Analysis of Illumina microarray data

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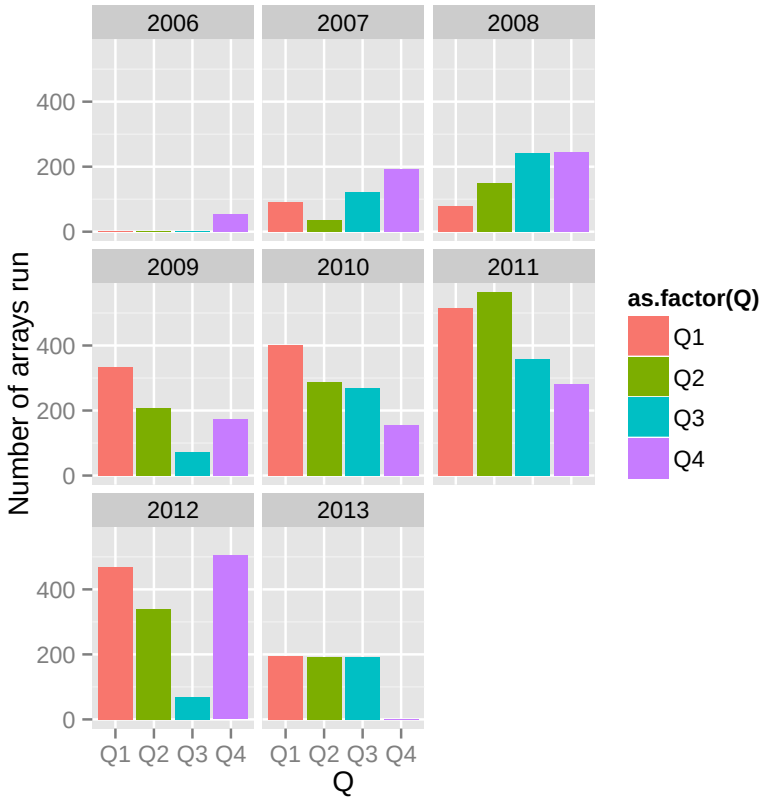
Outline

A brief history of Illumina

Now best-known as a sequencing company, Illumina are just one of several companies offering whole-genome microarrays.

- Hapmap (genotyping)
- GeneVar (expression)
- Metabric (expression)
- TCGA (methylation, genotyping)
- iCOGS (genotyping)

They have been the technology of choice at CRI since 2006



Are they still relevant?

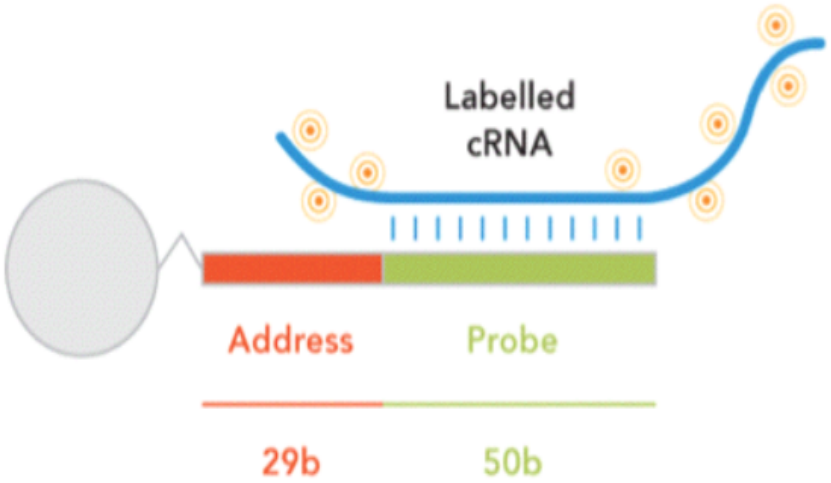
- Wealth of data available online e.g. on GEO
- Useful as a validation platform
- Methods are established and well-understood
- Cheaper? And easier access to equipment
- Some of the lessons learnt from microarray analysis can be applied to sequencing data
- Useful introduction to the world of Bioconductor

Microarray recap

- Microarrays use *hybridisation* to measure abundance of mRNA transcripts
- Pre-designed *probes* attached to solid surface
- Use *labels* to measure hybridization intensity
- *Scanned* image translated into probe intensities

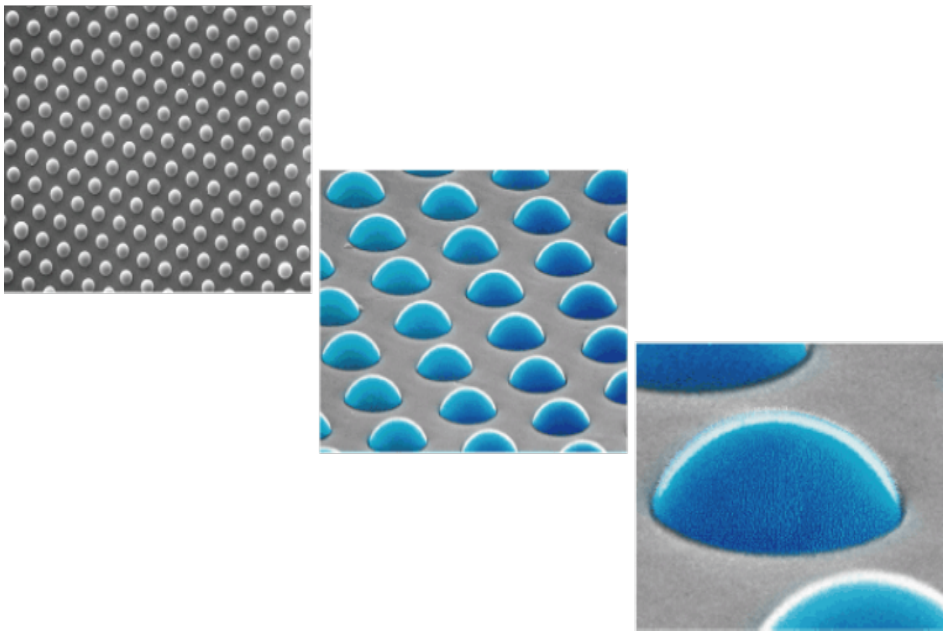
Technology overview

- Single-channel microarrays
- 50 base-pair 'probes' with the same sequence are attached to a bead
- Beads that have the same probe sequence are known as a *bead-type*
- Beads are identified by Illumina using a decoding sequence



Randomly-arranged arrays

- Arrays of randomly-arranged beads are constructed
- The number of replicates of each bead-type follows a normal distribution
- Around 20 replicates on most-recent arrays

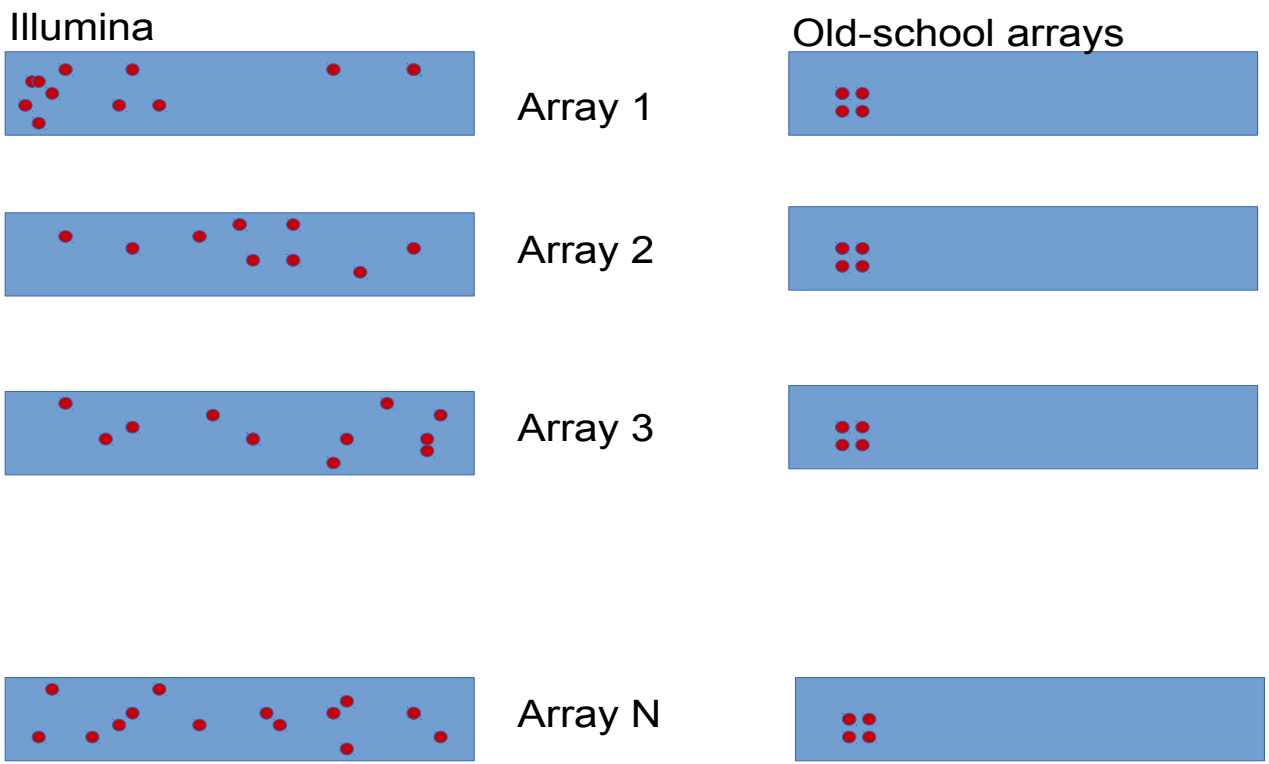


Combinations of arrays and nomenclature

- 'Refseq' chips with 24,000 bead-types per array, 8 arrays per chip
- WG- chips with 48,000 bead-types per array, 6 arrays per chip
- HT-12 chips with 48,000 bead-types per array, 12 arrays per chip
- Each chip also has an annotation 'version' (v1, v2, v3, v4)

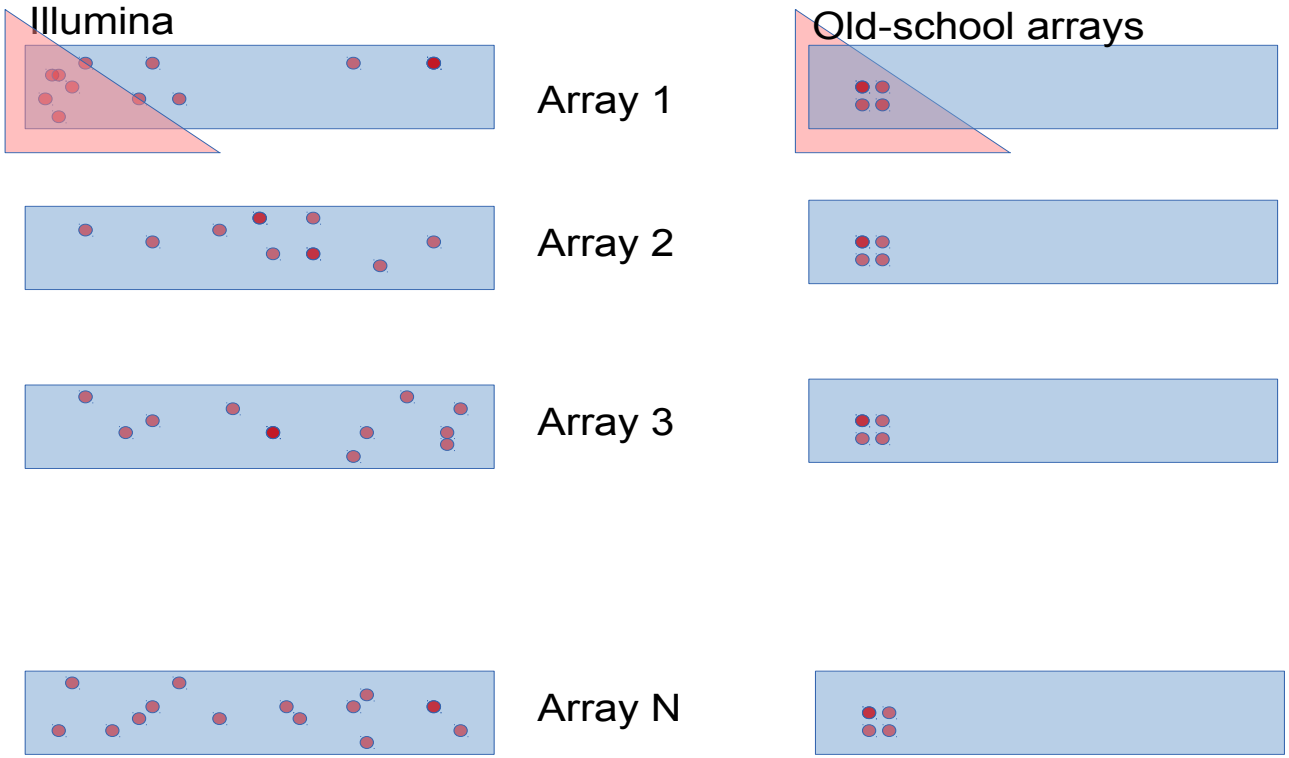
Example

Observations for Gene X on Illumina and older array technologies



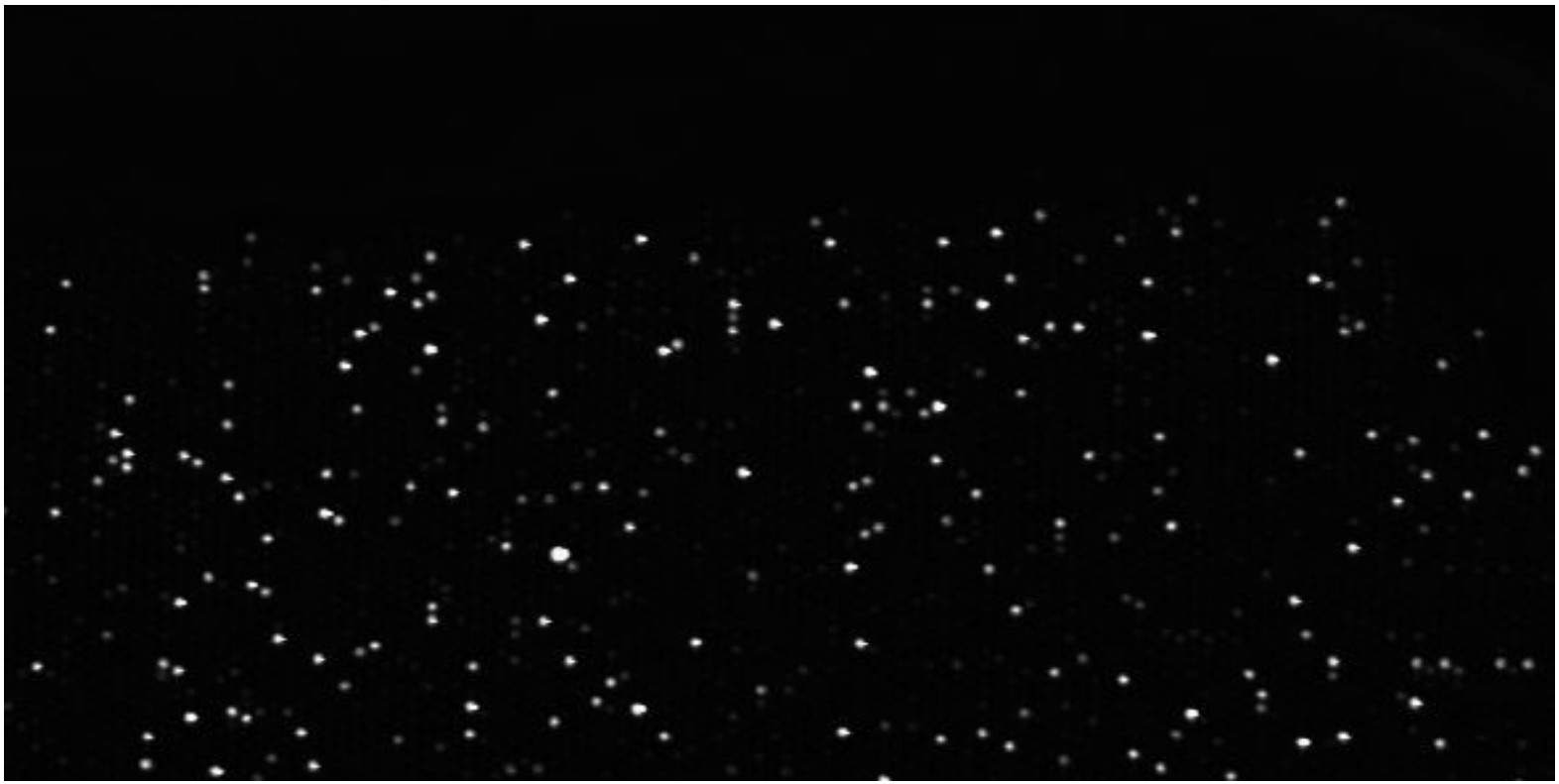
Example

Consider a technical failure on one of the arrays



Output formats

TIFF image representation of the array surface



Output formats

A text description of the bead locations, unique to each array.

```
Code....Grn..GrnX...GrnY
0010008 0934 1649.4 3650.0
0010008 0504 1866.5 3583.5
0010008 0607 0368.3 3075.2
0010008 1052 1908.5 5868.4
0010008 0437 0340.1 1465.9
0010008 0640 1974.0 7747.5
0010008 0798 1779.6 5615.7
0010008 0589 1952.5 5658.5
0010008 0508 1978.4 1144.0
0010008 1018 1691.3 4703.5
0010008 0701 0865.5 2197.7
0010008 0831 1572.3 7449.7
0010008 0700 0579.5 2328.0
0010010 0105 1184.2 4347.6
0010010 0108 1850.7 1129.3
.....
.....
Over 1,000,000 Lines
.....
.....
7650767 0300 0641.4 2720.7
7650767 0337 0298.4 1518.4
7650767 0256 0306.3 2944.1
7650767 0319 0848.0 1184.5
7650767 0198 1589.5 0399.0
```

Know as *bead-level* data.

Output formats

annotation file from Illumina that gives the probe sequences. Shared for experiments using the same chip-type.

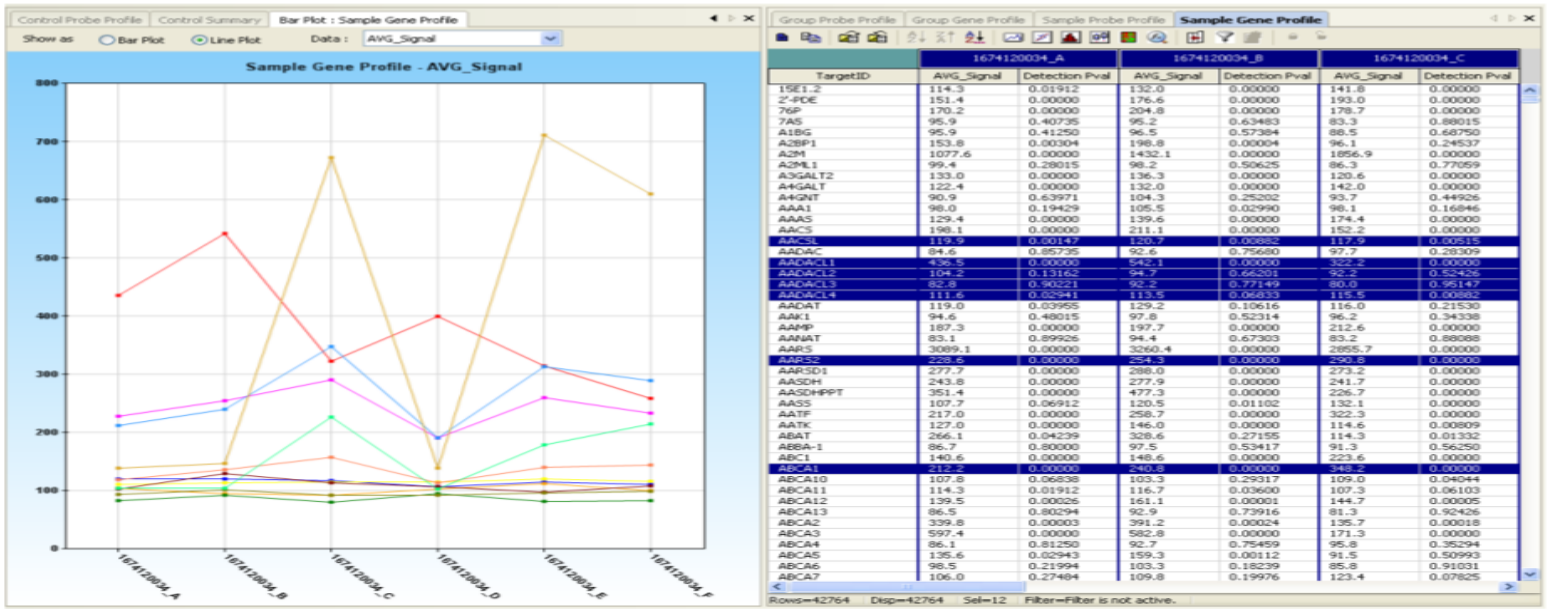
```
##

##      Code                  Sequence Symbol
## 1 7650767 ACGACCTCCTGTTGGAC.....   LAYN
```

We will revisit these later...

Summary-level data

Additionally, BeadStudio or GenomeStudio software can be used on the scanned data

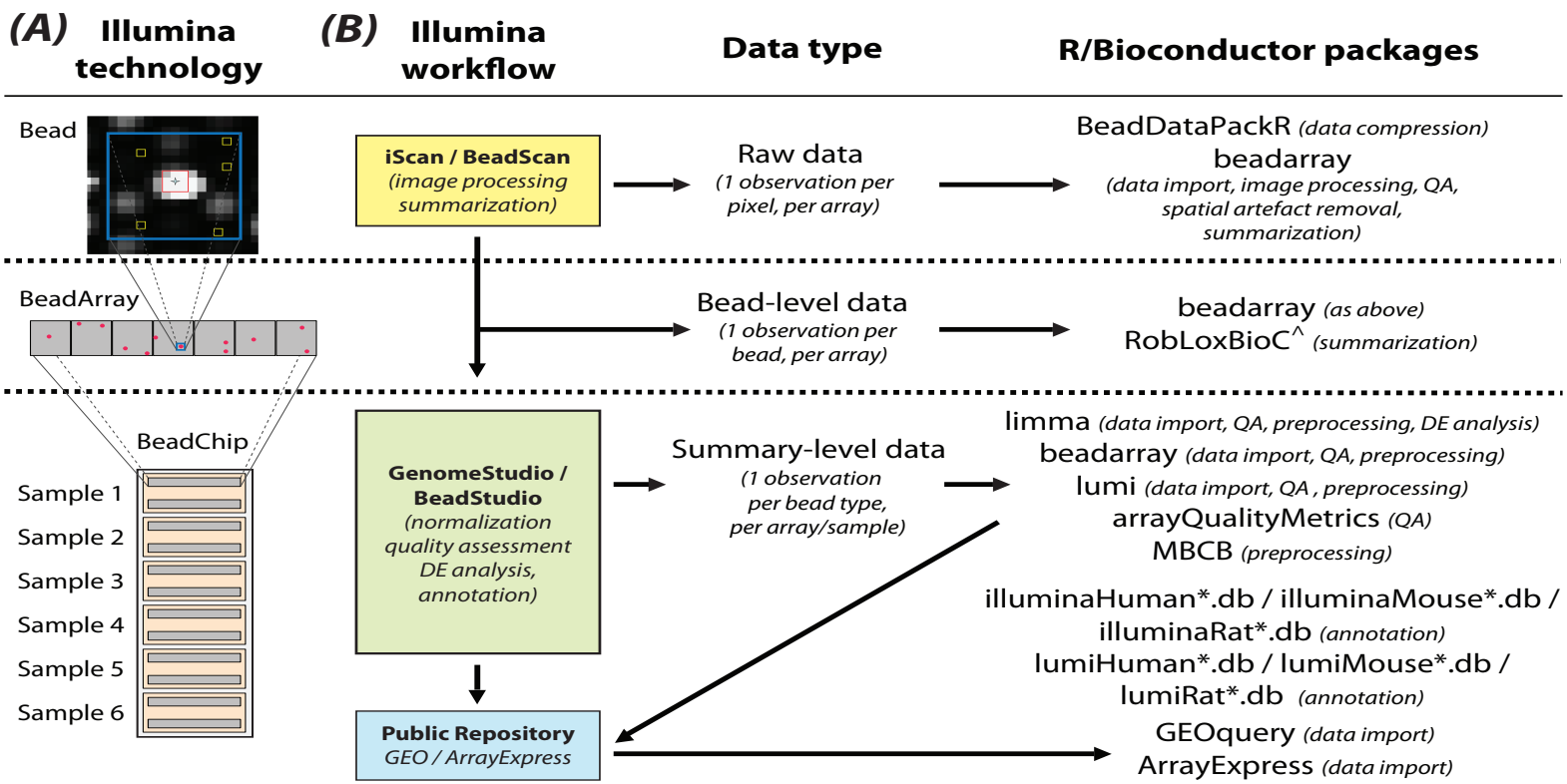


Starting point for analysis

The analyst may have a choice over what data to take as a starting point

- Raw images
- Bead-level data - vary number of observations for a bead-type on each array and pre-computed intensities
- Bead-summary - One value for each bead-type on each array. i.e. an 'Expression Matrix'
- Starting with raw, or bead-level data gives more flexiblity in analysis
- Bead-summary data fit in with standard Bioconductor tools

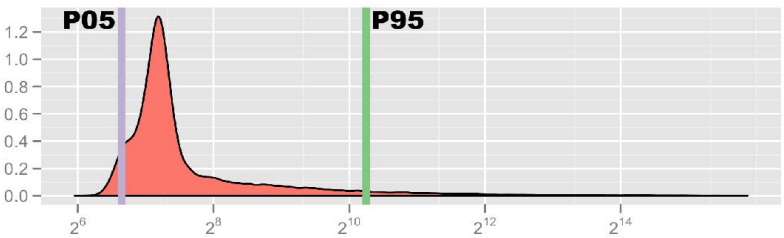
Workflow Overview



From Ritchie et al

Scanner Metrics

- The scanner can give lots of useful quality control information
- P95 = 95th intensity percentile (x-axis)
- P05 = 5th intensity percentile (y-axis)
- P95:P05 Ratio or signal to noise ratio, red line = ideal



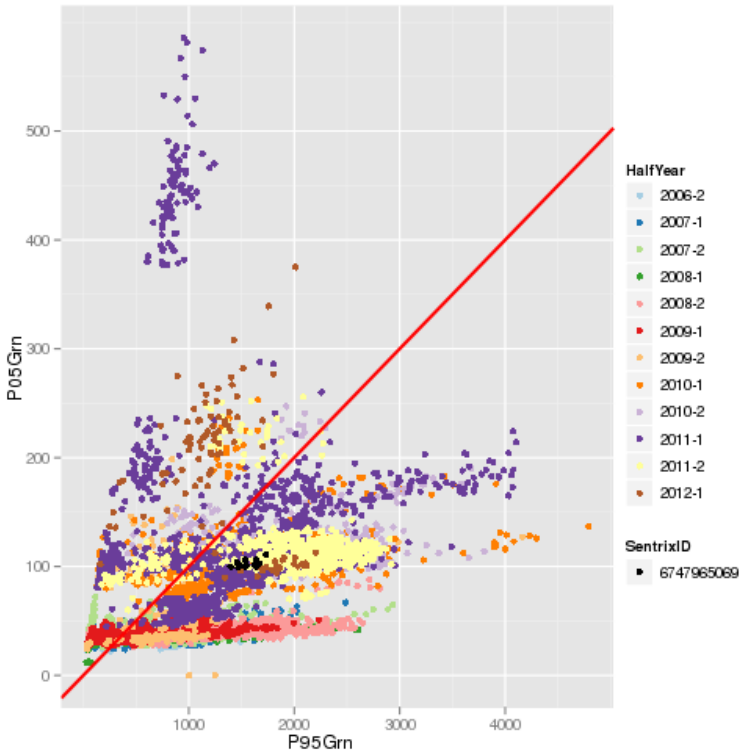
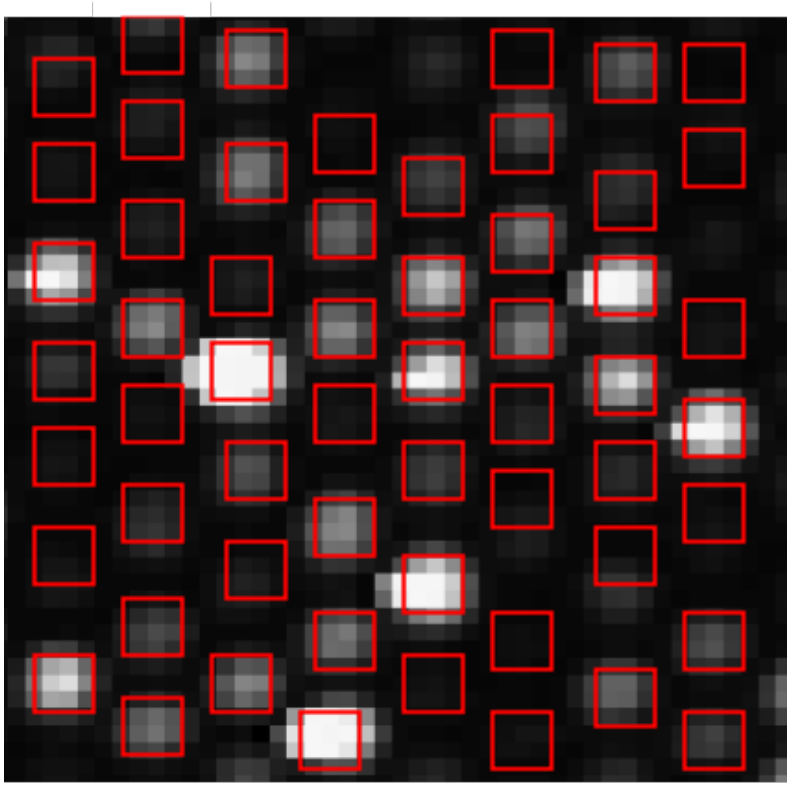


Image Processing

- Calculate foreground & background
- Background correct
- The resulting values are found in the text files
- The steps can be repeated in beadarray
- *Usually we start with these values*

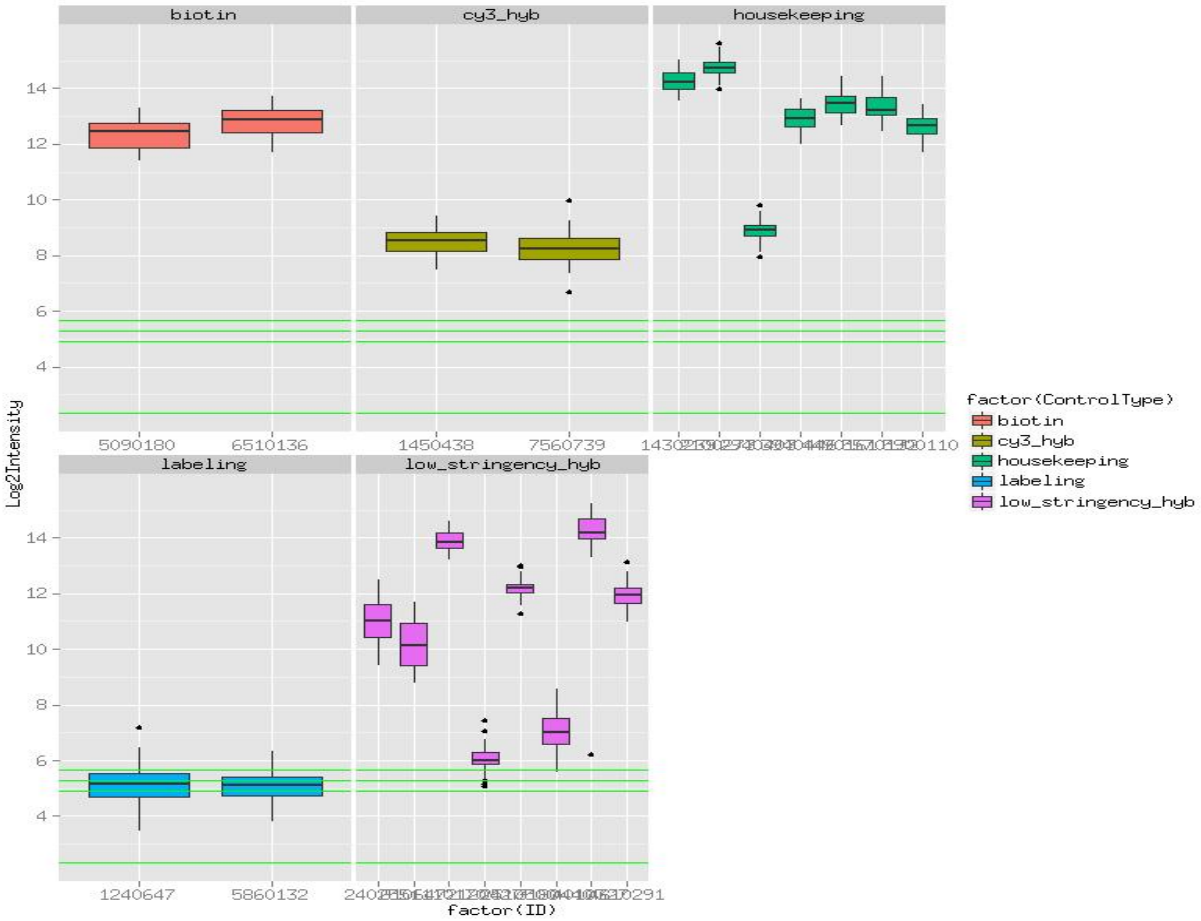
Full, gory, details in Smith et al



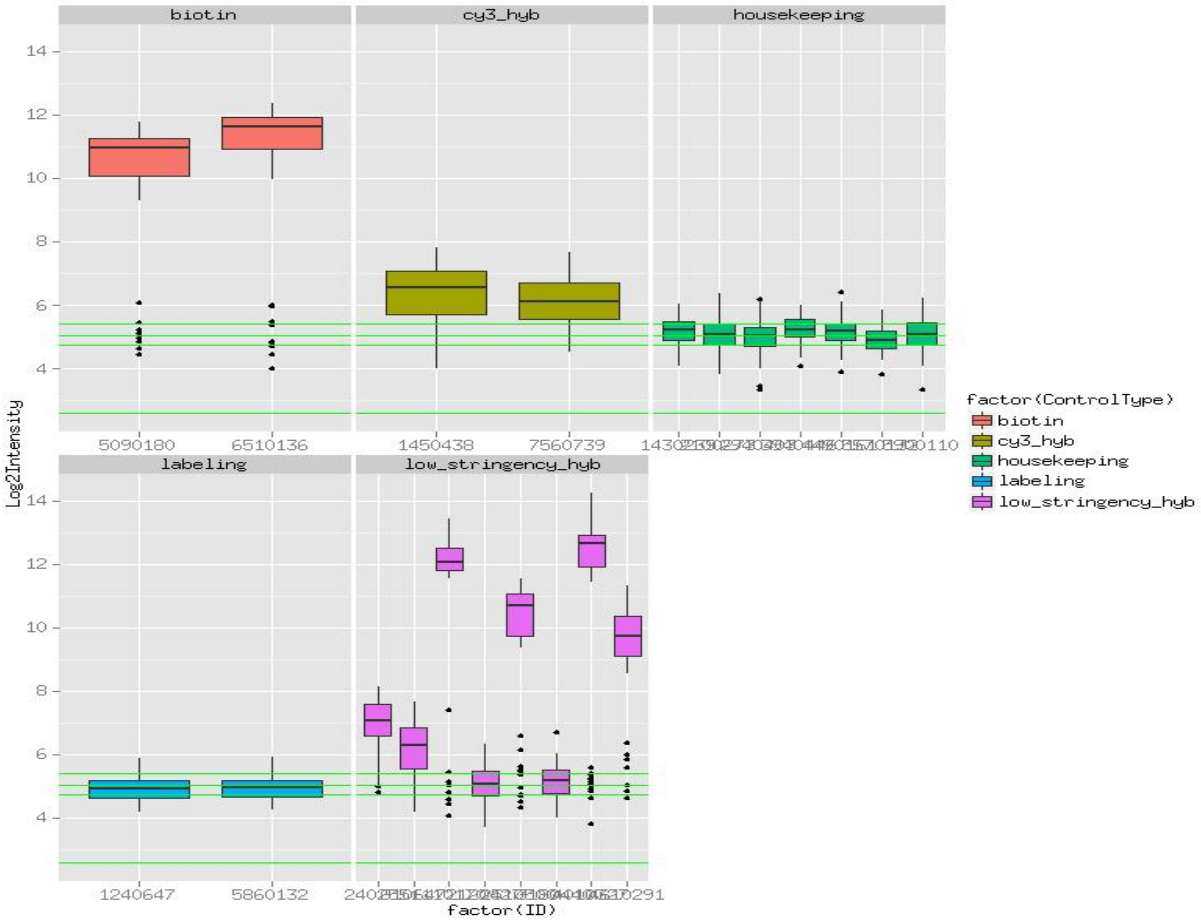
Control Plots

- Illumina design probes for housekeeping genes **Positive controls**
- They also have probes that should not hybridise to the target genome **Negative controls**
- The difference between these controls is a measure of signal level and the potential to detect differential expression
- The negative controls are also use to assess whether a given gene-probe is expressed / detected.

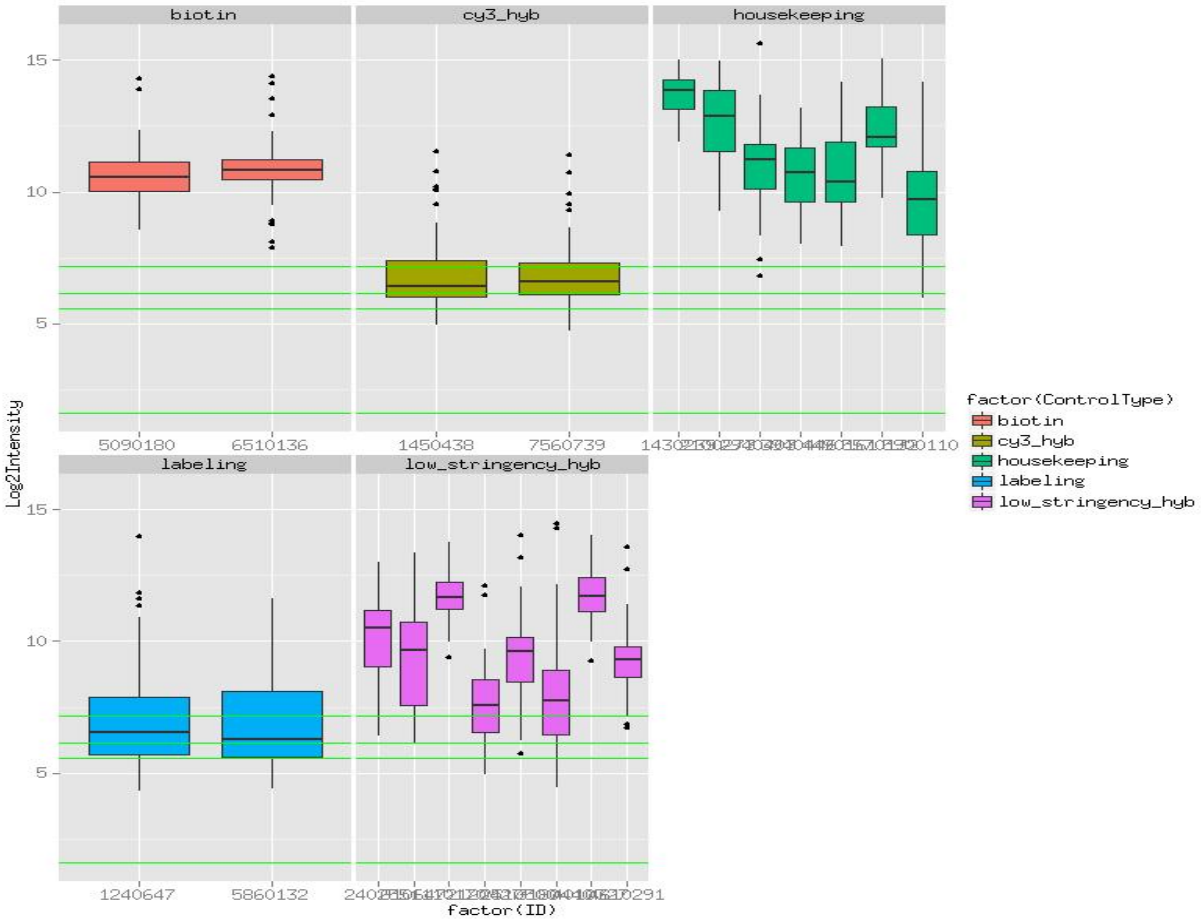
The good..


biotin
cy3_hyb
housekeeping
labeling
low_stringency_hyb
Log2Intensity
14
12
10
8
6
4
5090180
6510136
1450438
7560739
1240647
5860132
factor(ID)
factor(ControlType)
biotin
cy3_hyb
housekeeping
labeling
low_stringency_hyb

The bad..

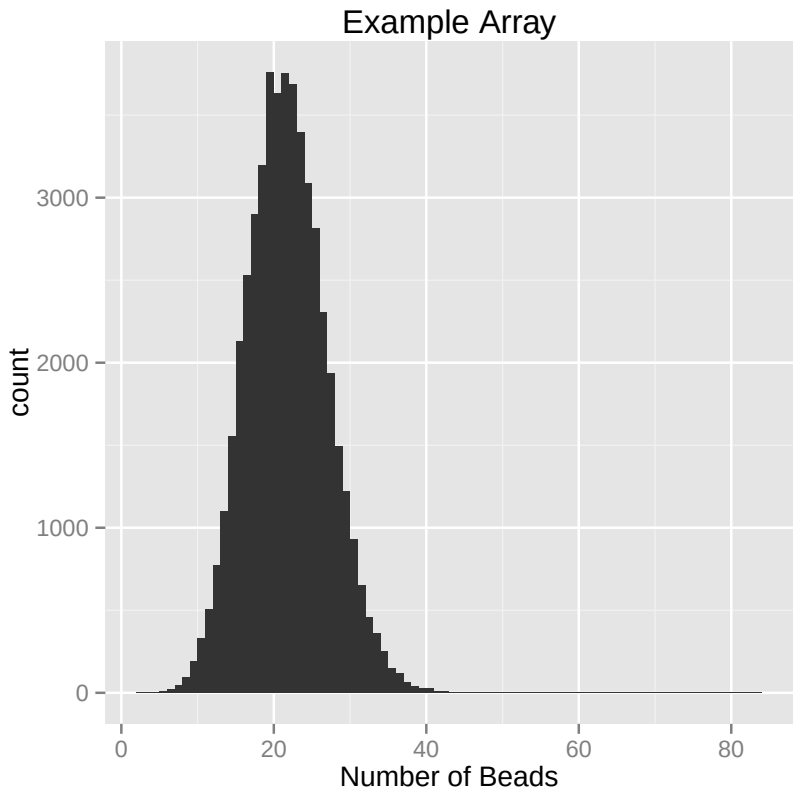

biotin
cy3_hyb
housekeeping
labeling
low_stringency_hyb
Log2Intensity
14
12
10
8
6
4
5090180
6510136
1450438
7560739
1240647
5860132
factor(ID)
factor(ControlType)
biotin
cy3_hyb
housekeeping
labeling
low_stringency_hyb

and the ugly..


biotin
cy3_hyb
housekeeping
labeling
low_stringency_hyb
Log2Intensity
15
10
5
5090180
6510136
1450438
7560739
1240647
5860132
factor(ID)
factor(ControlType)
biotin
cy3_hyb
housekeeping
labeling
low_stringency_hyb

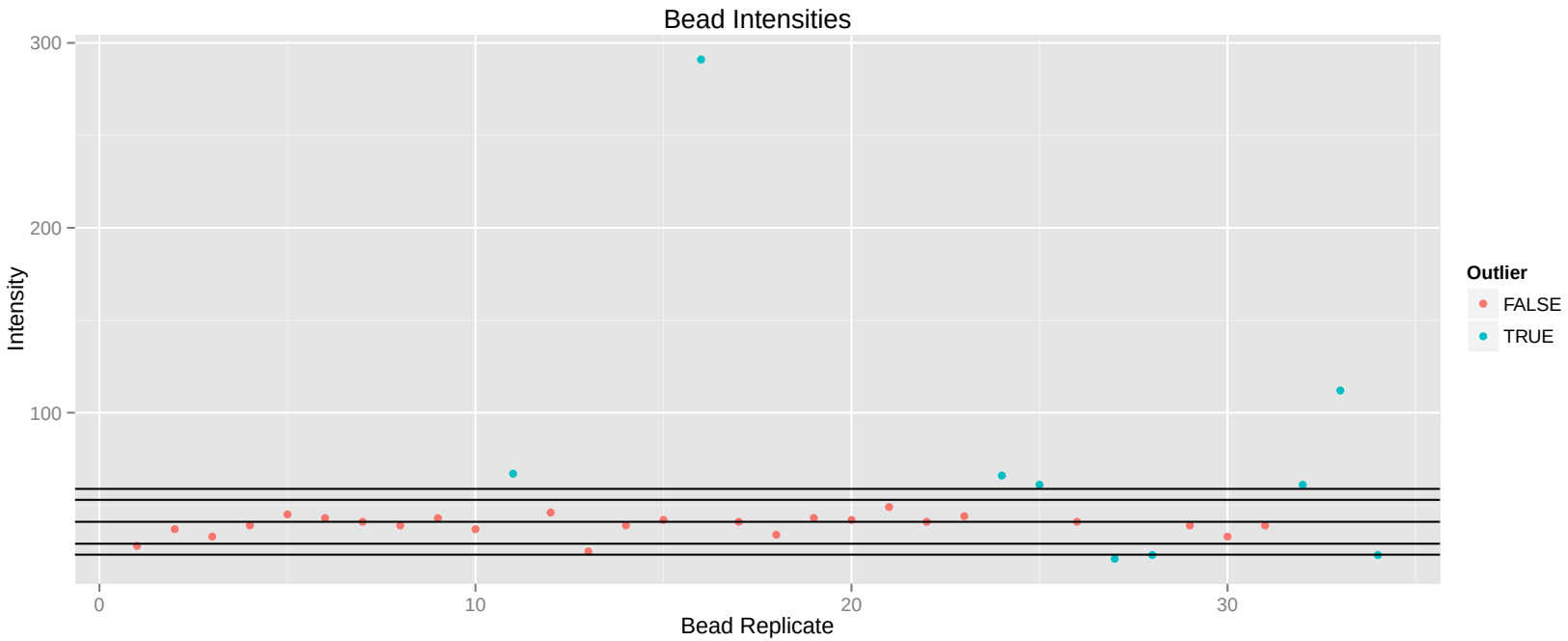
Dealing with multiple beads

- Having many replicates of each bead-type is one of the strengths of the BeadArray platform
- As is random array design
- But ultimately we want to compare one observation for each bead type between arrays



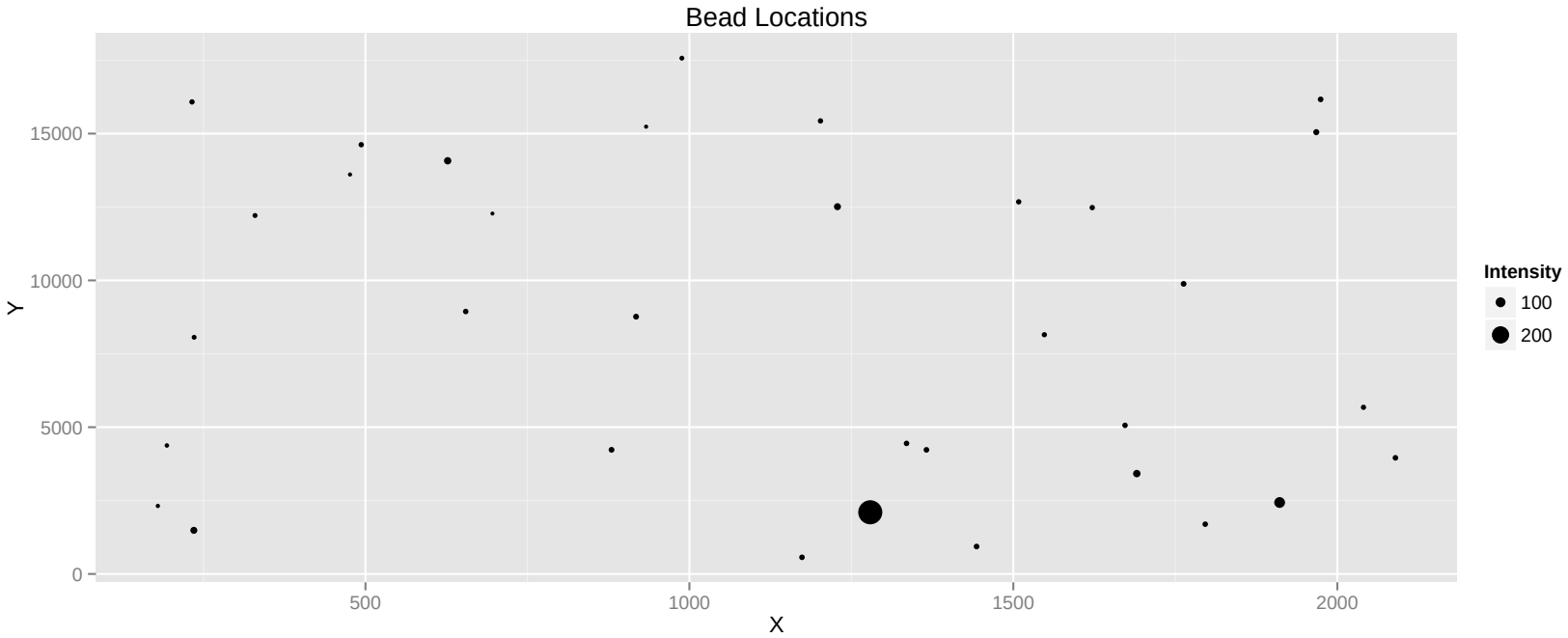
Illumina outlier removal

Illumina use an algorithm for removing unreliable beads using difference from the un-logged median. We prefer to use this method on the $\log_2$ scale



Illumina outlier removal

Where the outliers are located could also give clues about potential artefacts



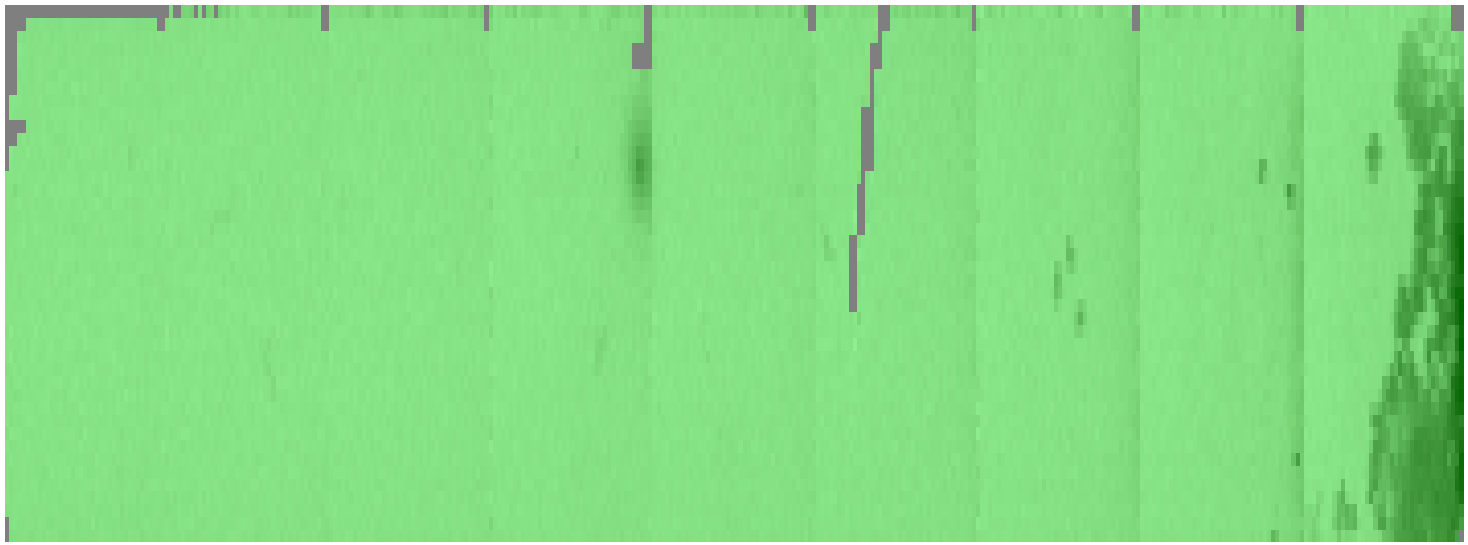
Spatial artefacts

Sometimes obvious artefacts can be apparent on the TIFF image itself

Would you trust any measurements taken on the right of this array?

Tools for assessing spatial artefacts

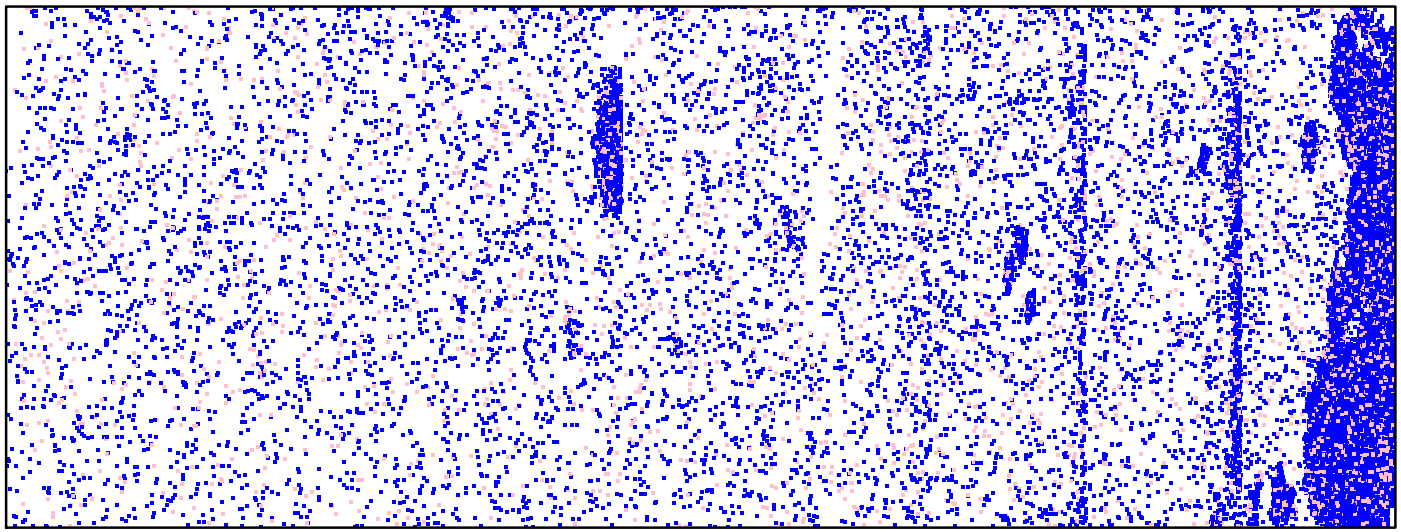
The `imageplot` function can create a false-colour representation of the array surface. Intensities of beads within a window are averaged and displayed.



Tools for assessing spatial artefacts

Outliers for each bead type can be calculated and plotted

```
## 50752  outliers found on the section
```

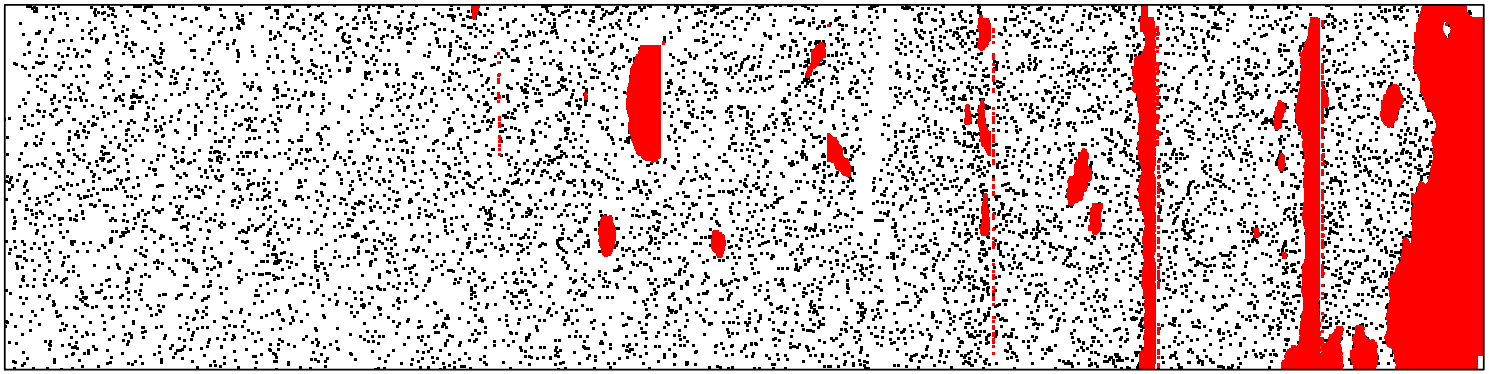


BASH

- The BASH method can be used to mask beads within spatial artefacts
- Adapted from Harshlight method first applied to Affymetrix arrays
- BASH looks for clusters of outliers close together
- Weights are assigned to each bead; 0 = exclude and 1 = include

BASH

4158323231_B_1

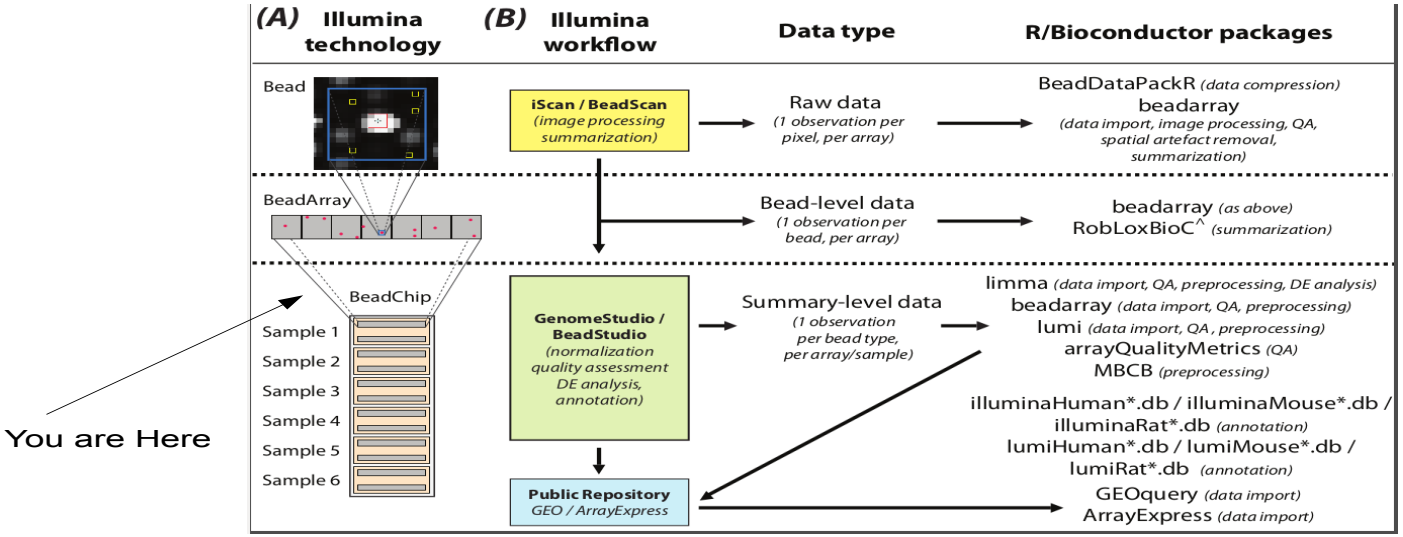


- Spatial investigations only possible with bead-level data
- May be missed as the cause of unreliable data if dealing with summary data
- Persistent manufacture or processing defects may also be overlooked

Producing summary values

For each array...

- Identify spatial artefacts and exclude any beads found within
- Group bead intensities according to Code (corresponding to a bead-type / gene)
- For each Code
  - log2 transform intensities
  - Calculate mean and standard error
- Repeat for next array

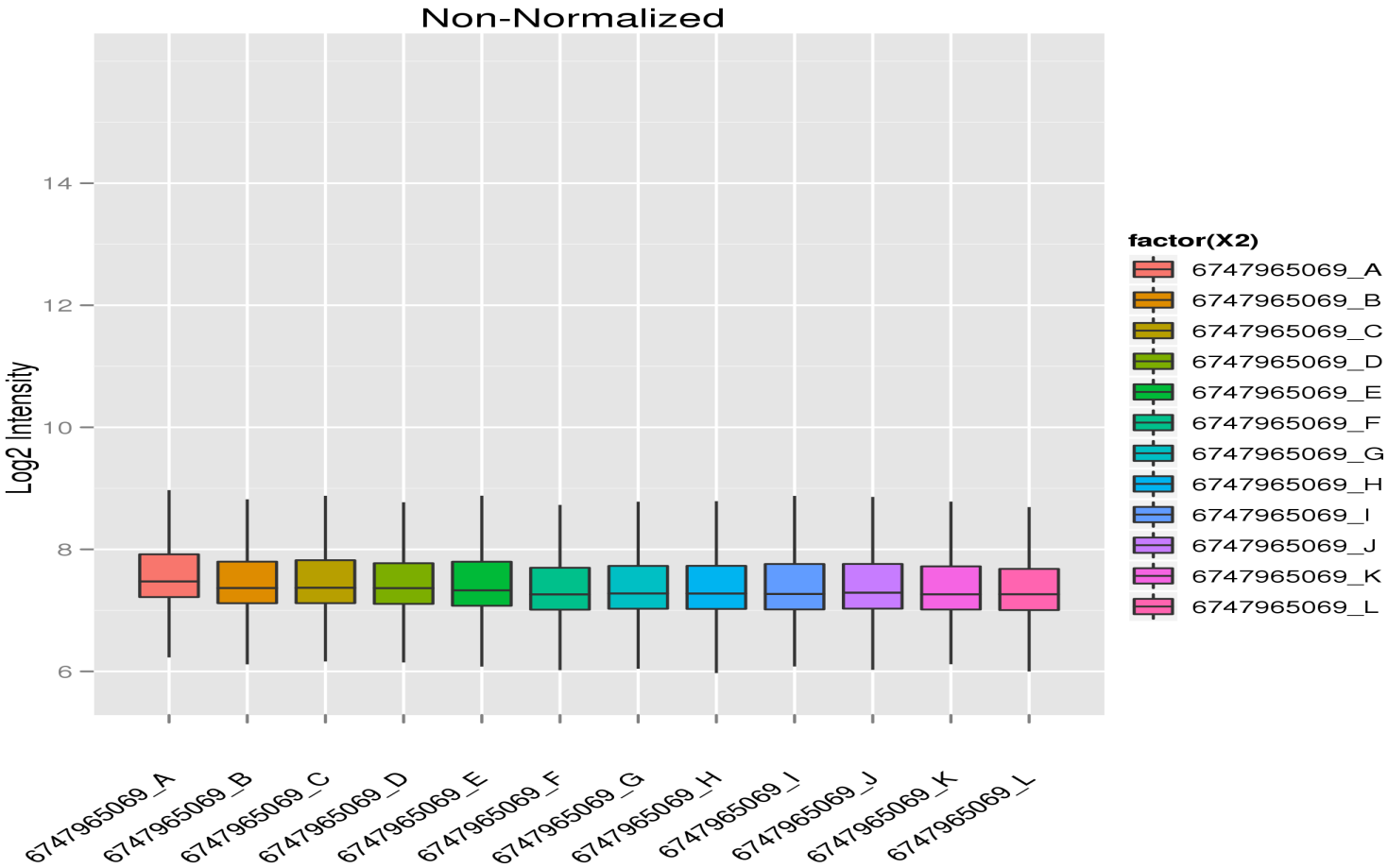


Entry-point for other microarray analysis packages

Starting with summarized data

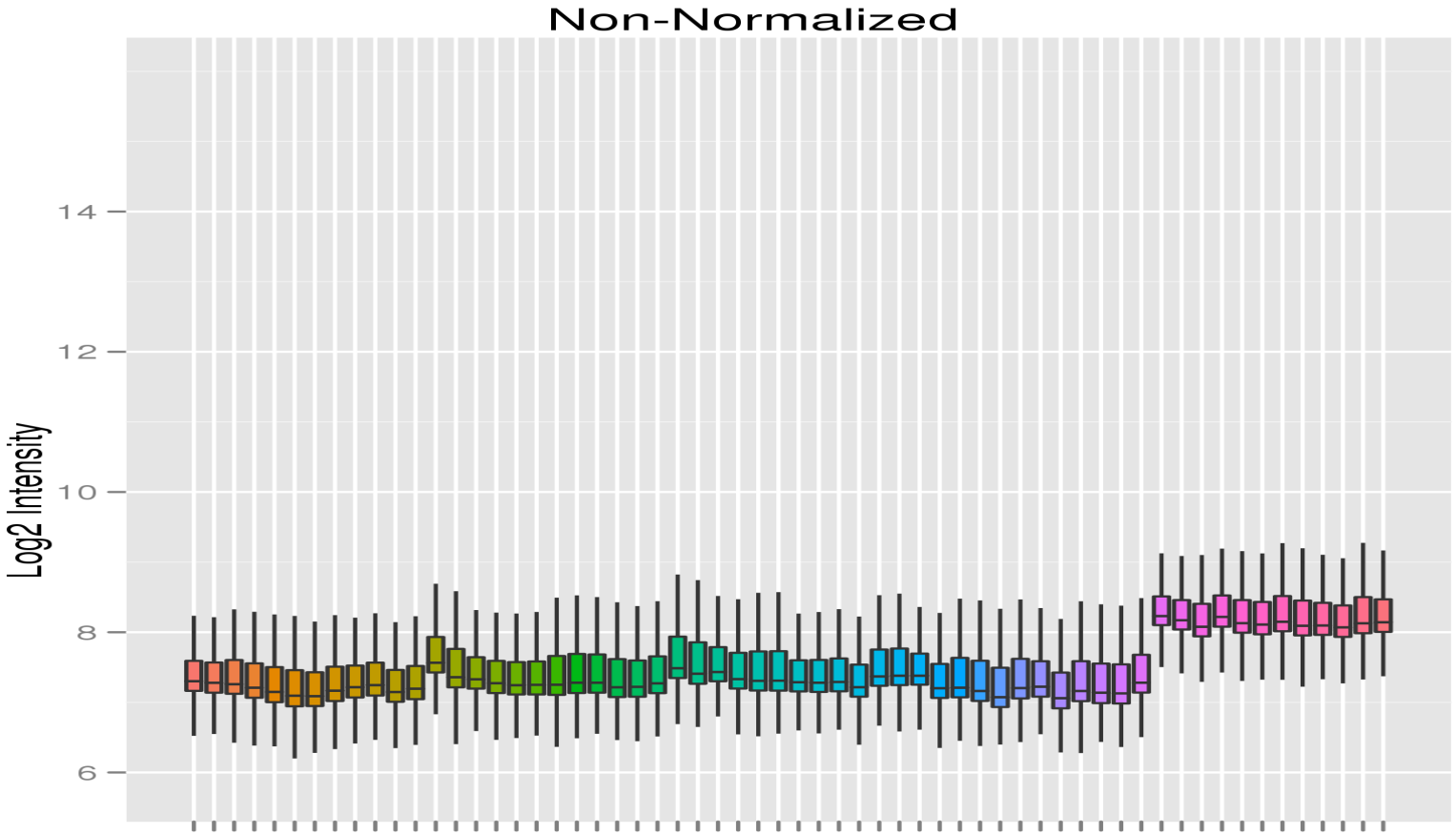
- Data exported from GenomeStudio are already summarized, but un-logged
- Ensure that data are not normalised
- Choose *probe-profile* data
- Summarized data can be read using limma, lumi or beadarray
- **Same analyses can be used on data summarized using beadarray, or exported from GenomeStudio**
- Summarized data can also be obtained from online repositories GEO or ArrayExpress

Quality Control - Summarised beads



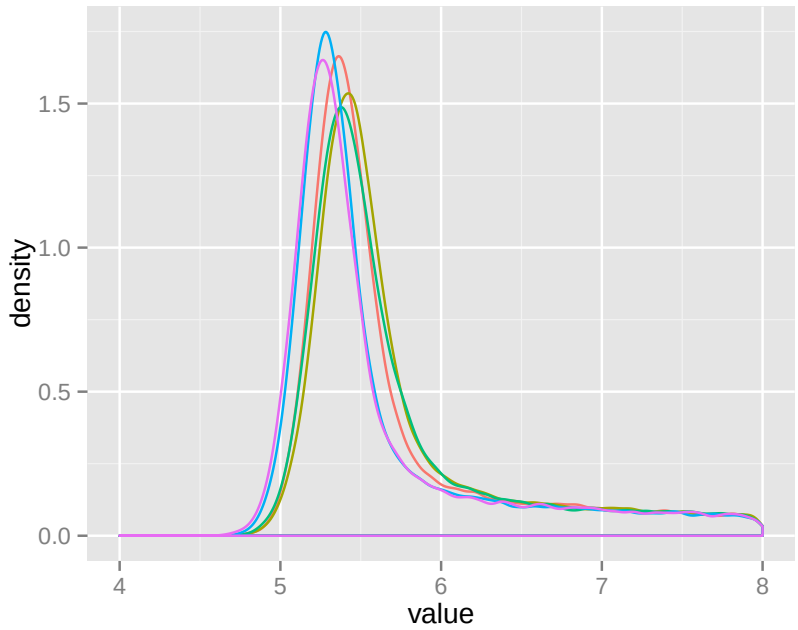
Why normalise?

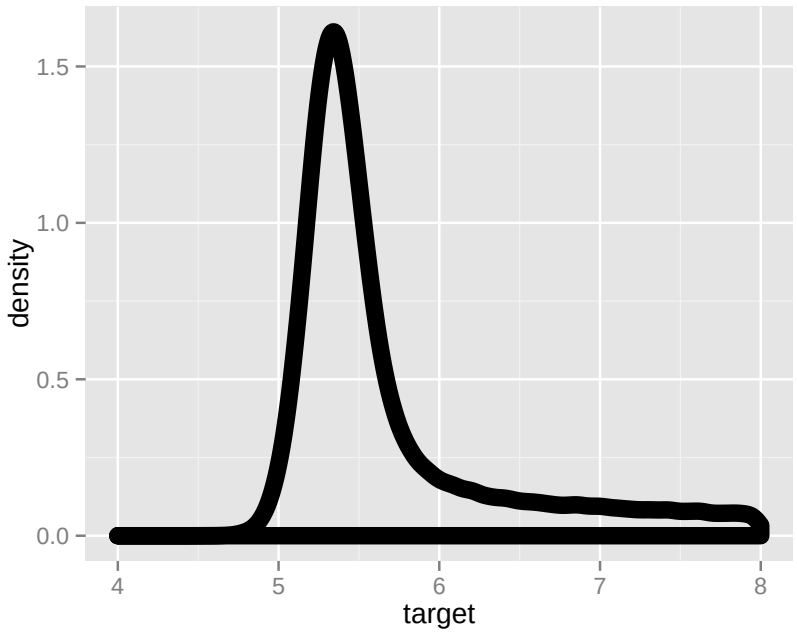
We want to be measuring *biological* rather than *technical* variation.



Normalisation of Illumina arrays

- Schmid et al review available methods and give guidelines for choosing most-appropriate method.
- The most popular is probably **Quantile normalisation**, or some variation on this.





Quantile Normalization

Consider the following matrix of values to be normalised

```
##   Array1 Array2 Array3
## A      1      3      9
## B      3      4      1
## C      9      2      5
## D      2      1      7
## E      4      9      6
```

Genes A, B, C, D and E measured on three arrays

Quantile Normalization - Step 1

Determine ranks of each column

```
##   Array1 Array2 Array3
## A      1      3      9
## B      3      4      1
## C      9      2      5
## D      2      1      7
## E      4      9      6
```

```
##      Array1   Array2   Array3
## [1,] "Rank1" "Rank3" "Rank5"
## [2,] "Rank3" "Rank4" "Rank1"
## [3,] "Rank5" "Rank2" "Rank2"
## [4,] "Rank2" "Rank1" "Rank4"
## [5,] "Rank4" "Rank5" "Rank3"
```

Quantile Normalization - Step 2

Sort each column Largest...Smallest

Original data

```
##   Array1 Array2 Array3
## A      1      3      9
## B      3      4      1
## C      9      2      5
## D      2      1      7
## E      4      9      6
```

Sorted data

```
##      Array1 Array2 Array3
## [1,]      1      1      1
## [2,]      2      2      5
## [3,]      3      3      6
## [4,]      4      4      7
## [5,]      9      9      9
```

Then calculate target distribution by averaging the sorted rows

```
## Rank1 Rank2 Rank3 Rank4 Rank5
##     1     3     4     5     9
```

Quantile Normalization - Step 3

Go back to the rank matrix

```
##      Array1  Array2  Array3
## [1,] "Rank1" "Rank3" "Rank5"
## [2,] "Rank3" "Rank4" "Rank1"
## [3,] "Rank5" "Rank2" "Rank2"
## [4,] "Rank2" "Rank1" "Rank4"
## [5,] "Rank4" "Rank5" "Rank3"
```

```
##      Array1  Array2  Array3
## [1,] "1"     "Rank3" "Rank5"
## [2,] "Rank3" "Rank4" "Rank1"
## [3,] "Rank5" "Rank2" "Rank2"
## [4,] "Rank2" "Rank1" "Rank4"
## [5,] "Rank4" "Rank5" "Rank3"
```

Substitue with values from the target distribution

```
## Rank1 Rank2 Rank3 Rank4 Rank5
##     1     3     4     5     9
```

Quantile Normalization - Step 3

Go back to the rank matrix

```
##      Array1  Array2  Array3
## [1,] "1"     "Rank3" "Rank5"
## [2,] "Rank3" "Rank4" "Rank1"
## [3,] "Rank5" "Rank2" "Rank2"
## [4,] "Rank2" "Rank1" "Rank4"
## [5,] "Rank4" "Rank5" "Rank3"
```

```
##      Array1  Array2  Array3
## [1,] "1"     "Rank3" "Rank5"
## [2,] "Rank3" "Rank4" "Rank1"
## [3,] "Rank5" "Rank2" "Rank2"
## [4,] "3"     "Rank1" "Rank4"
## [5,] "Rank4" "Rank5" "Rank3"
```

Substitue with values from the target distribution

```
## Rank1 Rank2 Rank3 Rank4 Rank5
##     1     3     4     5     9
```

Quantile Normalization - Step 3

Go back to the rank matrix

```
##      Array1  Array2  Array3
## [1,] "1"     "Rank3" "Rank5"
## [2,] "Rank3" "Rank4" "Rank1"
## [3,] "Rank5" "Rank2" "Rank2"
## [4,] "3"     "Rank1" "Rank4"
## [5,] "Rank4" "Rank5" "Rank3"
```

```
##      Array1  Array2  Array3
## [1,] "1"     "Rank3" "Rank5"
## [2,] "4"     "Rank4" "Rank1"
## [3,] "Rank5" "Rank2" "Rank2"
## [4,] "3"     "Rank1" "Rank4"
## [5,] "Rank4" "Rank5" "Rank3"
```

Substitue with values from the target distribution

```
## Rank1 Rank2 Rank3 Rank4 Rank5
##     1     3     4     5     9
```

Quantile Normalization - Step 3

Go back to the rank matrix

```
##      Array1  Array2  Array3
## [1,] "1"     "Rank3" "Rank5"
## [2,] "4"     "Rank4" "Rank1"
## [3,] "Rank5" "Rank2" "Rank2"
## [4,] "3"     "Rank1" "Rank4"
## [5,] "Rank4" "Rank5" "Rank3"
```

```
##      Array1  Array2  Array3
## [1,] "1"     "Rank3" "Rank5"
## [2,] "4"     "Rank4" "Rank1"
## [3,] "Rank5" "Rank2" "Rank2"
## [4,] "3"     "Rank1" "Rank4"
## [5,] "5"     "Rank5" "Rank3"
```

Substitue with values from the target distribution

```
## Rank1 Rank2 Rank3 Rank4 Rank5
##     1     3     4     5     9
```

Quantile Normalization - Step 3

Go back to the rank matrix

```
##      Array1  Array2  Array3
## [1,] "1"     "Rank3" "Rank5"
## [2,] "4"     "Rank4" "Rank1"
## [3,] "Rank5" "Rank2" "Rank2"
## [4,] "3"     "Rank1" "Rank4"
## [5,] "5"     "Rank5" "Rank3"
```

```
##      Array1 Array2  Array3
## [1,] "1"    "Rank3" "Rank5"
## [2,] "4"    "Rank4" "Rank1"
## [3,] "9"    "Rank2" "Rank2"
## [4,] "3"    "Rank1" "Rank4"
## [5,] "5"    "Rank5" "Rank3"
```

Substitue with values from the target distribution

```
## Rank1 Rank2 Rank3 Rank4 Rank5
##     1     3     4     5     9
```

Quantile Normalization - Summary

We then repeat to get the normalized matrix

Original data

```
##   Array1 Array2 Array3
## A      1      3      9
## B      3      4      1
## C      9      2      5
## D      2      1      7
## E      4      9      6
```

Normalized data

```
##   Array1 Array2 Array3
## A      1      4      9
## B      4      5      1
## C      9      3      3
## D      3      1      5
## E      5      9      4
```

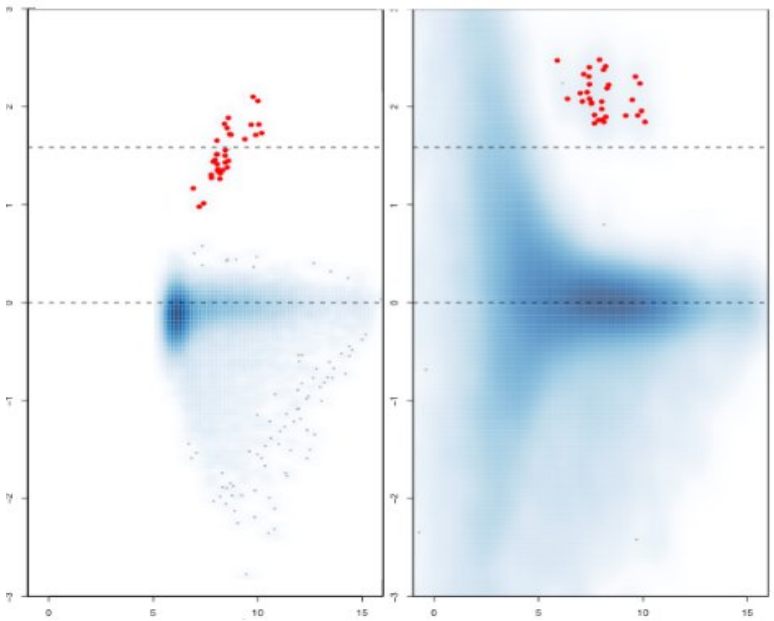
The code

```
df <- data.frame(Array1 = c(1, 3, 9, 2, 4), Array2 = c(3,
    4, 2, 1, 9), Array3 = c(9, 1, 5, 7, 6))
rownames(df) <- LETTERS[1:nrow(df)]
rks <- apply(df, 2, function(x) paste("Rank", rank(x,
    ties.method = "min"), sep = ""))
target <- round(rowMeans(apply(df, 2, sort)), 3)
names(target) <- paste("Rank", 1:length(target), sep = "")
for (i in 1:ncol(df)) {
    for (nm in names(target)) {
        rks[, i] <- gsub(nm, target[nm], rks[, i])
    }
}
norm <- as.data.frame(rks)
```

Background correction

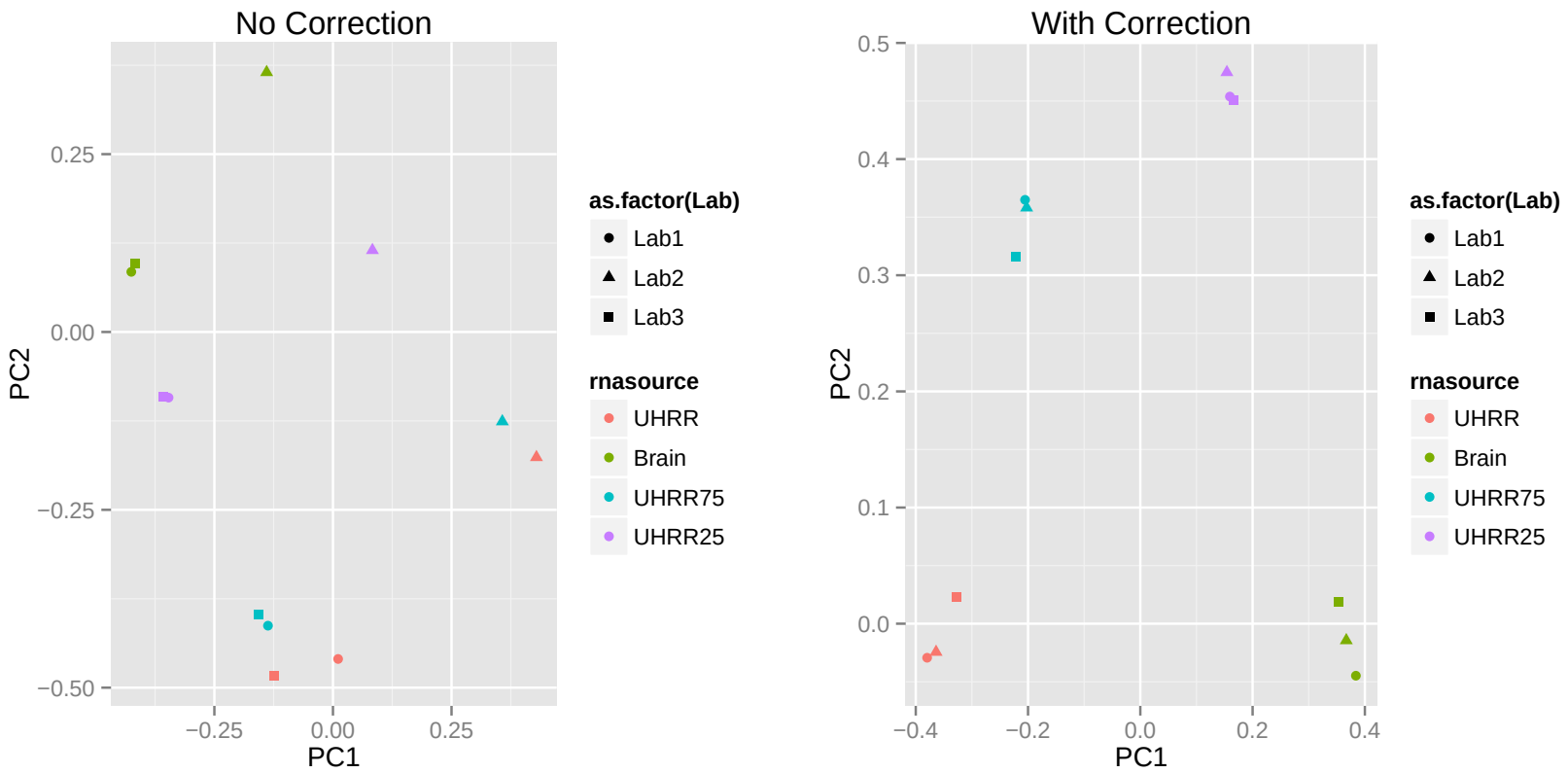
See Shi et al

- If a gene is not being expression, then its intensity on the array would be zero
- ...but it isn't
- Illumina attempt to measure *non-specific binding* using negative controls
- but they do a bad job (see right)



Batch Effects

Experiment with Brain and Reference RNA hybridised at different labs



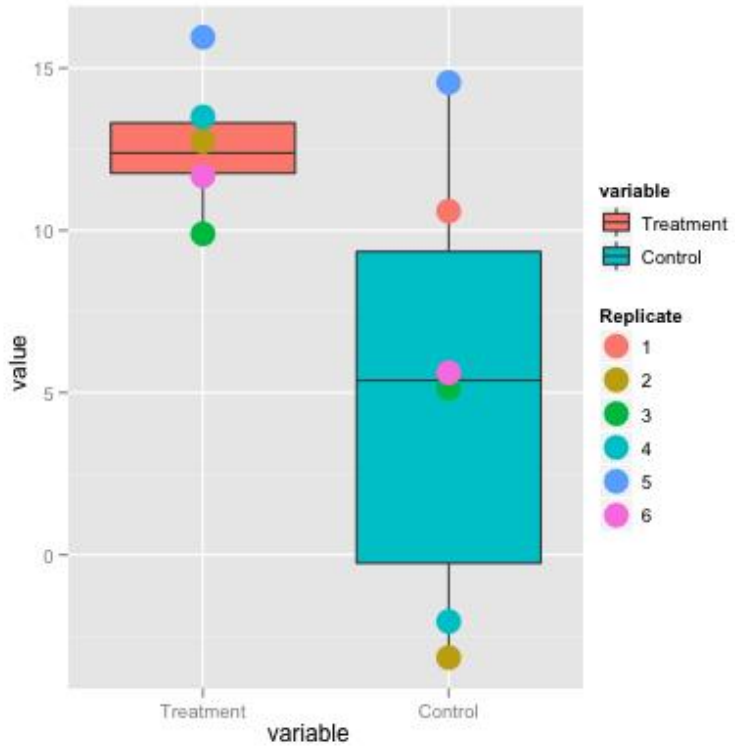
See Leek et al for comprehensive review

Normalisation Options

- The `neqc` method from limma combines quantile normalisation with a more sophisticated way of dealing with non-specific binding
- Also `vst` and `rsn` in lumi
- Ideally one would evaluate different methods
- May also need batch effect correction - see `sva` package in Bioconductor
- Case-study of batch effects on Illumina data provided by Kitchen et al

Differential Gene Expression

- Differential expression is done on per-probe basis
- Compare values in each sample group using (modified version of) 't-test'
  - fold-change
  - p-value
  - log-odds or B-statistic



1. Understanding the data

Normalised, processed data tend to be represented in a standard way in Bioconductor.

- `assayData` a series of N by S matrices
  - exprs
  - se.exprs
- `featureData` matrix of `N` rows to hold probe annotation accessed using `fData`
- `phenoData` matrix of `S` rows to hold sample annotation accessed using `pData`

assayData

```
exprs(normData)[1:5, 1:3]

             4613710017_B 4613710052_B
ILMN_1802380        8.430        8.445
ILMN_1893287        5.411        5.392
ILMN_1736104        5.288        5.427
ILMN_1792389        6.760        7.073
ILMN_1854015        5.584        5.680
             4613710054_B
ILMN_1802380        8.297
ILMN_1893287        5.179
ILMN_1736104        5.036
ILMN_1792389        6.827
ILMN_1854015        5.570
```

featureData

Each row matches a row in the expression matrix. Can have as many columns as you like.

```
fData(normData)[1:5, ]
```

```
                Row.names ArrayAddressID
ILMN_1802380 ILMN_1802380         10008
ILMN_1893287 ILMN_1893287         10010
ILMN_1736104 ILMN_1736104         10017
ILMN_1792389 ILMN_1792389         10019
ILMN_1854015 ILMN_1854015         10020
                IlluminaID  Status SYMBOL
ILMN_1802380 ILMN_1802380 regular   RERE
ILMN_1893287 ILMN_1893287 regular   <NA>
ILMN_1736104 ILMN_1736104 regular   <NA>
ILMN_1792389 ILMN_1792389 regular RNF165
ILMN_1854015 ILMN_1854015 regular   <NA>
                          GENOMICLOCATION
```

phenoData

Each row matches a **column** in the expression matrix. Can have as many columns as you like. Sample group information is commonly stored in this matrix.

```
pData(normData)[1:3, ]
```

```
                sampleID SampleFac
4613710017_B 4613710017_B      UHRR
4613710052_B 4613710052_B      UHRR
4613710054_B 4613710054_B      UHRR
```

2. Build the design matrix

In my analysis I want to compare Brain and UHRR groups

```
library(limma)
grps <- pData(normData)[, 2]
grps
```

```
##  [1] "UHRR"  "UHRR"  "UHRR"  "UHRR"  "UHRR"
##  [6] "UHRR"  "Brain" "Brain" "Brain" "Brain"
```

```
design <- model.matrix(~0 + as.factor(grps))
head(design)
```

```
##   as.factor(grps)Brain as.factor(grps)UHRR
## 1                    0                   1
## 2                    0                   1
## 3                    0                   1
## 4                    0                   1
## 5                    0                   1
```

3. Fit the linear model

Fit the linear model to the expression matrix

```
fit <- lmFit(exprs(normData), design)
fit
```

```
## An object of class "MArrayLM"
## $coefficients
##                Brain  UHRR
## ILMN_1802380 9.930 8.464
## ILMN_1893287 5.507 5.348
## ILMN_1736104 5.377 5.231
## ILMN_1792389 8.280 7.120
## ILMN_1854015 5.839 5.653
## 49571 more rows ...
##
## $stdev.unscaled
##                Brain   UHRR
## ILMN_1802380     0.5 0.4082
## ILMN_1893287     0.5 0.4082
```

3. Fit the linear model

Fit the linear model to the expression matrix

```
names(fit)

##  [1] "coefficients"     "stdev.unscaled"
##  [3] "sigma"            "df.residual"
##  [5] "cov.coefficients" "pivot"
##  [7] "genes"            "Amean"
##  [9] "method"           "design"

head(fit$coefficients, 2)

##                Brain  UHRR
## ILMN_1802380 9.930 8.464
## ILMN_1893287 5.507 5.348
```

A sanity check

```
grps
```

```
##  [1] "UHRR"  "UHRR"  "UHRR"  "UHRR"  "UHRR"
##  [6] "UHRR"  "Brain" "Brain" "Brain" "Brain"
```

```
mean(exprs(normData)[1, 1:6])
```

```
## [1] 8.464
```

```
mean(exprs(normData)[1, 7:10])
```

```
## [1] 9.93
```

```
fit$coefficients[1, ]
```

```
## Brain  UHRR
## 9.930 8.464
```

4. Make the contrast Matrix

```
cMat <- makeContrasts(DE = Brain - UHRR, levels = design)
cMat
```

```
##         Contrasts
## Levels   DE
##   Brain   1
##   UHRR   -1
```

```
fit2 <- contrasts.fit(fit, cMat)
head(fit2$coefficients, 2)
```

```
##                  Contrasts
##                         DE
##   ILMN_1802380 1.4654
##   ILMN_1893287 0.1588
```

```
head(fit2$coefficients, 2)

##                 Contrasts
##                        DE
##    ILMN_1802380 1.4654
##    ILMN_1893287 0.1588

fit$coefficients[1:2, 1] - fit$coefficients[1:2, 2]

## ILMN_1802380 ILMN_1893287
##       1.4654       0.1588
```

5. Annotate

For convenience, we attach the probe annotations to the data.

```
fit2$genes <- fData(normData)
```

How we dervie the annotations will be the subject of the next section...

5. Do the Empirical Bayes

```
efit <- eBayes(fit2)
names(efit)
```

```
##  [1] "coefficients"     "stdev.unscaled"
##  [3] "sigma"            "df.residual"
##  [5] "cov.coefficients" "genes"
##  [7] "Amean"            "method"
##  [9] "design"           "contrasts"
## [11] "df.prior"         "s2.prior"
## [13] "var.prior"        "proportion"
## [15] "s2.post"          "t"
## [17] "df.total"         "p.value"
## [19] "lods"             "F"
## [21] "F.p.value"
```

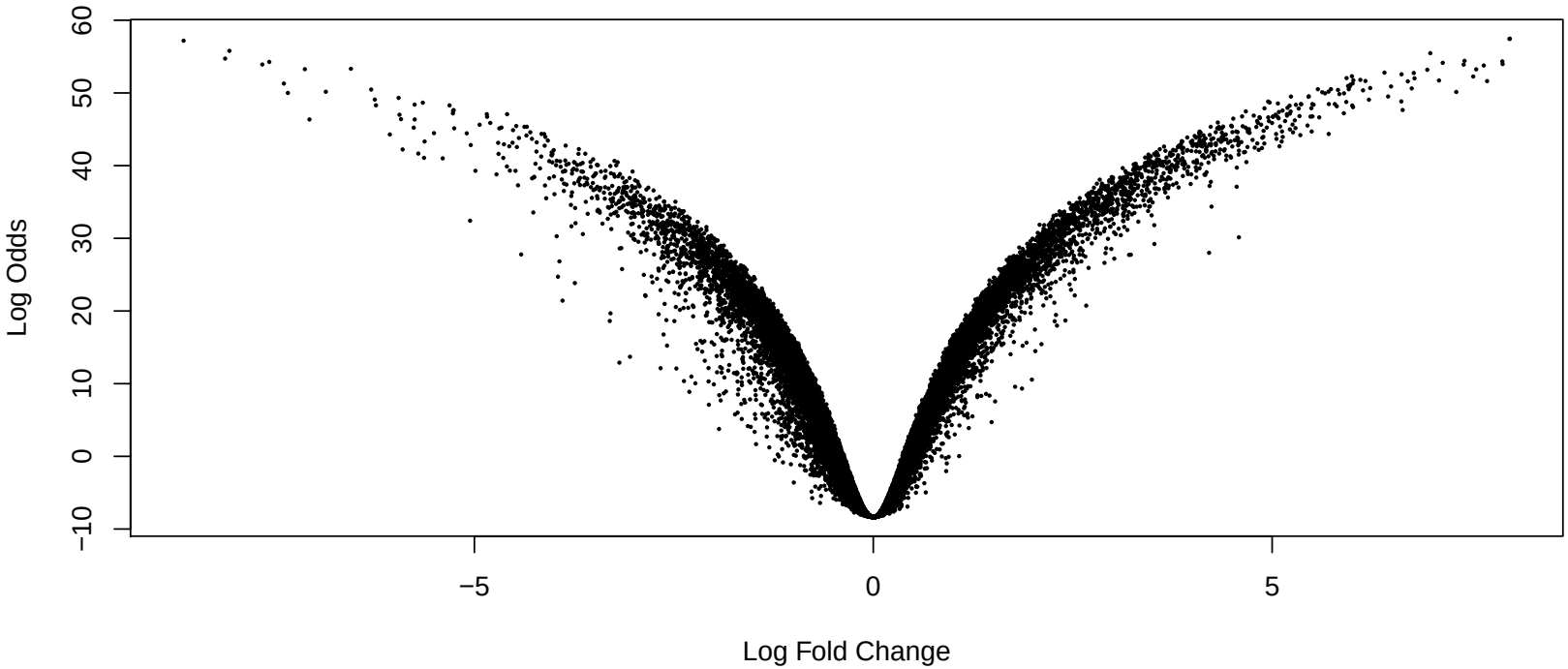
6. Tabulate the results

```
topTable(efit, n = 2)
```

```
##          Row.names ArrayAddressID   IlluminaID
## 19910 ILMN_1675947       3060273 ILMN_1675947
## 5703  ILMN_1795679        830672 ILMN_1795679
##        Status SYMBOL           GENOMICLOCATION
## 19910 regular    MT3 chr16:56624846:56624895:+
## 5703  regular  STMN2  chr8:80549059:80549108:+
##       PROBEQUALITY logFC AveExpr     t   P.Value
## 19910      Perfect 7.980   8.822 107.9 5.849e-30
## 5703       Perfect 7.976   9.152 107.7 6.136e-30
##       adj.P.Val     B
## 19910 1.365e-25 57.47
## 5703  1.365e-25 57.43
```

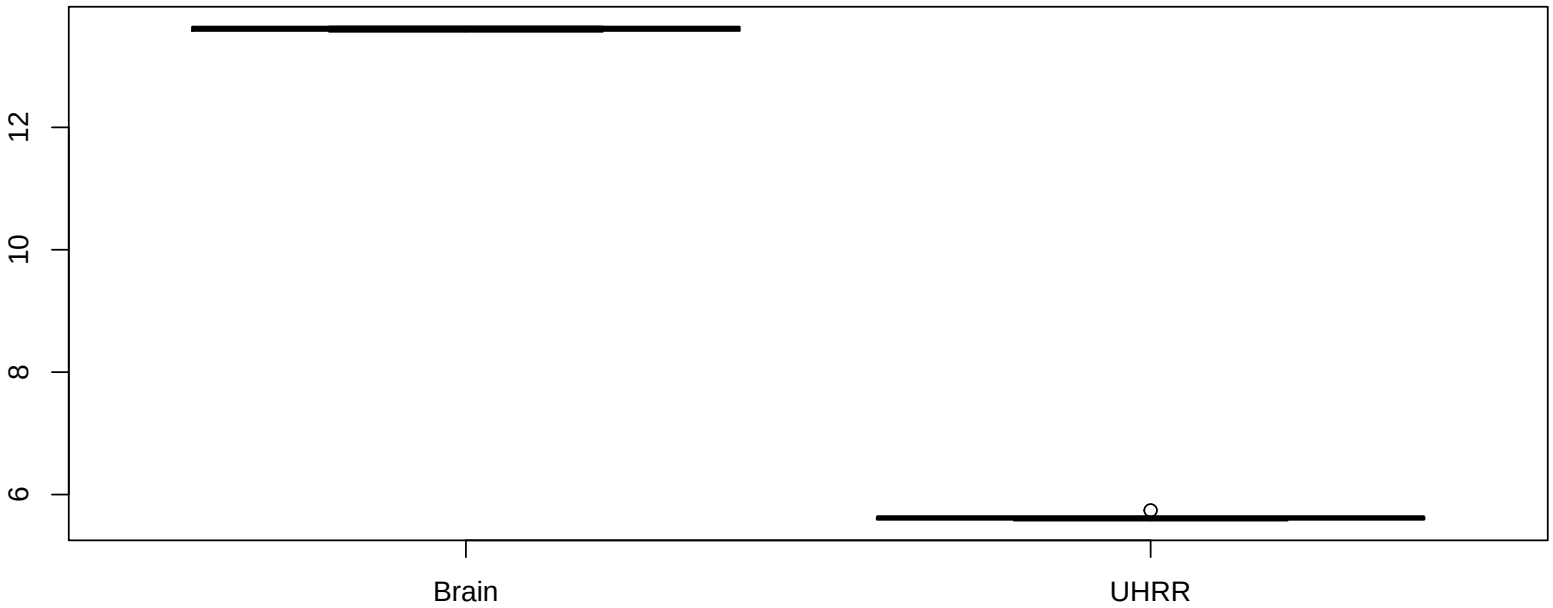
7. Visualise

```
volcanoplot(efit)
```

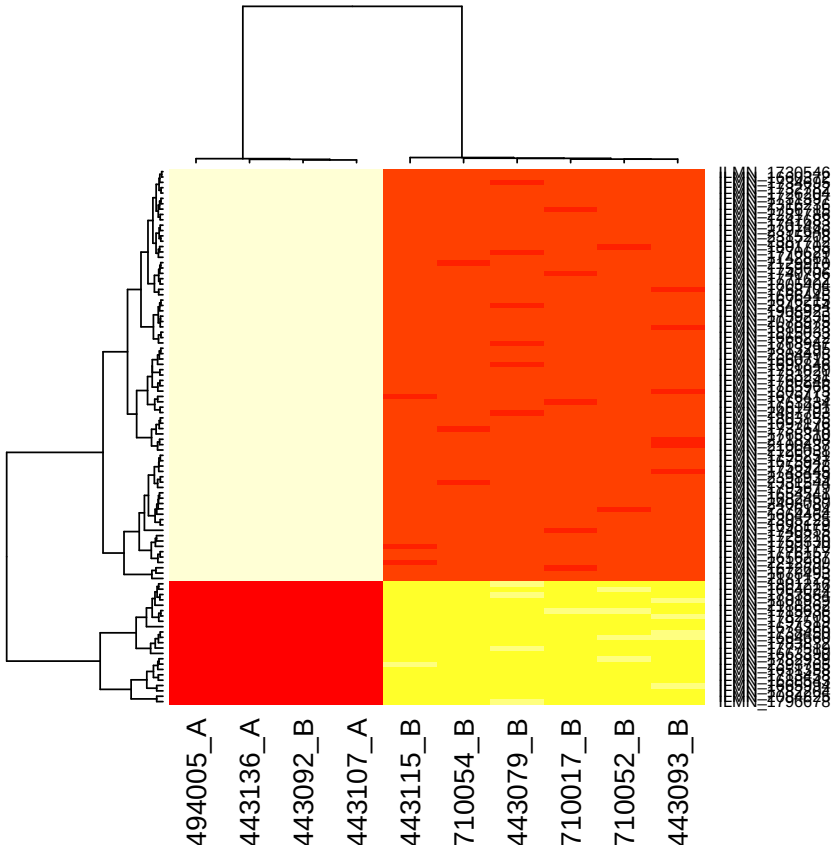


Another sanity check

```
boxplot(exprs(normData)["ILMN_1675947", ] ~ grps)
```



```
topDE <- order(efit$lods, decreasing = T)[1:100]
heatmap(as.matrix(exprs(normData)[topDE, ]))
```

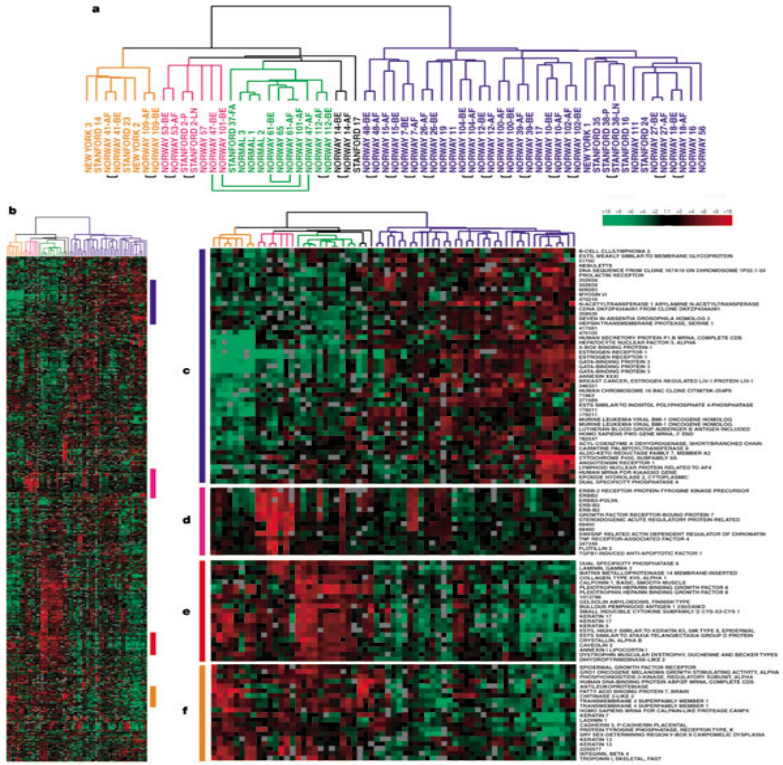


Why annotate probes?

- By default, each probe is assigned a numeric Code (*ArrayAddress*) if analysed at the bead-level, or a manufactuer identifer (*ILMN_*)
- The IDs need to be converted so that we could recognise our favourite genes in the data. e.g. TP53, BRCA1
- Or to relate the findings to biological function
- Is this the end of the story?

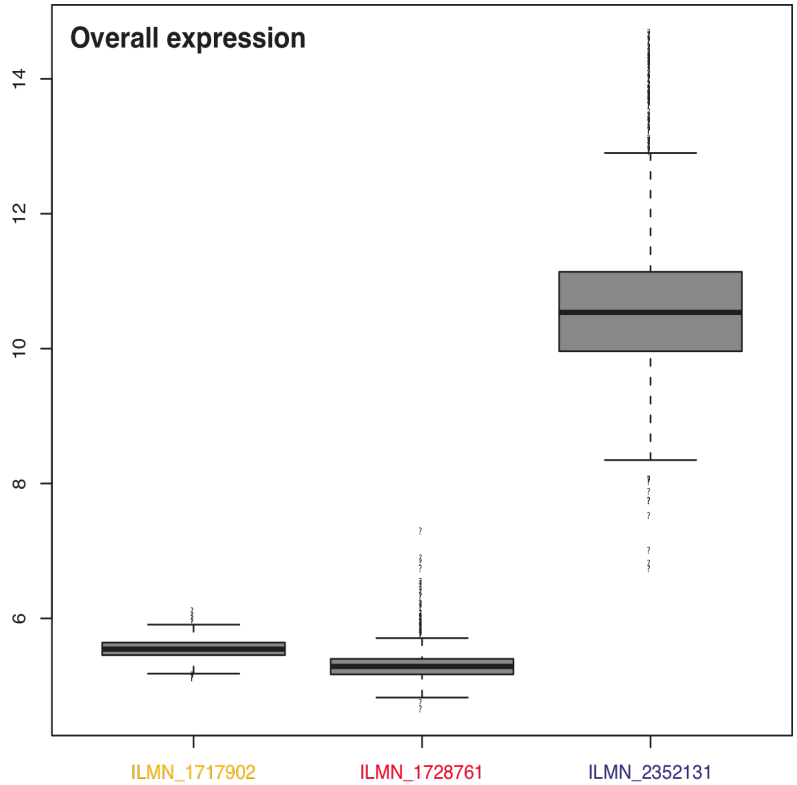
A motivating example

- Starting with Perou et al (right) various papers have classified breast cancer based on microarray profiles. They seem to agree on five subtypes, which have different prognosis and treatment options

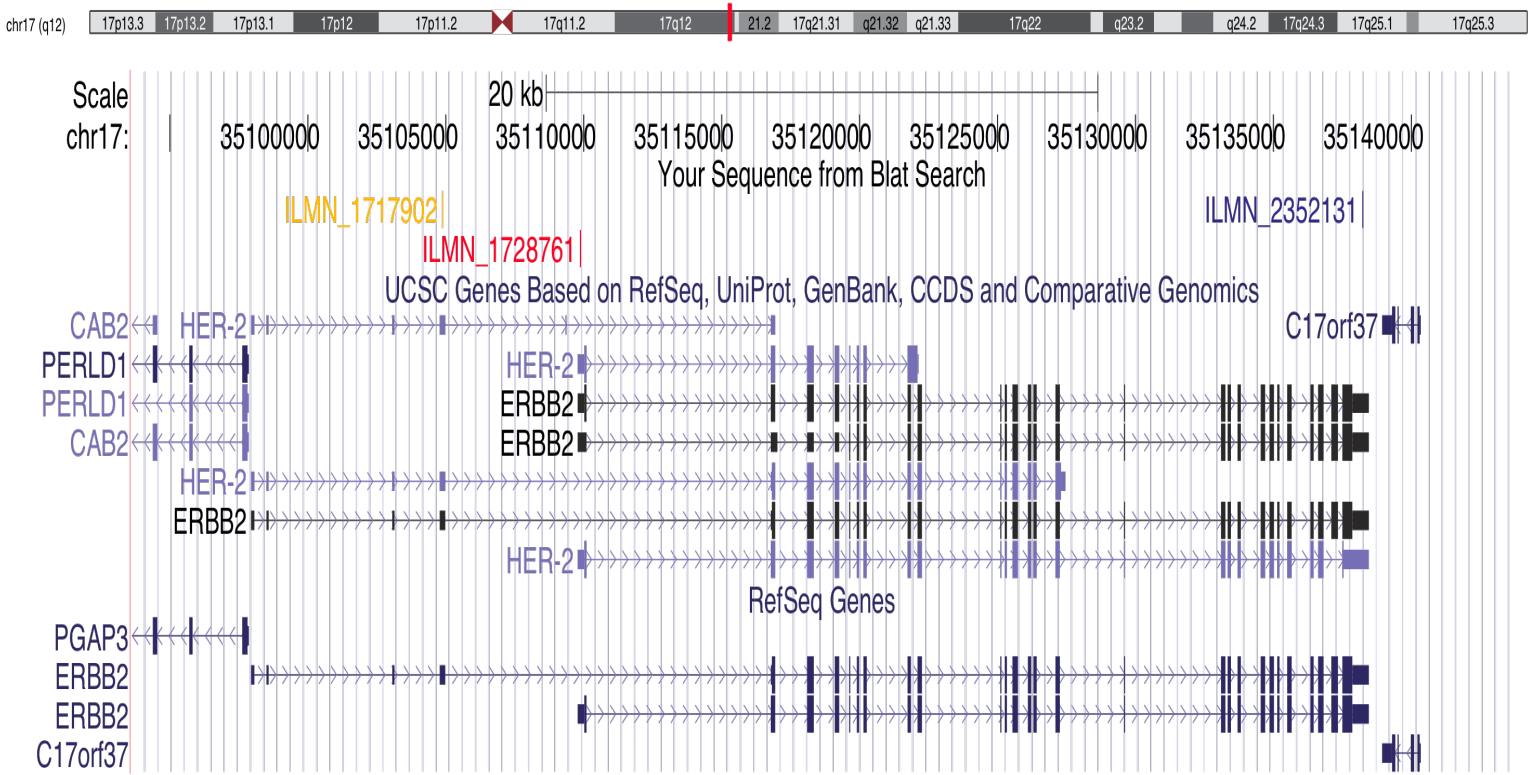


Expression of ERBB2 in a breast cancer study

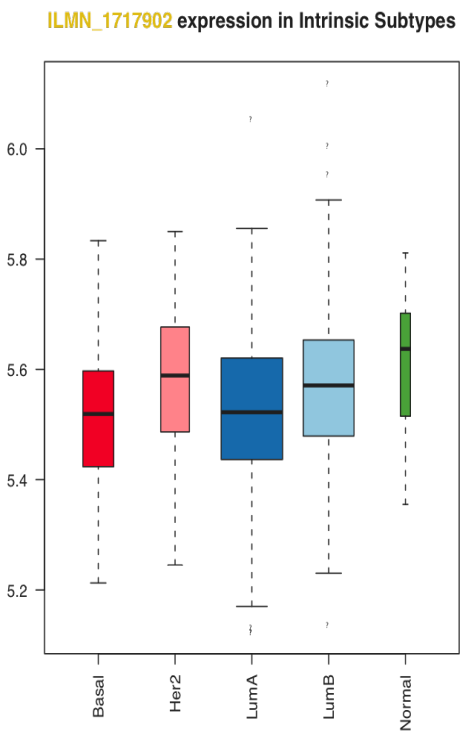
- The ERBB2 oncogene is important in breast cancer, so we want to be able to measure it accurately
- In the METABRIC study, three probes for ERBB2 on the microarray
- Simple approach would be to average values for all three probes

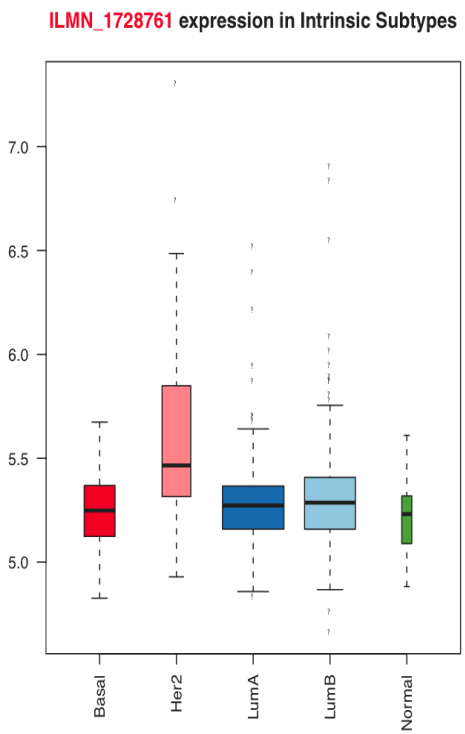


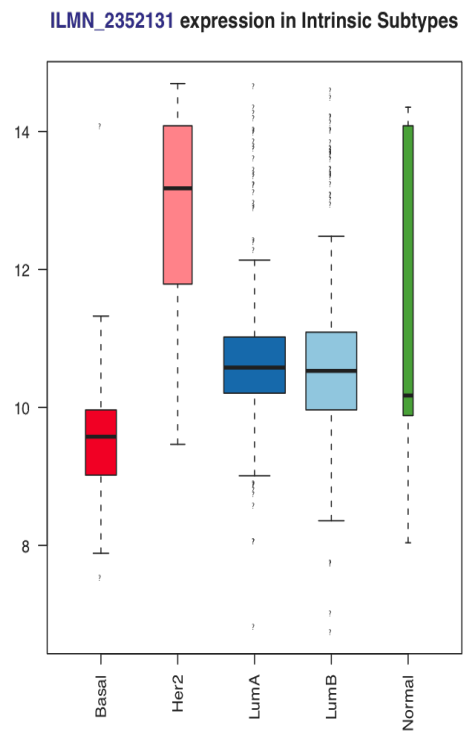
Location of ERBB2 probes



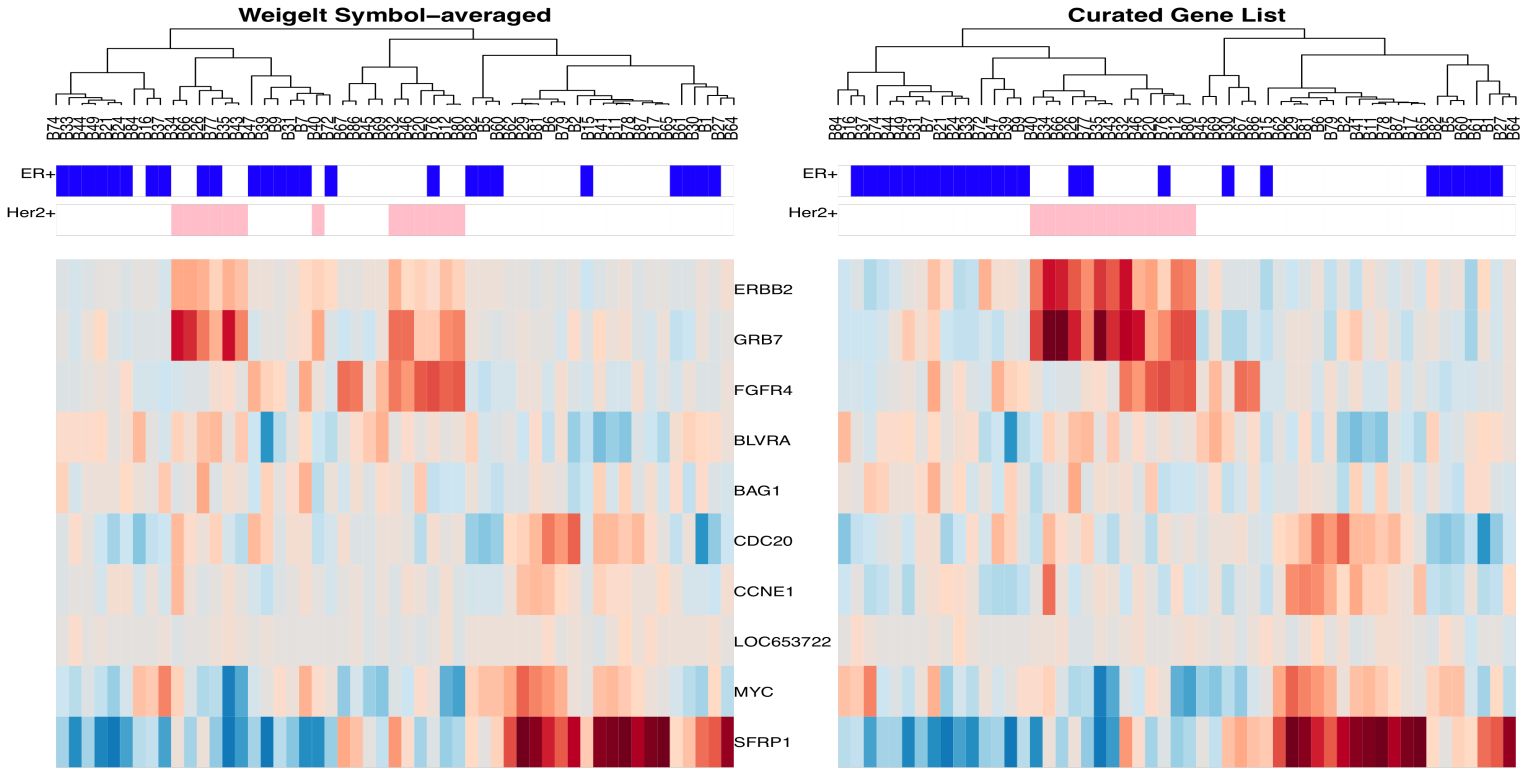
Difference between subtypes



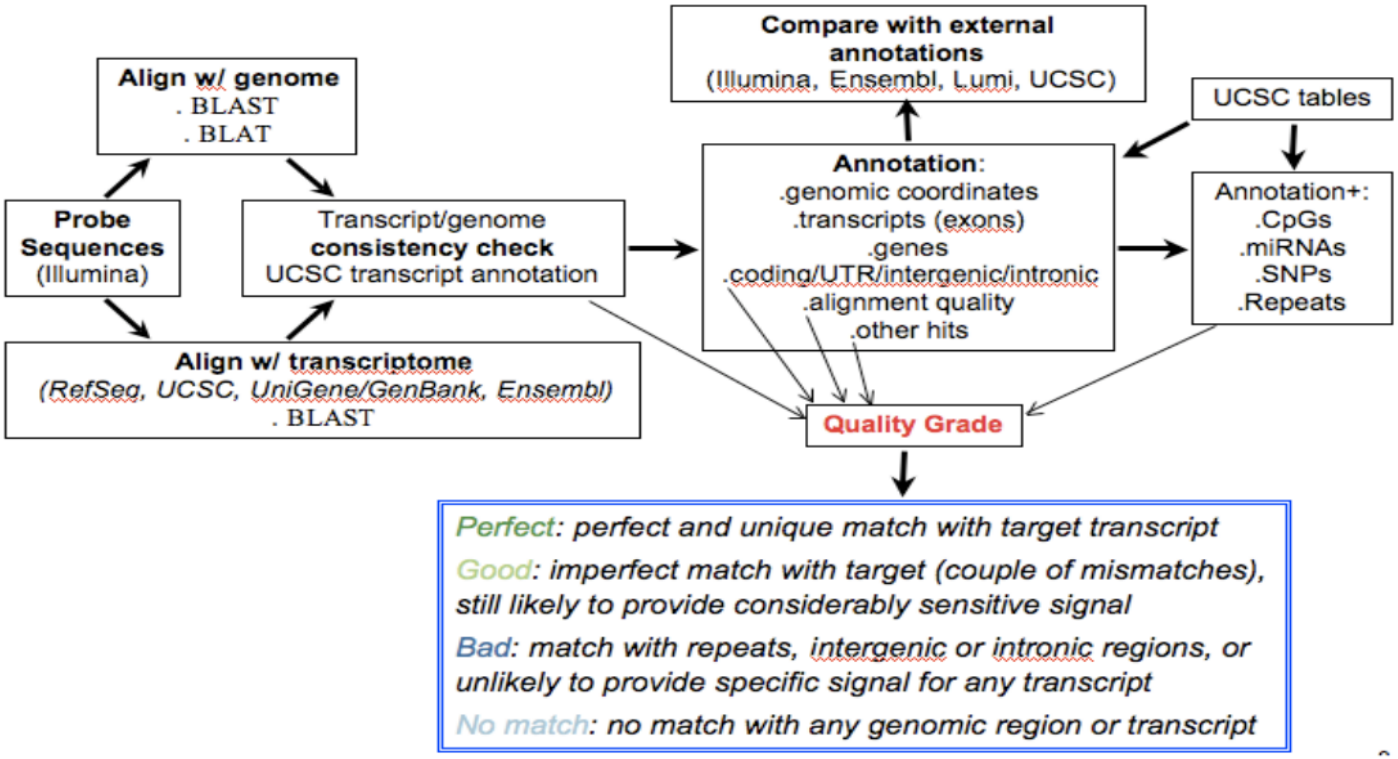




Using external data

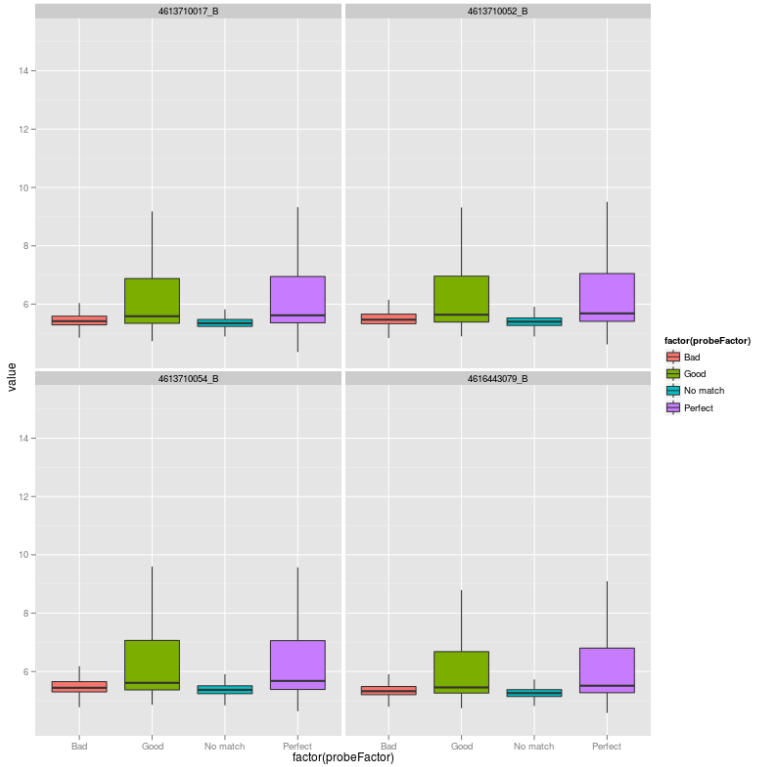


An annotation pipeline



Effect on intensity

- As expected Perfect and Good probes highest intensity
- Less signal for Bad and No match
- Although some exceptions..
- Associations also possible due to SNPs within probes



Annotation conclusions

- Annotation quality is known in advance
- Annotation can be used to remove uninformative data for filtering and reducing number of tests performed
- Similar in spirit to removing non-expressed probes
- Surprising results could be explained by annotation problems; missing genes from top hits, or unexpected findings
- If you *must* collapse to the gene-level, consider the annotation whilst doing so. **Please don't just average**

Similar issues for Affymetrix arrays

In practice

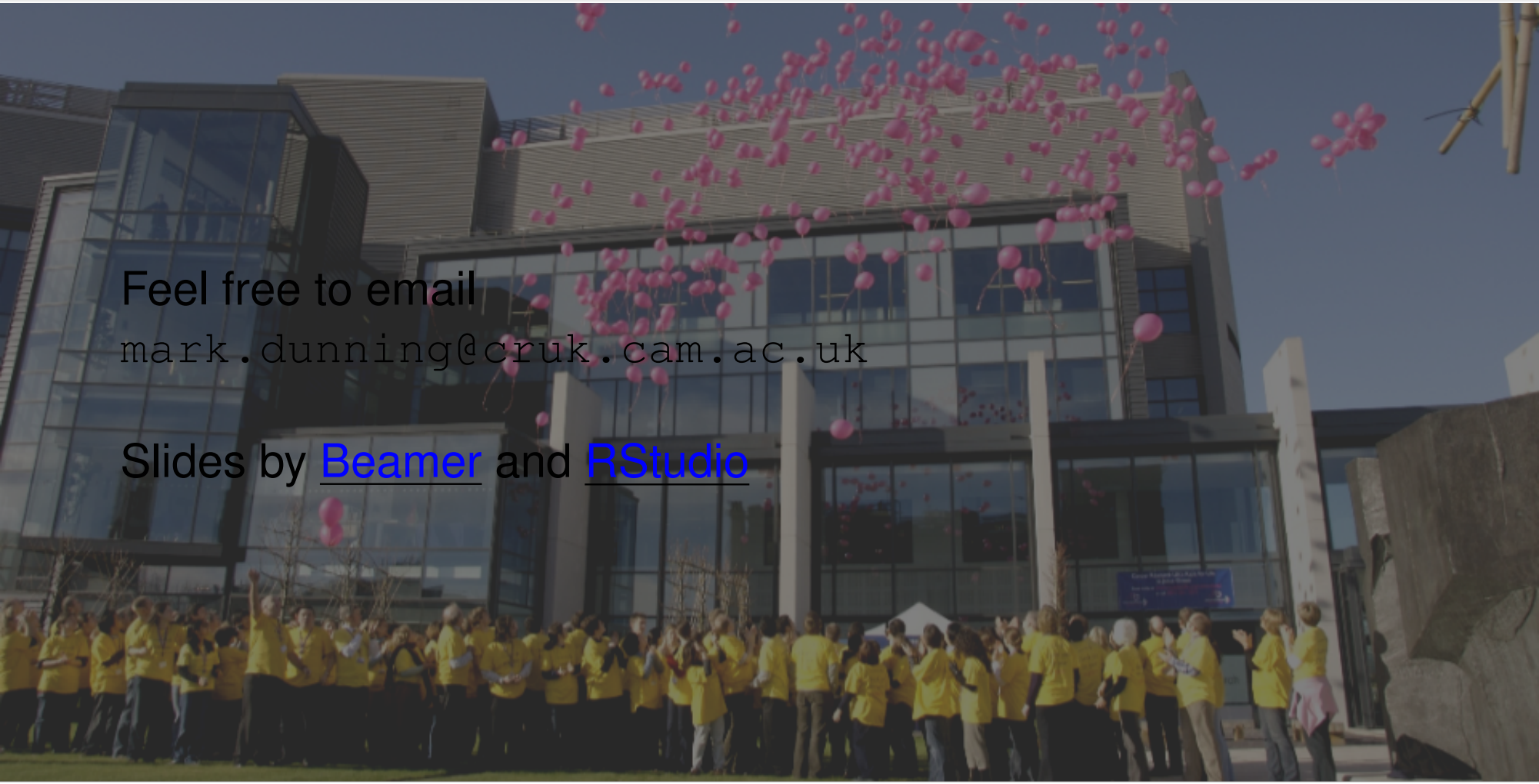
What I *should* have done before

```
quals <- fData(normData)$PROBEQUALITY
bestProbes <- quals == "Perfect"
fit.new <- lmFit(exprs(normData)[bestProbes, ], design)
```

Where does the annotation come from?

- The annotation slot in the bead-level data is the link to the correct annotation package
- If unsure, the `suggestAnnotation` function will try to advise
- `Humanv3` tells beadarray to use illuminaHumanv3.db for annotation
- If installed, the `illuminaHumanv3.db` will be loaded
- The control probes can then be identified and IDs converted automatically

The End



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Slides by Beamer and RStudio