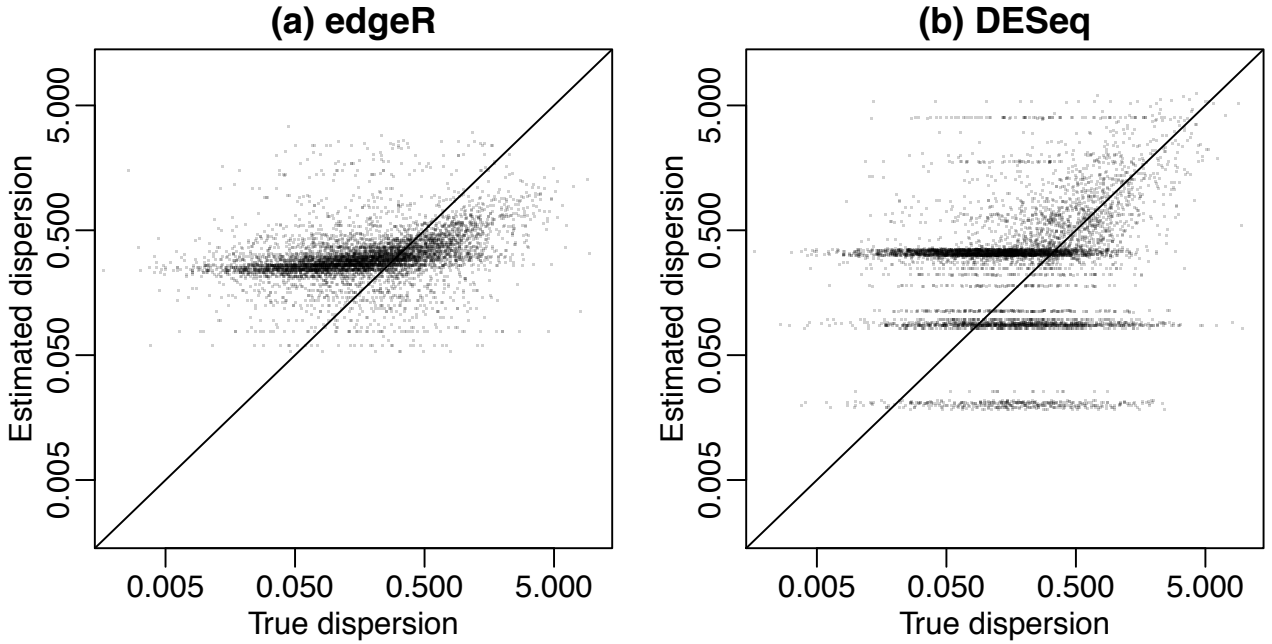
Bioconductor package edgeR

- Inputs are the same as DEseq: an integer matrix for counts and column labels for design.

```
library(edgeR)
d = DGEList(counts=data, group=c(0,0,0,1,1,1),
          lib.size=colSums(data))
d = calcNormFactors(d)
d = estimateCommonDisp(d)
d = estimateTagwiseDisp(d, trend=TRUE)
fit.edgeR = exactTest(d)
pval.edgeR = fit.edgeR$table$p.value
```

DSS (Wu *et al.* 2013, *Biostatistics*)

- Found that the shrinkage from DESeq and edgeR are too strong.



A hierarchical model for the data

$$Y_{gi}|\theta_{gi} \sim \text{Poisson}(\theta_{gi} s_i)$$

$$\theta_{gi}|\phi_g \sim \text{Gamma}(\mu_{g,k(i)}, \phi_g)$$

$$\phi_g \sim \text{log-normal}(m_0, \tau^2)$$

- Y_{gi}: observed counts for gene *g*, sample *i*
- Θ_{gi}: unobserved true expression for gene *g*, sample *i*
- Φ_g: dispersion (related biological variance) for gene *g*.
- s_i: library size for sample *i*.

The posterior

- Negative binomial is parameterized by mean and dispersion, then the posterior for dispersion is:

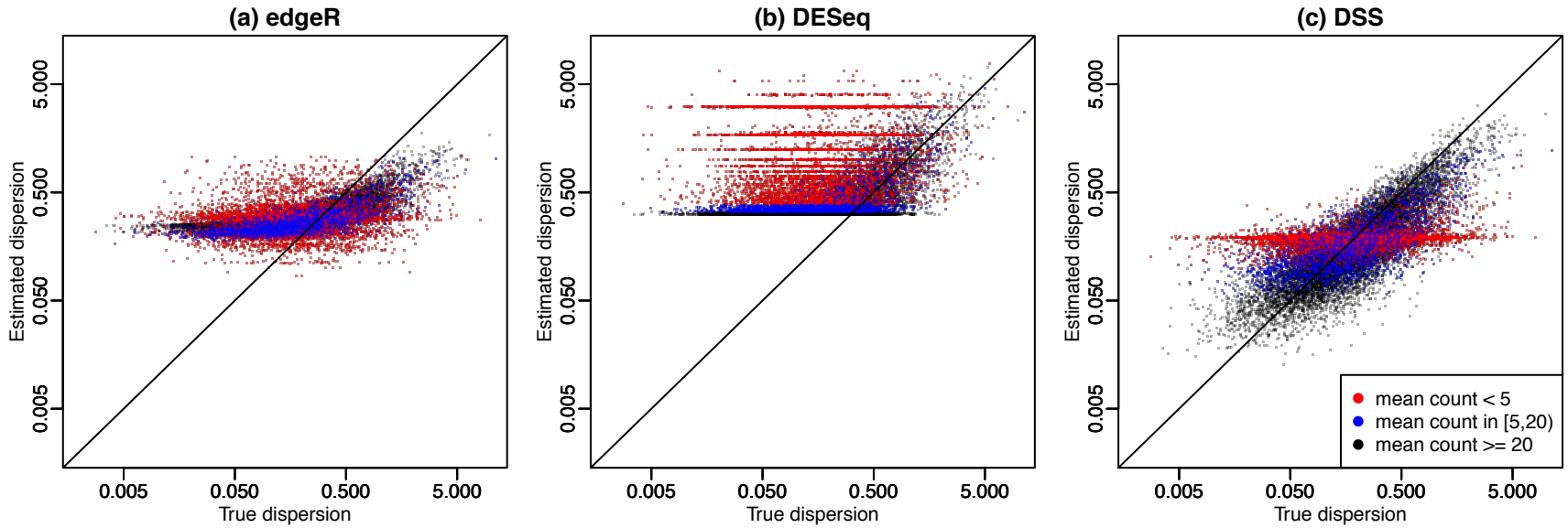
$$\log[p(\phi_g|Y_{gi},\nu_{gi},i=1,\ldots,n)] \propto \sum_i \psi(\phi_g^{-1}+Y_{gi}) - n\psi(\phi_g^{-1}) - \phi_g^{-1}\sum_i \log(1+\nu_{gi}\phi_g)$$
$$+ \sum_i Y_{gi}[\log(\nu_{gi}\phi_g) - \log(1+\nu_{gi}\phi_g)]$$
$$- \frac{[\log(\phi_g)-m_0]^2}{2\tau^2} - \log(\phi_g) - \log(\tau), \qquad (4.1)$$

- Here, $\nu_{gi} = \mu_{g,k(i)} s_i$ is the expected value for Y_{gi}.
- It's a penalized likelihood to penalize (1) dispersions far away from prior mean; and (2) large dispersions.

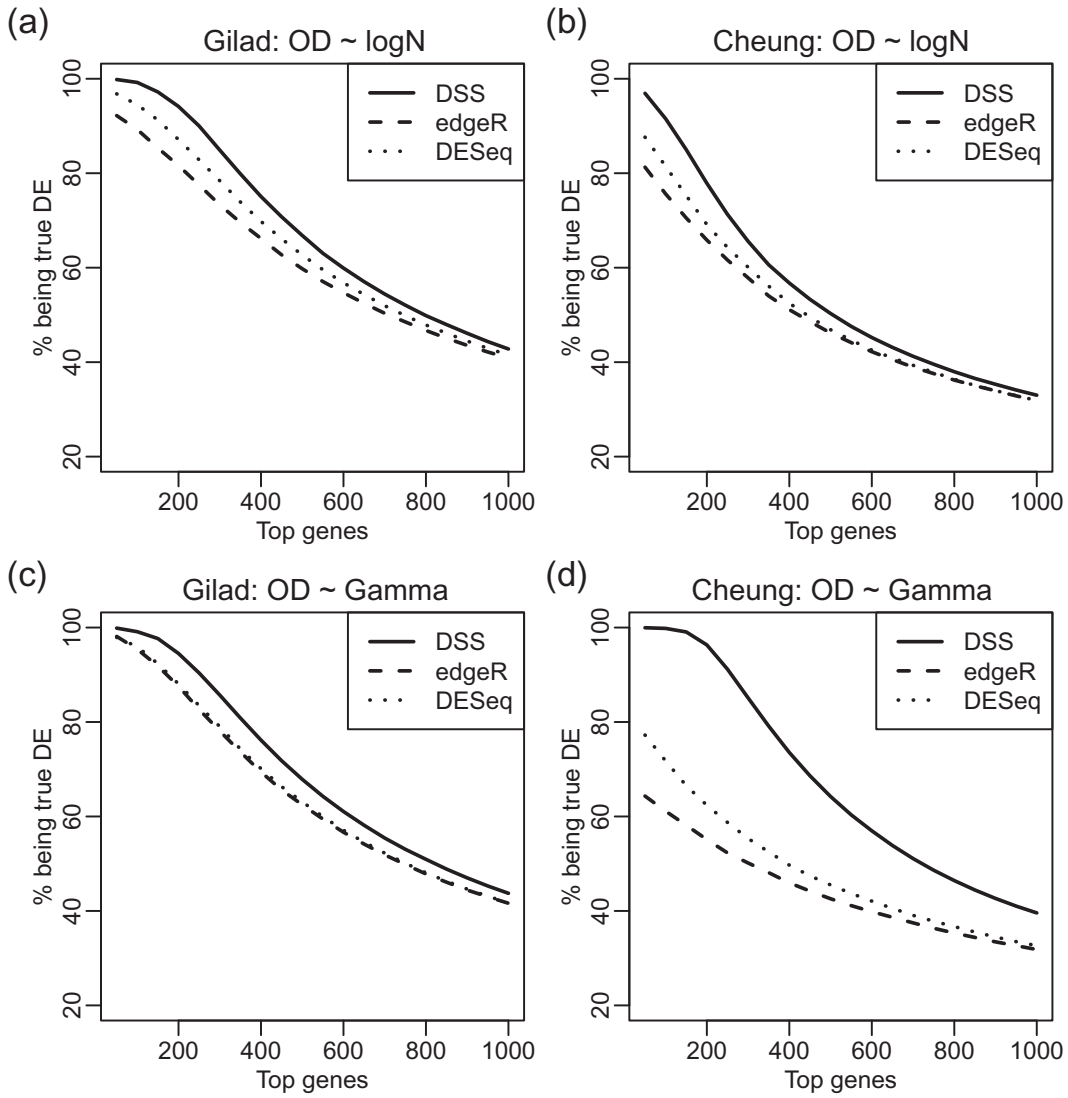
Testing and inference in two-group comparison

- Wald test: $t_g = \frac{\hat{\mu}_{g,1} - \hat{\mu}_{g,2}}{\sqrt{\hat{\sigma}^2_{g,1} + \hat{\sigma}^2_{g,2}}}$
  - With dispersions, variances can be computed according to NB distribution: $var = \mu + \mu^2\phi$
  - The variance for $\hat{\mu}_{g,1}$ is: $\hat{\sigma}^2_{g,1} \equiv \frac{1}{n_1^2}\left[\hat{\mu}_{g,1}\left(\sum_{j:k(j)=1}\frac{1}{s_j}\right) + n_1\hat{\mu}^2_{g,1}\tilde{\phi}_g\right]$
- Inferences: use normal P-values and local FDR.

Results: estimation of dispersions



Simulations on DE detection



DSS Bioconductor package

- Inputs are the same as DEseq and edgeR: an integer matrix for counts and column labels for design.

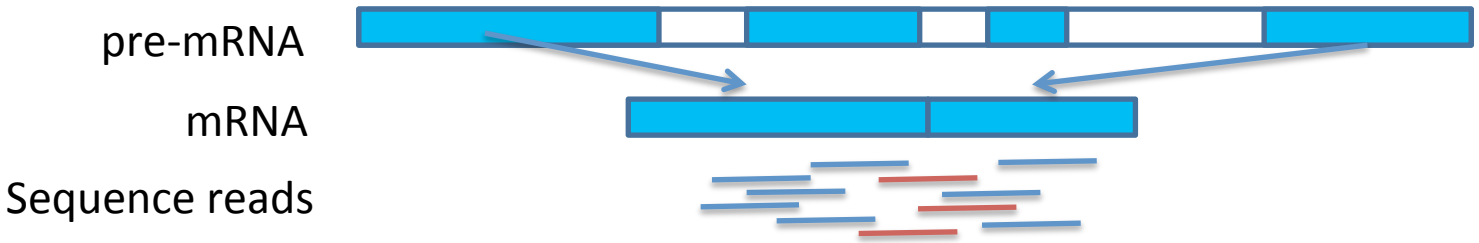
```
conds=c(0,0,0,1,1,1)
seqData=newSeqCountSet(X, conds)
seqData=estNormFactors(seqData)
seqData=estDispersion(seqData)
result=waldTest(seqData, 0, 1)
```

Summary for DE test

- Based on my experiences and simulation results:
  - All methods provide very similar results when the dispersion (biological variance) is small.
  - DSS performs better when dispersion is large.
- The methods we talked about are based on the gene counts. DESeq and edgeR are the most popular software for that.
- There are other methods perform transcript level expression estimation and DE analysis: cufflink and cuffdiff.

Alternative splicing

- RNA-seq can detect alternative splicing patterns by analyzing the junction reads.



- The exon junctions are not in the reference genome so special alignment methods are needed.
- Usually reads are aligned to known junctions.
- There are methods to detect new junctions (*ab initio* splicing detection).

Using tophat

- Based on bowtie, aligns RNA-Seq reads to a genome in order to identify exon-exon splice junctions.
- Runs on Linux and Mac OSX
- Command:

```
tophat -o out_dir bowtid_index input.fastq
```

- Output:
  - accepted_hits.sam: read alignments in SAM format.
  - junctions.bed: junction reads in BED format.

Estimate isoform expressions

- Isoform: different transcripts from the same gene, caused by alternative splicing.
- Different isoforms could have different expression levels.
- A toy example for a gene with 3 exons:
  - It was known the gene has two isoforms: exon1+exon2, and exon1+exon3.
  - The read counts from the exons are 10, 7, 5.
  - What are the expression level for the two isoforms?

Some approaches

- A Poisson model : Jiang et al. (2009) *Bioinformatics*.
  - Underlying Poisson rate is a linear combination of isoform expressions, then derive joint data likelihood.
  - Compute MLE for the isoform expressions by maximizing Joint likelihood through numerical methods.
- Solas: Poisson model with EM algorithm: Richard et al. (2010) NAR.
- Cufflink Trapnell et al. (2010) *NBT*: a product of Bernoulli model with multivariate normal prior, then use Bayesian method to report maximum a posteriori (MAP).

Use cufflink

- Runs on Linux or Mac OSX
- Input is alignment result from tophat.
- Command:

```
cufflinks -o output_dir  accepted_hits.sam
```

Summary for isoform expression

- Mostly for known isoforms (the combination patterns of exons).
- MLE approaches for estimation.
- Number of junction reads are not very well utilized, so there might be some rooms for improvement.

The Tuxedo Suite: bowtie, tophat and cufflink

- Developed by the same group headed by Steven Selzberg (at Hopkins).
- bowtie: alignment
- tophat: alignment to exon junctions
- cufflink: estimate isoform expressions.

Review

- RNA-seq provides information for:
  - expression.
  - Alternative splicing.
  - Structural variation, e.g., gene fusion.
- Statistical problems include:
  - Summarization.
  - Normalization.
  - differential expression testing.
  - isoform expression estimation.
- Some rooms for method developments.