limma, Affymetrix, RMA, Independent filtering

- Journal club signup
- Some project ideas
- Affymetrix arrays + RMA → robust linear regression
- Some of the basics of mapping (mostly practical, theory comes next week)

Project ideas: Consulting/Research

1. **Long-read RNA-seq**: in-house dataset, compare a new protocol (longer fragments sequenced with MiSeq) of RNA-seq data to existing protocols using a qPCR independent truth dataset. Involves running various algorithms, comparing them to truth and to existing datasets.
2. **Male-female differential expression analysis** (RNA-seq): non-model insect for which the *de novo* assembled transcriptome is currently being built. In principle, it would be a standard differential expression analysis.
3. **Splicing changes in cancer** (RNA-seq): dataset from Unispital Zurich (~40 patients). Would augment standard differential expression analyses with recurrent splice changes. Apply 1 or 2 methods.
4. **Myeloid cell classification**. Partial re-analysis of Becher et al. 2014 http://www.ncbi.nlm.nih.gov/pubmed/25306126 (e.g. Figure 5)

Project ideas: some papers used in past years

1. http://www.ncbi.nlm.nih.gov/pubmed/23029029 -- take the raw reads, map to genome, count reads by gene, do a differential expression analysis between cell types
2. http://www.ncbi.nlm.nih.gov/pubmed/22988256 -- recreate some of the analyses (some real datasets, some simulations) that compared normalization methods
3. http://www.ncbi.nlm.nih.gov/pubmed/22965124 -- recreate some of the analyses that compared RNA-seq to microarrays
4. http://www.ncbi.nlm.nih.gov/pubmed/21824971 -- using some independent simulated RNA-seq, add a new method to an existing comparison

Affymetrix probe design

Early platforms (11 or 20 probes in a set), 25bp probes, 3' biased

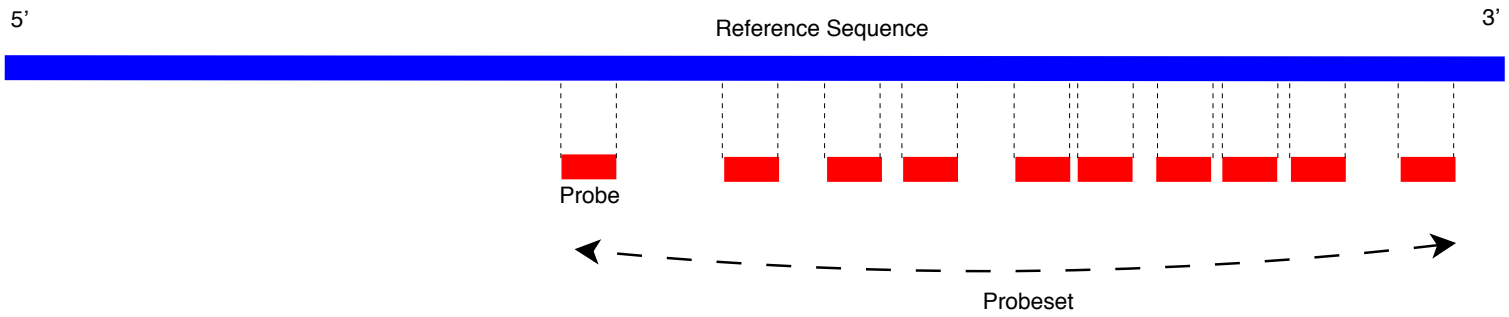


Figure 1.1: Multiple probes interrogating the sequence for a particular gene make up probesets.

Figure 1.2: Pefect Match and Mismatch Probes.

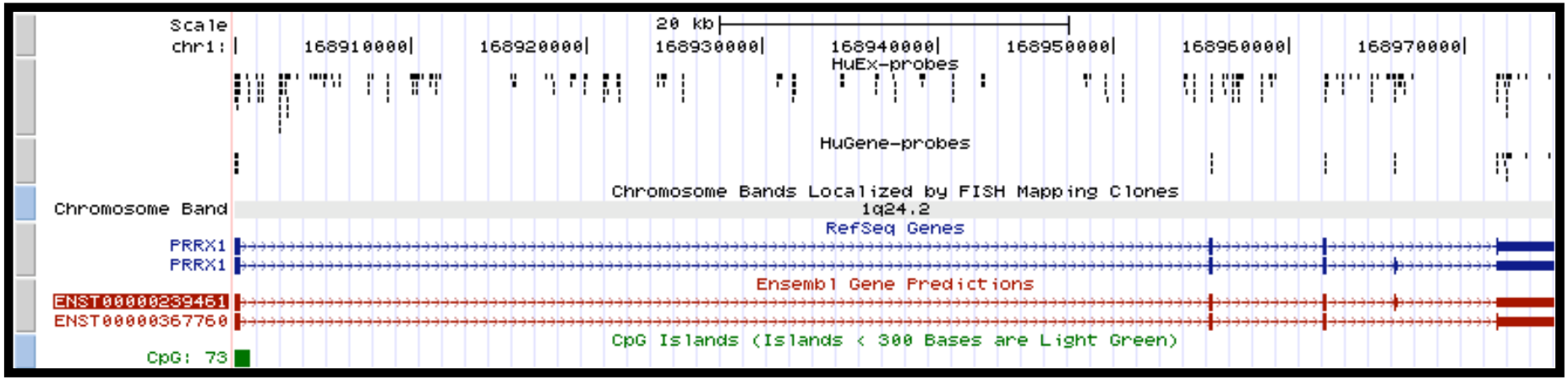
Latest Affymetrix design: “whole transcript” arrays

Still 25 base pair probes, multiple probes per transcript (“probesets”)

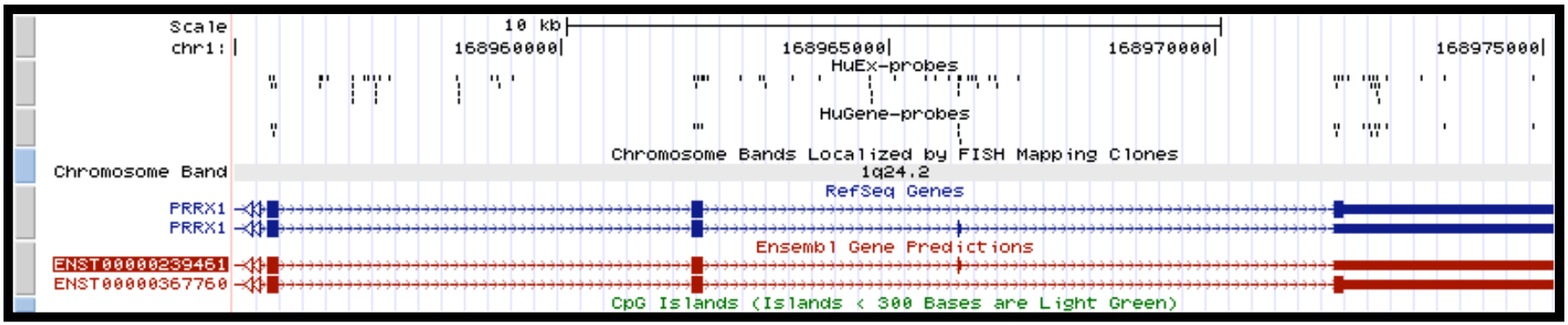
No more mismatch probes.

Reference Sequence

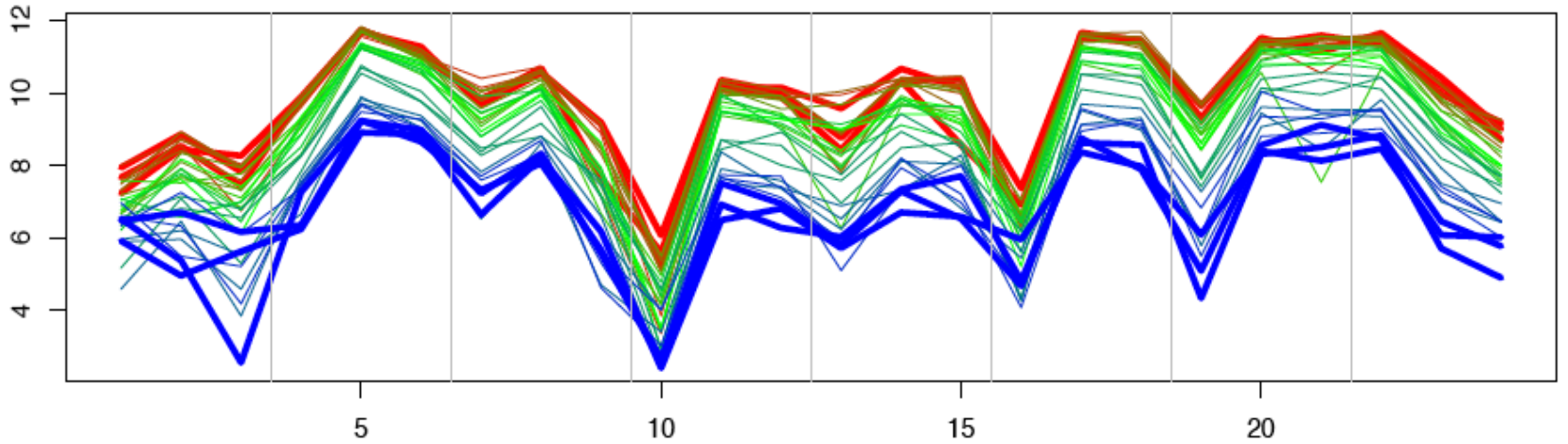
- HuExon: *Human **Exon** 1.0 ST* (~40 probes per gene, 4 probes per “exon”, annotated and predicted transcripts)
- HuGene: *Human **Gene** 1.0 ST* (~25 probes per gene, annotated genes only)
- NEW in 2013: HTA (Human Transcriptome Array): updated content + junction probes



The nature of Affymetrix Probe Level Data



HuGene data [red–heart,blue–brain,mixtures] 10 ENSG00000116132



- Data for one gene that is differentially expressed between heart (red is 100% heart) and brain (blue is 100% brain).
- 11 mixtures x 3 replicates = 33 samples (33 lines)
- Note the parallelism: probes have different **affinities**

"Summarization": Going from probesets to summarized expression level

MAS 4.0

$$AvDiff = \frac{1}{|A|} \sum_{j \in A} (PM_j - MM_j)$$

MAS 5.0

$$CT_j = \begin{cases} MM_j, & \text{if } MM_j < PM_j \\ \text{less than } PM_j, & \text{if } MM_j \geq PM_j \end{cases}$$

$$signal = TukeyBiweight\{log(PM_j - CT_j)\}$$

dChip (MBEI)

$$PM_{ij} - MM_{ij} = \theta_i \cdot \phi_j + \varepsilon_{ij}, \qquad \varepsilon_{ij} \sim N(0, \sigma^2)$$

θ_i expression index
ϕ_j probe-specific affinity
ε_{ij} noise component

RMA, GCRMA

Robust multichip analysis (RMA)

Exploration, normalization, and summaries of high density oligonucleotide array probe level data

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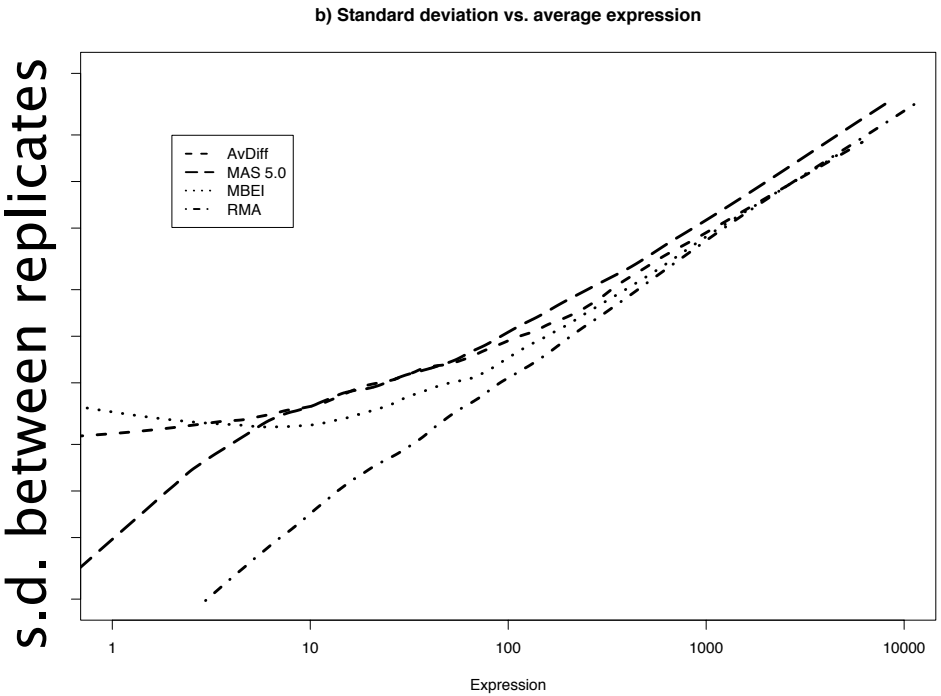
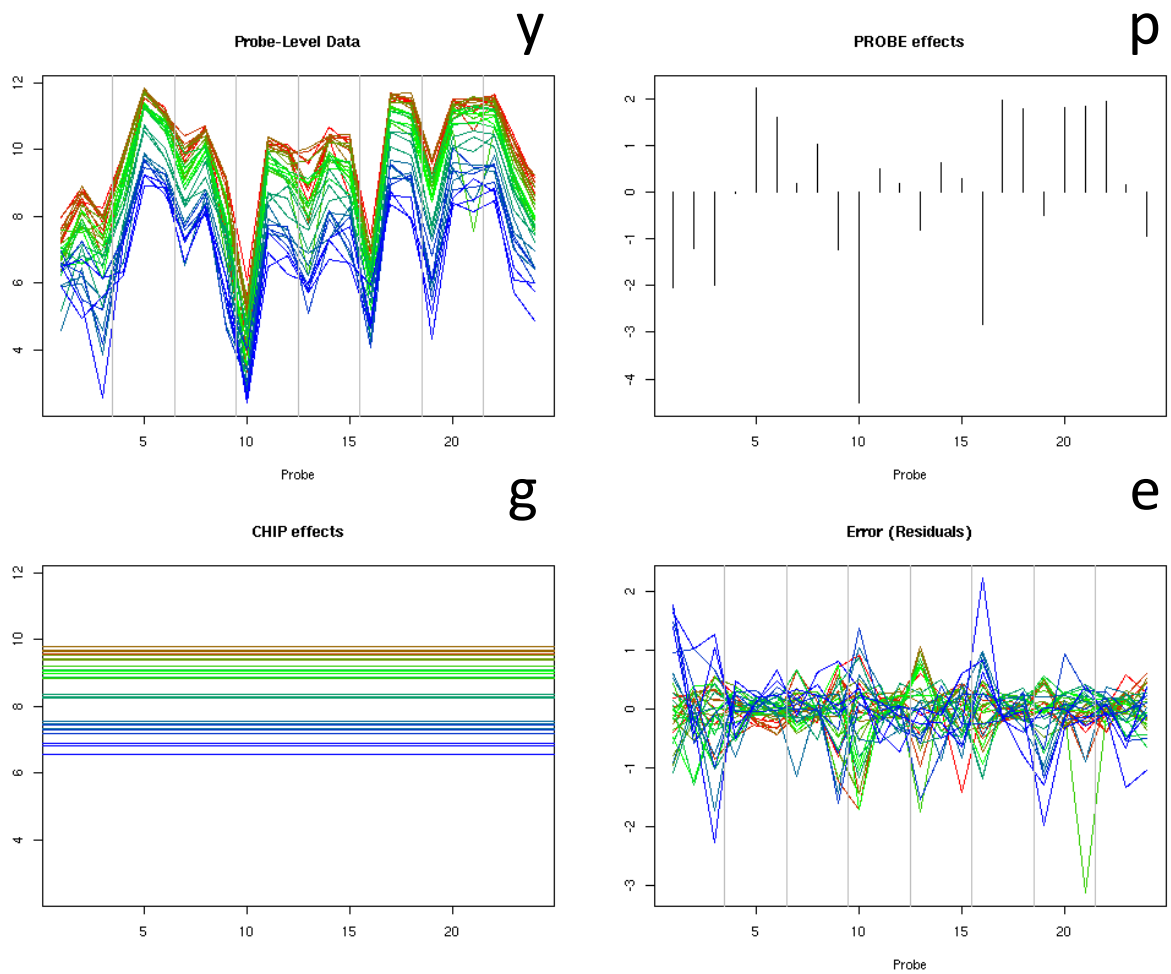
Biostatistics 2003

Encompasses 3 steps

- background correction
- normalization
- probe level model fit ("summarization")



Linear model decomposes the probe-level data into PROBE effects and CHIP effects



Linear model:

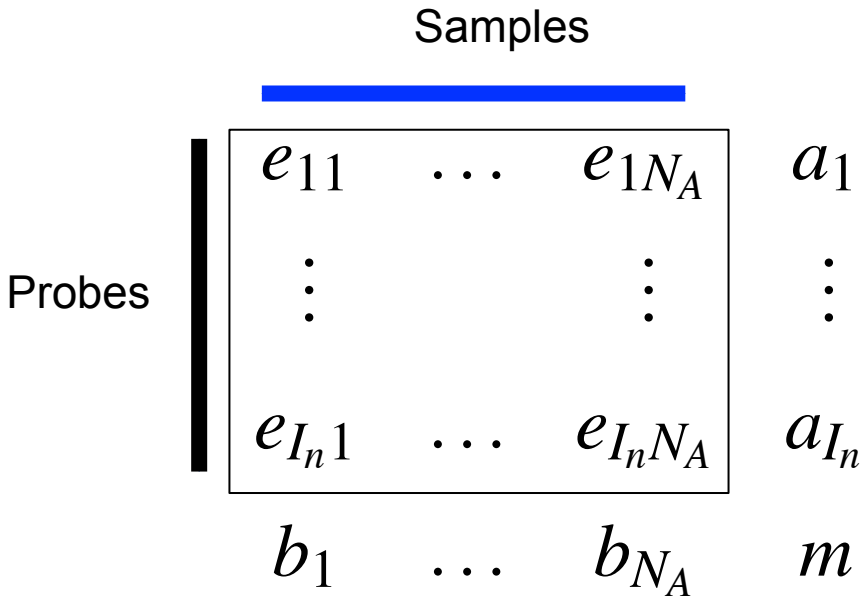
$$y_{ik} = g_i + p_k + e_{ik}$$

Robust Multichip Analysis (RMA) uses this model.
Irizarry et al. 2003, Biostatistics

Parameters are estimated robustly, meaning a small number of outliers have minimal effect

Tissue mixture dataset

Fitting the model – median polish

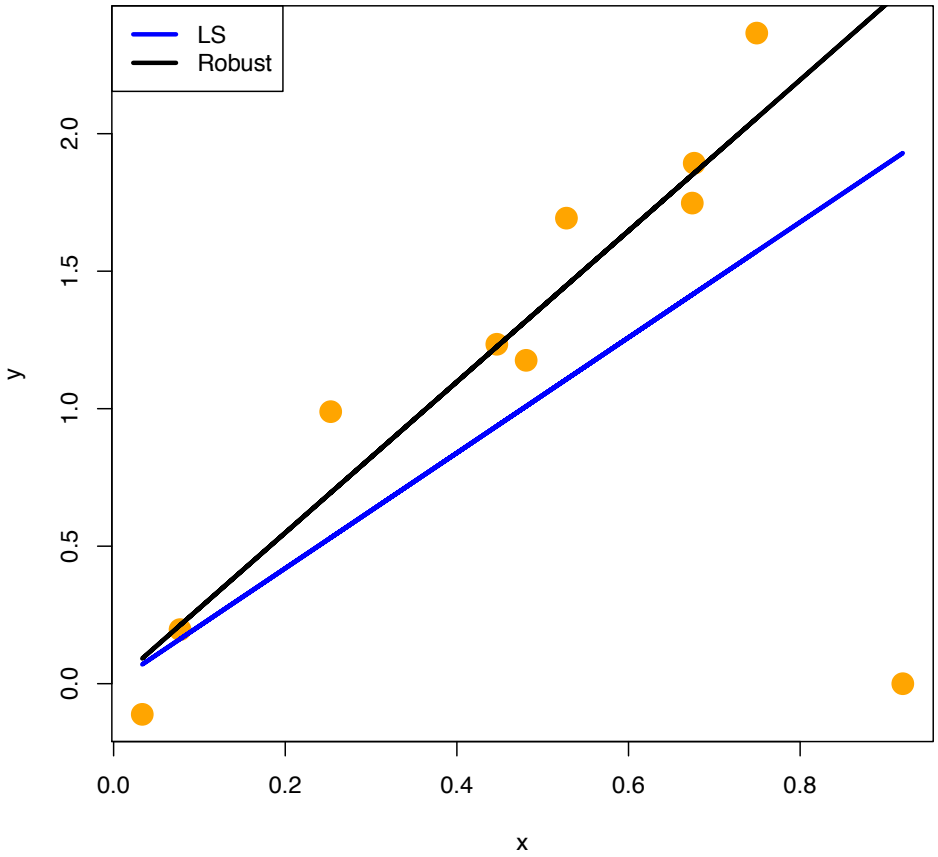



```
pe <- rnorm(11)
ce <- rnorm(8)+8
z <- outer(pe,ce,"+") +
     rnorm(length(pe)*length(ce),sd=.5)
e <- z
m <- a <- b <- 0
niter <- 3

for(i in 1:niter) {
  rm <- rowMedians(e)  # calc row medians
  e <- sweep(e,1,rm)   # subtract row medians
  a <- a + rm          # add row medians to a
  mb <- median(b)
  b <- b-mb
  m <- m+mb

  cm <- colMedians(e)  # calc col medians
  e <- sweep(e,2,cm)   # subtract col medians
  b <- b + cm          # add col medians to b
  ma <- median(a)
  a <- a-ma
  m <- m+ma
}

# a - "probe effects"
# m+b - "chip effects"
```

http://en.wikipedia.org/wiki/Ordinary_least_squares

Robust regression – motivating example

```
library(MASS)

n <- 10
x <- runif(n)
y <- 3*x + rnorm(n,sd=.2)
y[which.max(y)] <- 0  # add in outlier

f <- lm(y~0+x)
fr <- rlm(y~0+x)

plot(x,y,pch=19,col="orange",cex=2)
lines(x,predict(fr),lwd=3)
lines(x,predict(f),lwd=3,col="blue")
legend("topleft",c("LS","Robust"),
       lwd=3,lty=1,col=c("blue","black"))
```



OLS = ordinary least squares

The OLS estimator is … optimal in the class of linear unbiased estimators when the errors are homoscedastic and serially uncorrelated … OLS provides minimum-variance mean-unbiased estimation when the errors have finite variances.

Has good properties, when the data is “nice”.

Replace:

$$\arg\min_\beta \sum_{i=1}^{n} (y_i - f_i(\beta))^2$$

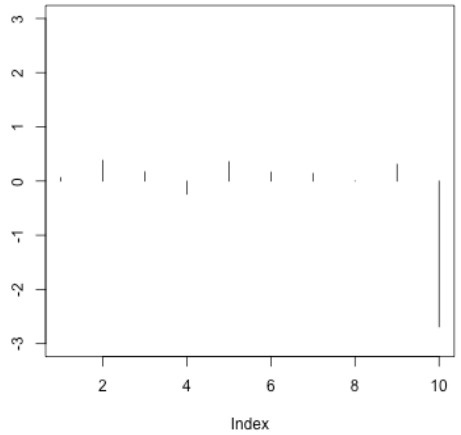
with:

$$\arg\min_\beta \sum_{i=1}^{n} w_i(\beta)(y_i - f_i(\beta))^2$$

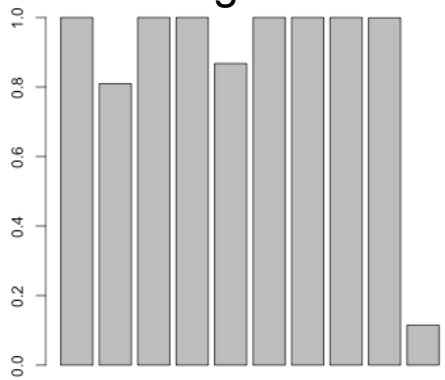
http://en.wikipedia.org/wiki/Iteratively_reweighted_least_squares

Robust regression – mechanics of iteratively reweighted least squares

Residuals



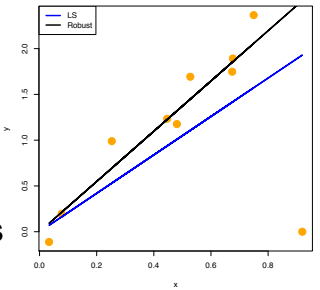
Weights



Sketch of IRLS:

Calculate initial estimates of parameters

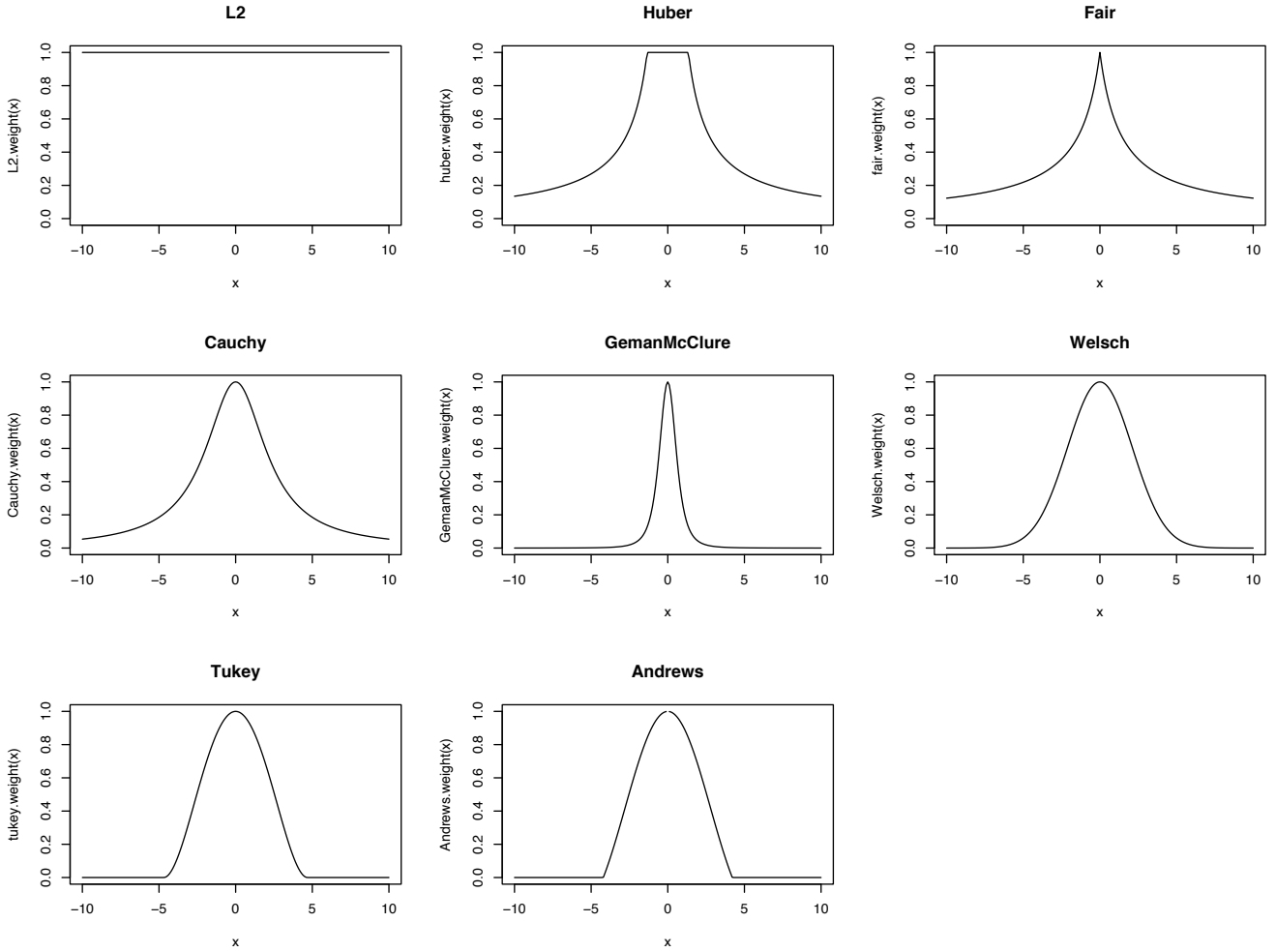
Repeat until very little change:
- Calculate residuals
- Using standardized residuals, weight observations
- Re-estimate parameters




```
# this construction only works for the
# 1-parameter no-intercept linear model
tukey <- function(r,k=1.345) {
  abs(r) < k + k/abs(r)*(abs(r)>k)
}

w <- 1
niter <- 2
b <- sum(w*y*x)/sum(w*x^2)

for(i in 1:niter) {
  r <- y-b*x
  w <- tukey( r/mad(r) )
  b <- sum(w*y*x)/sum(w*x^2)
}


par(mfrow=c(2,1))
plot(r,type="h",ylim=c(-3,3))
barplot(w)
```

← mad = median absolute deviation

More details – weight functions (as function of standardized residuals)



More details – weight functions (of normalized residuals)
Concept: influence / bounded influence

The estimated standard error for our estimators is thus given by

$$\mathrm{SE}\left(\hat{\beta}_j^{(n)}\right) = \frac{1}{\sqrt{I_n}} \sqrt{\frac{\sum_{i=1}^{I_n} \psi\left(\frac{\log_2\left(y_{ij}^{(n)}\right) - \hat{\beta}_j^{(n)}}{s}\right)^2 / I_n}{\left(\sum_{i=1}^{I_n} \psi'\left(\frac{\log_2\left(y_{ij}^{(n)}\right) - \beta_j^{(n)}}{s}\right) / I_n\right)^2}}.$$

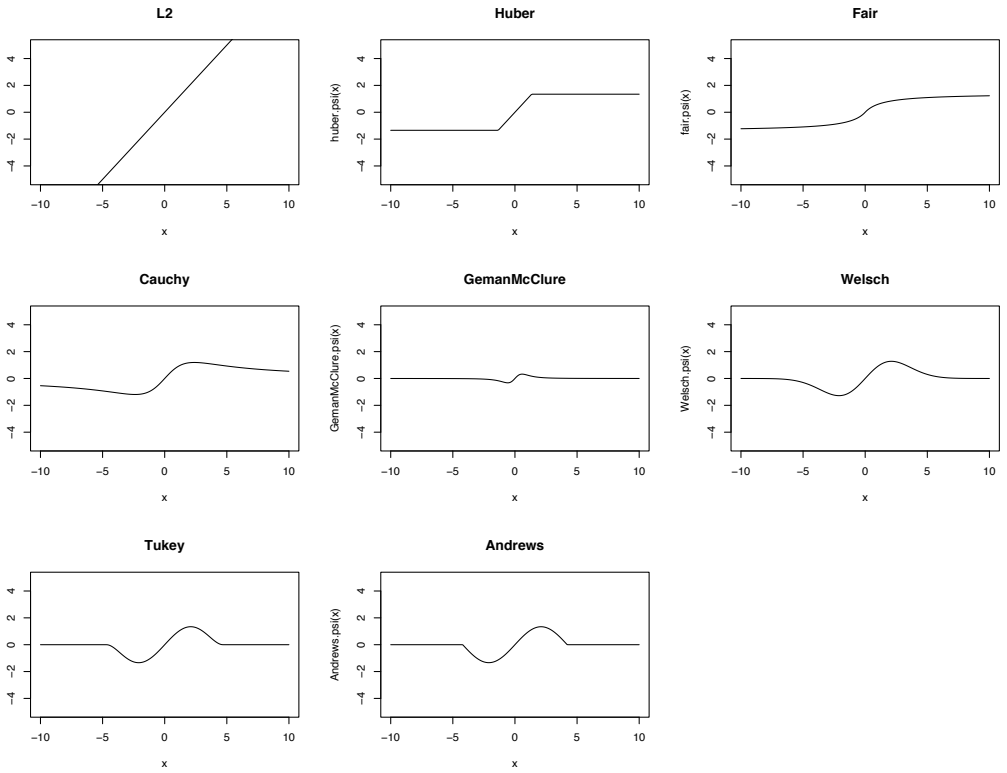
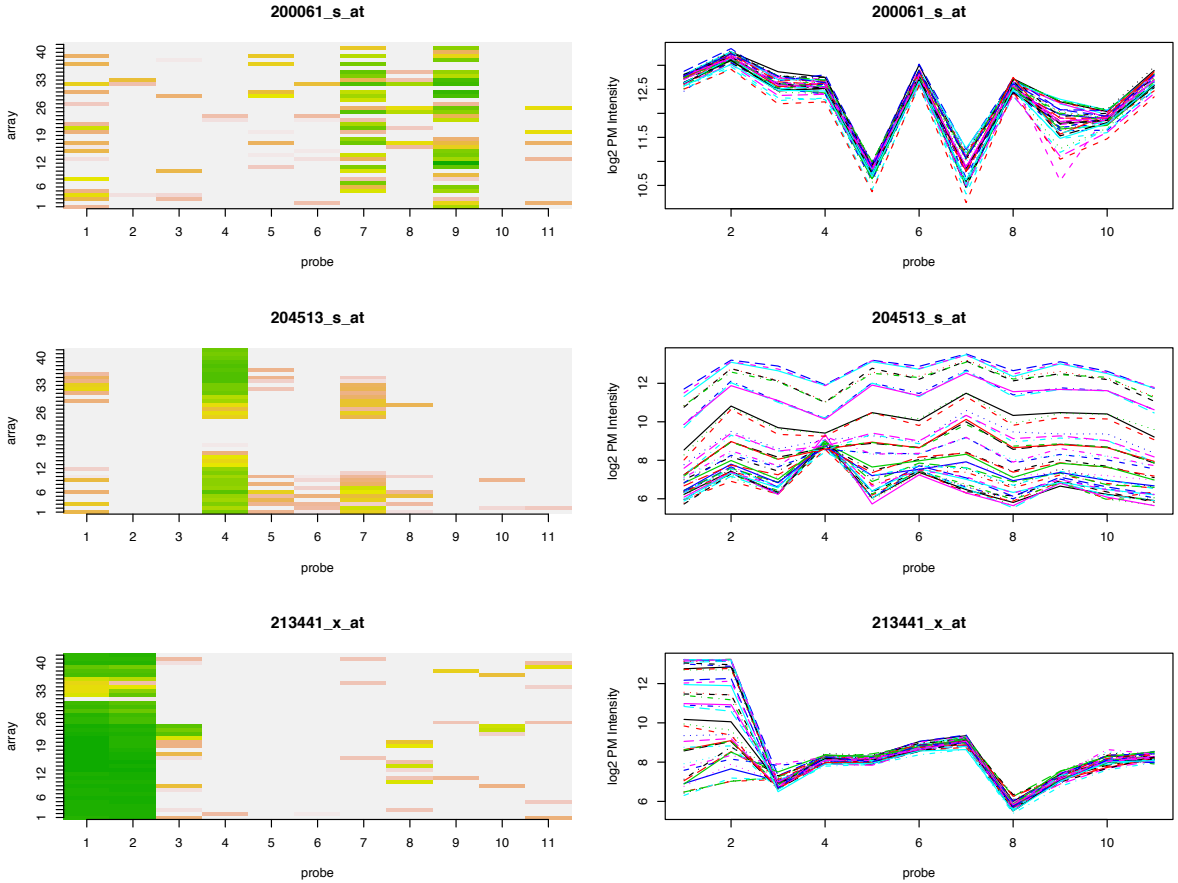


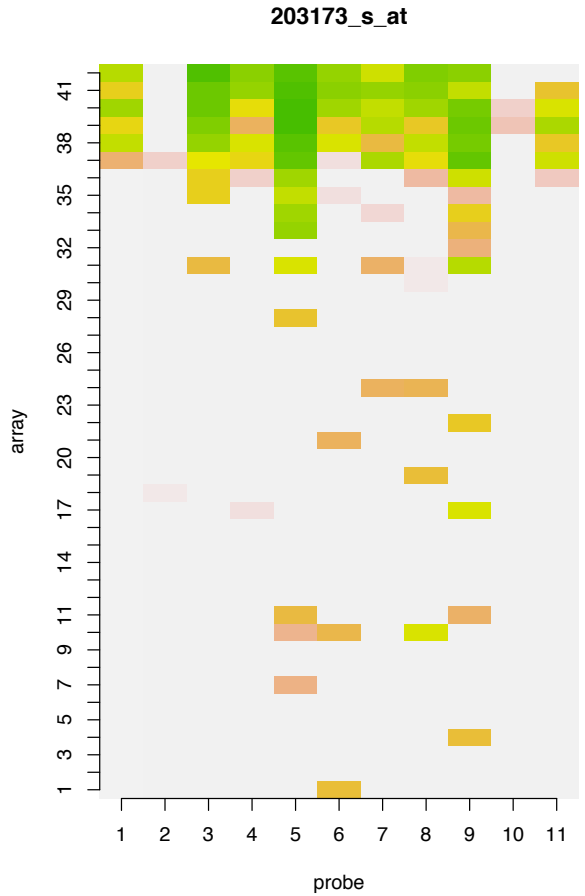
Figure 4.2: The ψ functions for some common M-estimators.

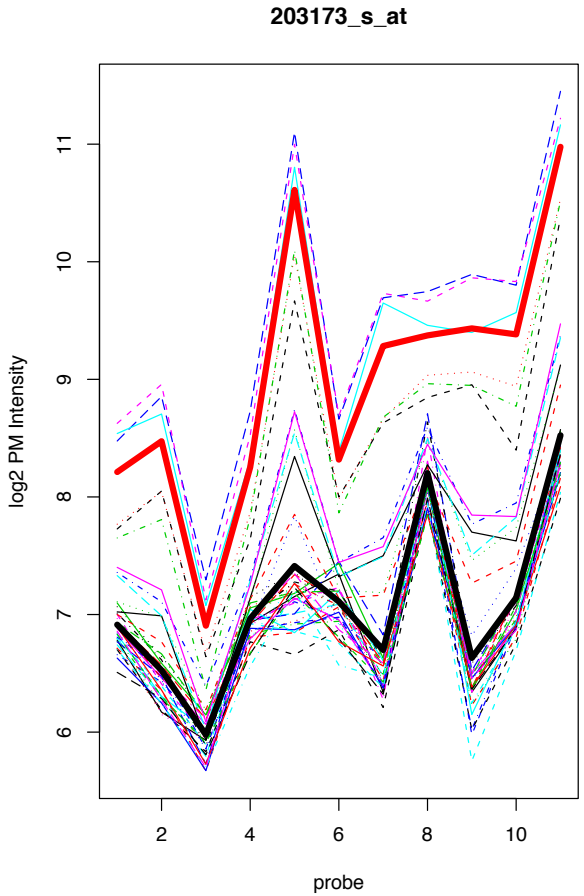
Robust regression leads to various quality assessment metrics



Identifies poor performing probes

Robust regression leads to various quality assessment metrics





Identifies poor performing samples

Relate to limma objects

$$\begin{bmatrix} y_1 \\ y_2 \\ y_3 \\ y_4 \\ y_5 \\ y_6 \end{bmatrix} = \begin{bmatrix} 1 & 0 & 0 \\ 1 & 0 & 0 \\ 0 & 1 & 0 \\ 0 & 1 & 0 \\ 0 & 0 & 1 \\ 0 & 0 & 1 \end{bmatrix} \begin{bmatrix} \alpha_1 \\ \alpha_2 \\ \alpha_3 \end{bmatrix} + \begin{bmatrix} \epsilon_1 \\ \epsilon_2 \\ \epsilon_3 \\ \epsilon_4 \\ \epsilon_5 \\ \epsilon_6 \end{bmatrix}$$

$E[y_1]=E[y_2]=\alpha_1$
$E[y_3]=E[y_4]=\alpha_2$
$E[y_5]=E[y_6]=\alpha_3$

$$\beta = C\alpha = \begin{bmatrix} -1 & 1 & 0 \\ 0 & -1 & 1 \end{bmatrix} \begin{bmatrix} \alpha_1 \\ \alpha_2 \\ \alpha_3 \end{bmatrix} = \begin{bmatrix} \alpha_2 - \alpha_1 \\ \alpha_3 - \alpha_2 \end{bmatrix}$$

```
> design
  alpha1 alpha2 alpha3
1      1      0      0
2      1      0      0
3      0      1      0
4      0      1      0
5      0      0      1
6      0      0      1
> cont.matrix <- makeContrasts(beta1="alpha2-alpha1",
                  beta2="alpha3-alpha2",levels=design)

> cont.matrix
         Contrasts
Levels    beta1 beta2
  alpha1     -1     0
  alpha2      1    -1
  alpha3      0     1

fit <- lmFit(y,design)

fit.c  <- contrasts.fit(fit, cont.matrix)
fit.c  <- eBayes(fit.c)

> head(round(y,2),3)
       [,1]   [,2]  [,3]  [,4]   [,5]   [,6]
[1,]  -1.62   1.49  2.50  1.57  -0.71   0.38
[2,]  -4.50  -4.95 -3.66 -7.83  -1.59   6.94
[3,] -10.17 -21.90 14.03  3.66 -12.21 -15.26

> head(round(fit$coef,2),3)
     alpha1 alpha2 alpha3
[1,]  -0.07   2.03  -0.16
[2,]  -4.73  -5.75   2.67
[3,] -16.04   8.85 -13.74

> head(round(fit.c$coef,2),3)
      Contrasts
       beta1  beta2
  [1,]  2.10  -2.20
  [2,] -1.02   8.42
  [3,] 24.89 -22.59
```

University of Zurich[UZH]

Institute of Molecular Life Sciences

MAPPING MILLIONS OF SHORT "READS" ONTO THE GENOME/TRANSCRIPTOME

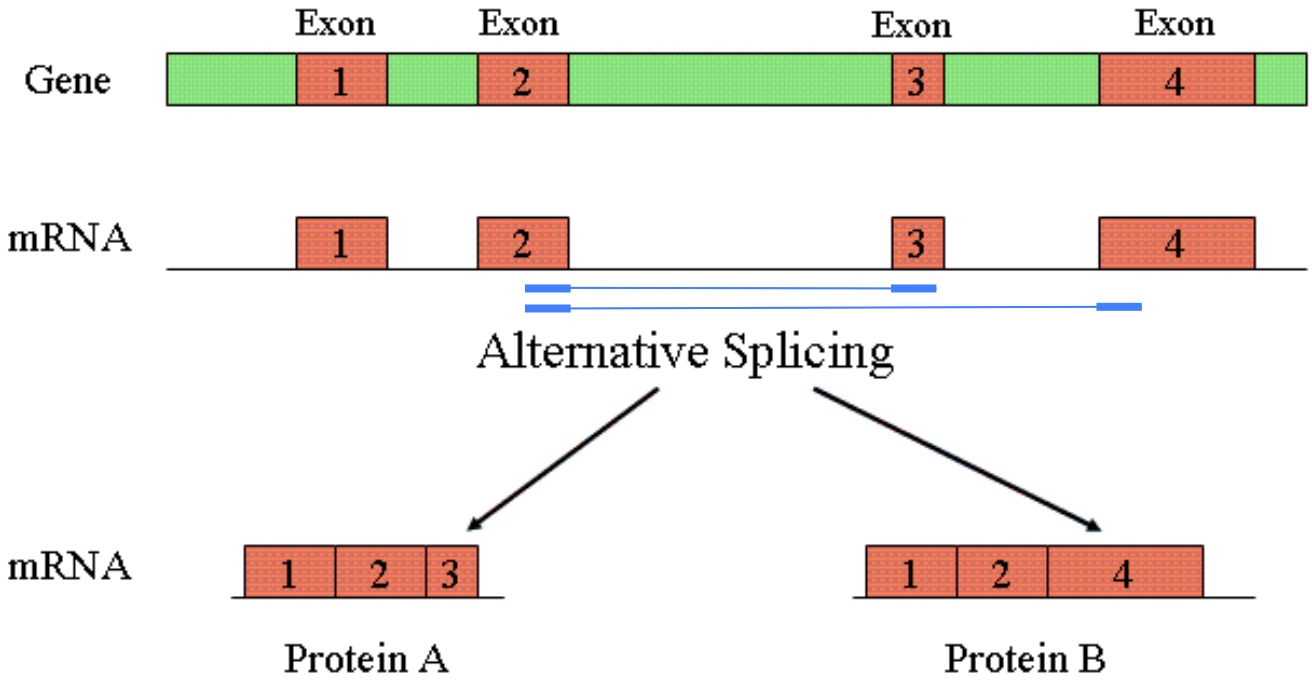
Mark D. Robinson, Statistical Genomics, IMLS

In a nutshell: Mapping reads onto genomes/ transcriptomes

- Computationally intense problem: DNA sequencing instruments produce millions-billions of short “reads” from genome (or cDNA population, etc.)
- Genomes are large: Human 3.3B characters
- Need to allow for mismatches (e.g. genetic polymorphisms, sequencing errors)
- Repetitive regions (report multiple equally good alignments?)
- BLAST – was workhorse of sequence alignment for many years (slightly different application: best substring)
- Additional challenges: RNA, paired-end reads, quality scores, bisulphite treatment, etc.

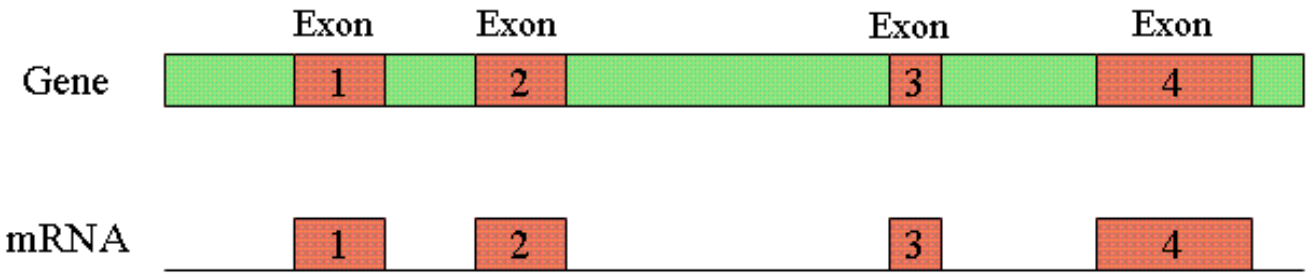
http://www.ncbi.nlm.nih.gov/Class/MLACourse/Modules/MolBioReview/alternative_splicing.html

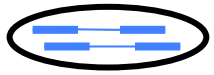
Mapping cDNA (from RNA) reads: needs a "splice-aware" aligner



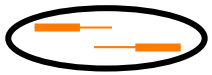
Solution: if you know where they are, add the junction sequence to the genome

Paired-end reads





Paired-end reads: Sequence both ends of DNA fragment



Single-end reads: Sequence one end of DNA fragment

Paired-end reads: costs more, but gives additional information (exact length of fragment, can map reads where one of pair hits repetitive region)

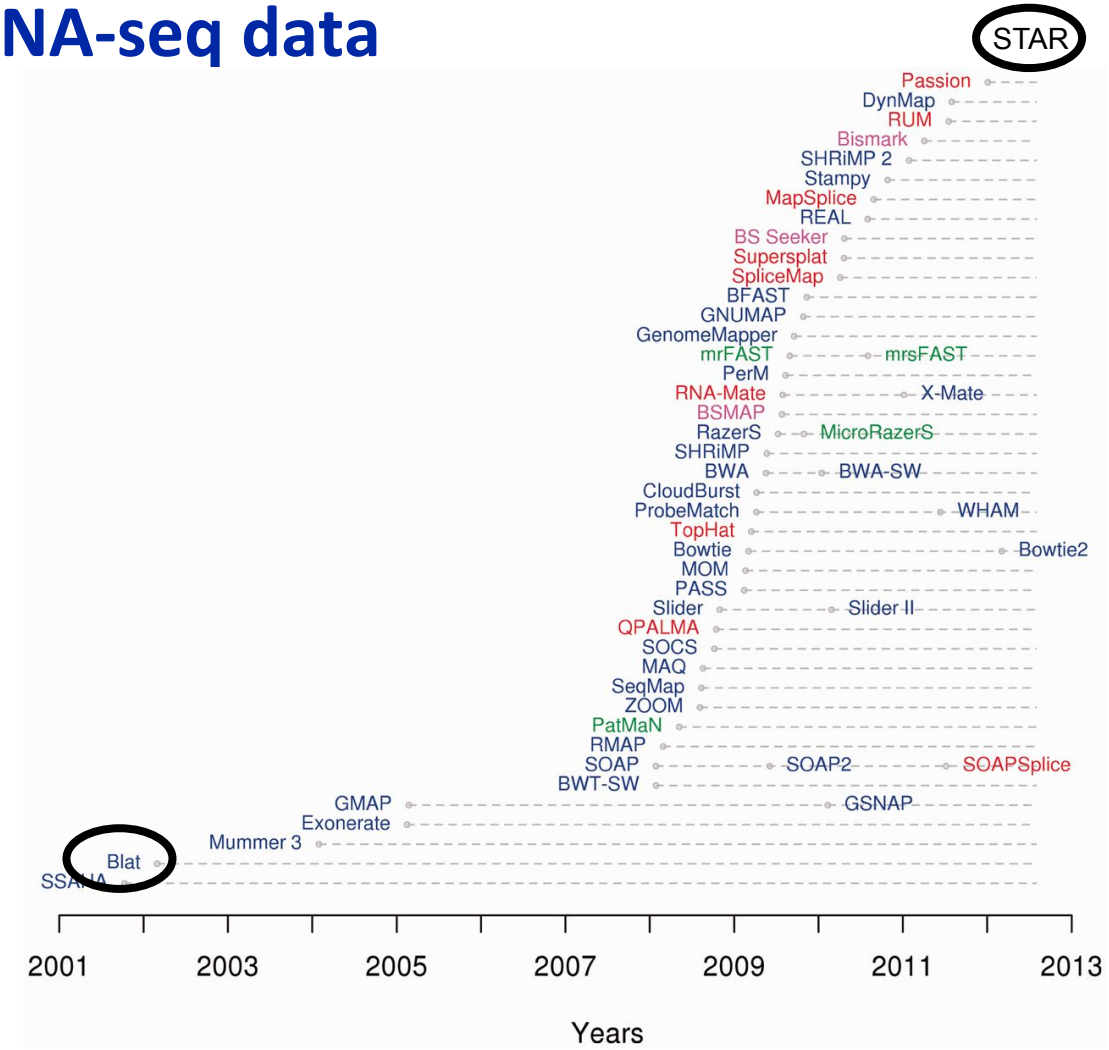
FASTA versus FASTQ formats

```
>ERR030881.107 HWI-BRUNOP16X_0001:2:1:13663:1096
ATCTTTTGTGGCTACAGTAAGTTCAATCTGAAGTCAAAACCAACCAATTT
>ERR030881.311 HWI-BRUNOP16X_0001:2:1:18330:1130
TCCATACATAGGCCTCGGGGTGGGGGAGTCAGAAGCCCCCAGACCCTGTG
```

```
@ERR030881.107 HWI-BRUNOP16X_0001:2:1:13663:1096#0/1
ATCTTTTGTGGCTACAGTAAGTTCAATCTGAAGTCAAAACCAACCAATTT
+
5.544,444344555CC?CAEF@EEFFFFFFFFFFFFFFFFEFFFEFFF
@ERR030881.311 HWI-BRUNOP16X_0001:2:1:18330:1130#0/1
TCCATACATAGGCCTCGGGGTGGGGGAGTCAGAAGCCCCCAGACCCTGTG
+
GFFFGFFBFCHHHHHHHHHHIHEEE@@@=GHGHHHHHHHHHHHHHHHHH
```

1 read

The world of mapping algorithms: focus here on RNA-seq data



Fonseca et al. 2012

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Exercises: Mapping reads to genomes/ transcriptomes, counting features

- Spot check mapping with BLAT
- Familiarize with the STAR pipeline → later: kallisto
- Understand the common file types
- Mapping a small example (2M bases of chr19 as the “genome”)

Mark D. Robinson, Statistical Genomics, IMLS

A small example

hs_ch19_subset.fa: Sequence from human chr19 from 2,000,000-4,000,000

→ imagine a genome that is only 2M bases long

galaxy_small.fastq: subset of reads from a real dataset

chr19_rescaled.gff: Table of annotation

→ Tell the aligner where the junctions should be

Pipeline + Filetypes

FASTA/FASTQ → SAM/BAM → "count" table

raw reads	aligned reads	matrix

Annotation is stored in:
GTF/GFF1/GFF2/GFF3

http://www.broadinstitute.org/igv/GFF

Getting help / info

STAR (RNA-seq):

https://code.google.com/p/rna-star/

IGV:

http://www.broadinstitute.org/software/igv/home

For DNA: bowtie2, bwa mem, …

IGV


chr19
11 kb
1,004 kb
1,006 kb
1,008 kb
1,010 kb
1,012 kb
1,014 kb
tophat2_mapped_s.bam Coverag
[0 - 3223]
tophat2_mapped_s.bam
chr19_rescaled.gff3
"NM_198969"
"NM_198970"
"NM_001130"