Concepts and Main Aspects of RNA-Seq

Wellcome Trust Centre for Human Genetics

25th, 28th and 29th April 2016

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Overview of Gene Expression Profiling and Experimental Design

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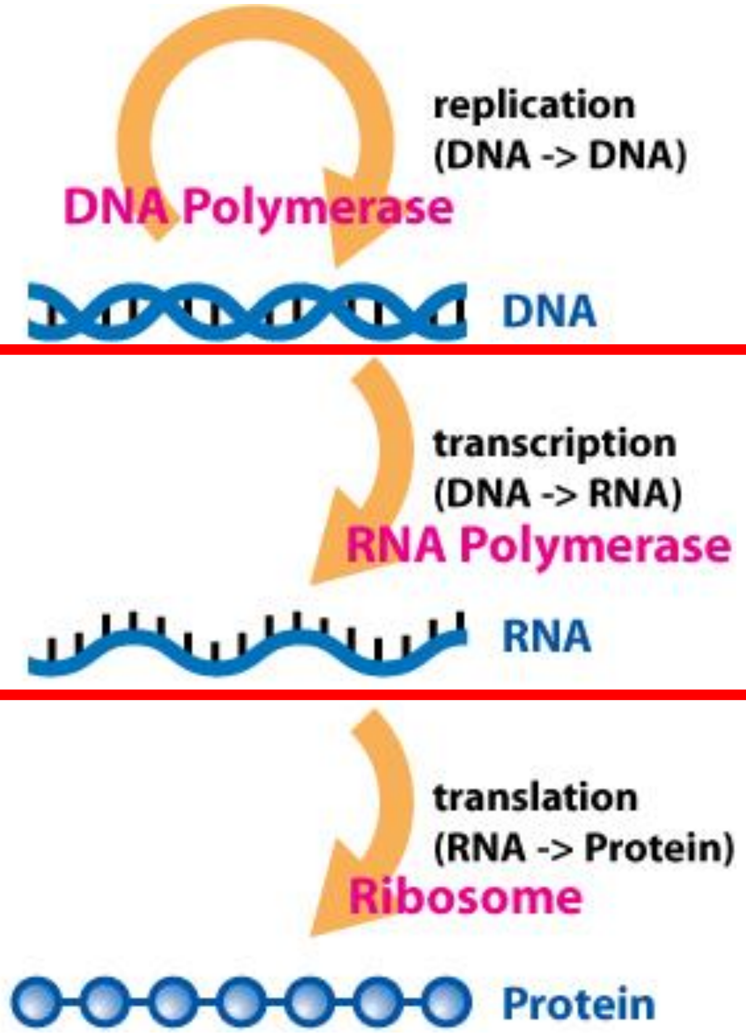
Day 2 Overview

- General remarks on gene expression data
- RNA-Seq and microarray technology
- Experimental design considerations
- Transcript quantification
- Data normalisation and quality
- Differential expression analysis
- Biological interpretation using pathway analysis

Schedule

Time	Topic
09.45-10.30	RNA-Seq experimental design
10.30-11.00	Transcript quantification, normalisation
11.00-11.15	Coffee break
11.15-12.00	Differential expression analysis
12.00-12.30	Pathway analysis
12.30-13.30	Lunch
13.30-16.00	Practical sessions (break at 15.00)

Transcriptome profiling



Entire transcriptome can be measured by microarrays or RNA-Seq

Widely-used techniques, provide insight into biological system, albeit a snapshot – highly dynamic and complex process (splicing, gene methylation, RNA stability/degradation, miRNA regulation etc)

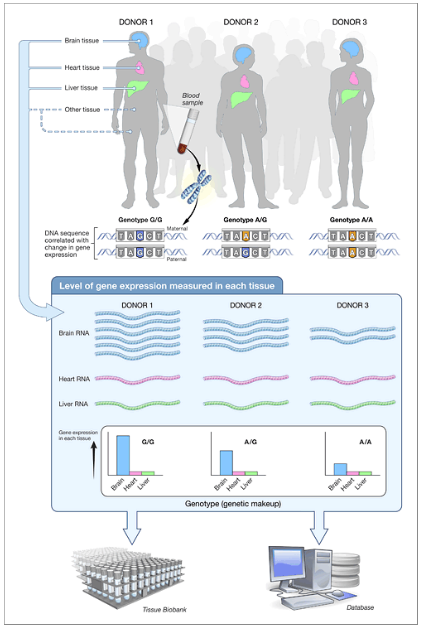
Examples of large gene expression projects

- ENCODE
- Allen brain atlas
- Genotype-Tissue Expression Project (GTEx)
- TGCA

ALLEN BRAIN ATLAS
DATA PORTAL

THE CANCER GENOME ATLAS

- Public repositories
  - Gene Expression Omnibus (GEO) http://www.ncbi.nlm.nih.gov/geo/
  - Sequence Read Archive (SRA)
  - http://www.ncbi.nlm.nih.gov/sra

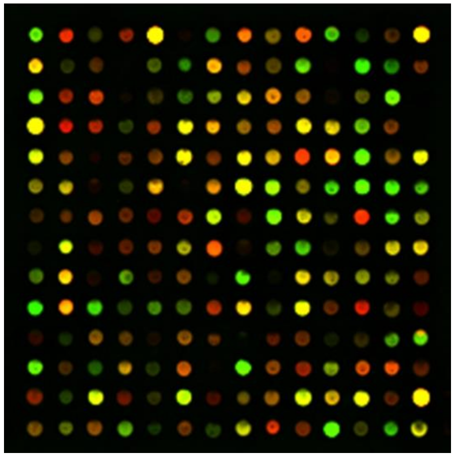


Typical expression profiling designs

- Disease vs control
- Gene knockdown/knockout vs wildtype
- Effect of treatment/stimulus/drug

- Clinical applications
  - Tumour-normal pairs
  - Good prognosis vs poor prognosis
  - Patient subgroups responding to different treatments
  - ‘Gene signature’ to predict who will respond well to a given treatment
- Time course
- Different tissues/stages of development

Premise of gene expression profiling



- Compare gene expression in different conditions
- Differentially expressed genes may provide some biological insight
- But not magical solutions! Large amounts of descriptive data generated – what to do next?

Limitations of gene expression data

- Comprehensive but inherently limited to descriptive results, no matter how well experiment performed or data analysed

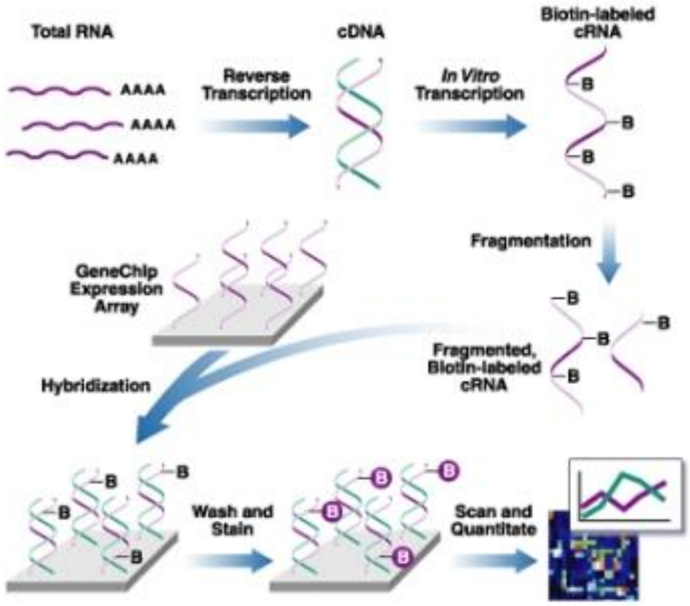
- Produce large amounts of information; subjective interpretation, can be mined in different ways, always much left untouched (often publically available)

- Expensive and time-consuming so often published as a stand-alone experiment

- However best used as starting point for further work - following up hypotheses from gene expression data to uncover mechanistic/causal effects can produce elegant studies

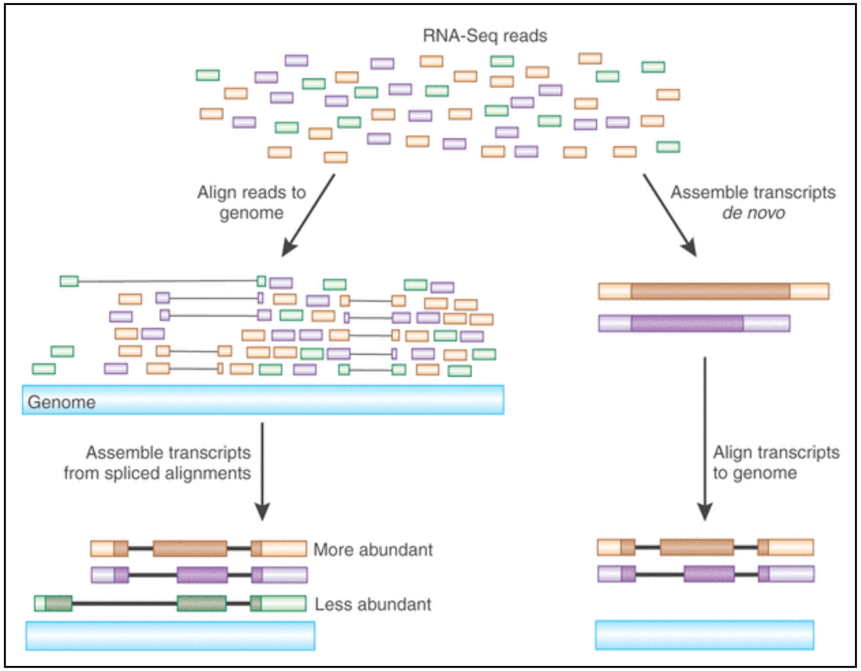
Two key technologies

Microarrays



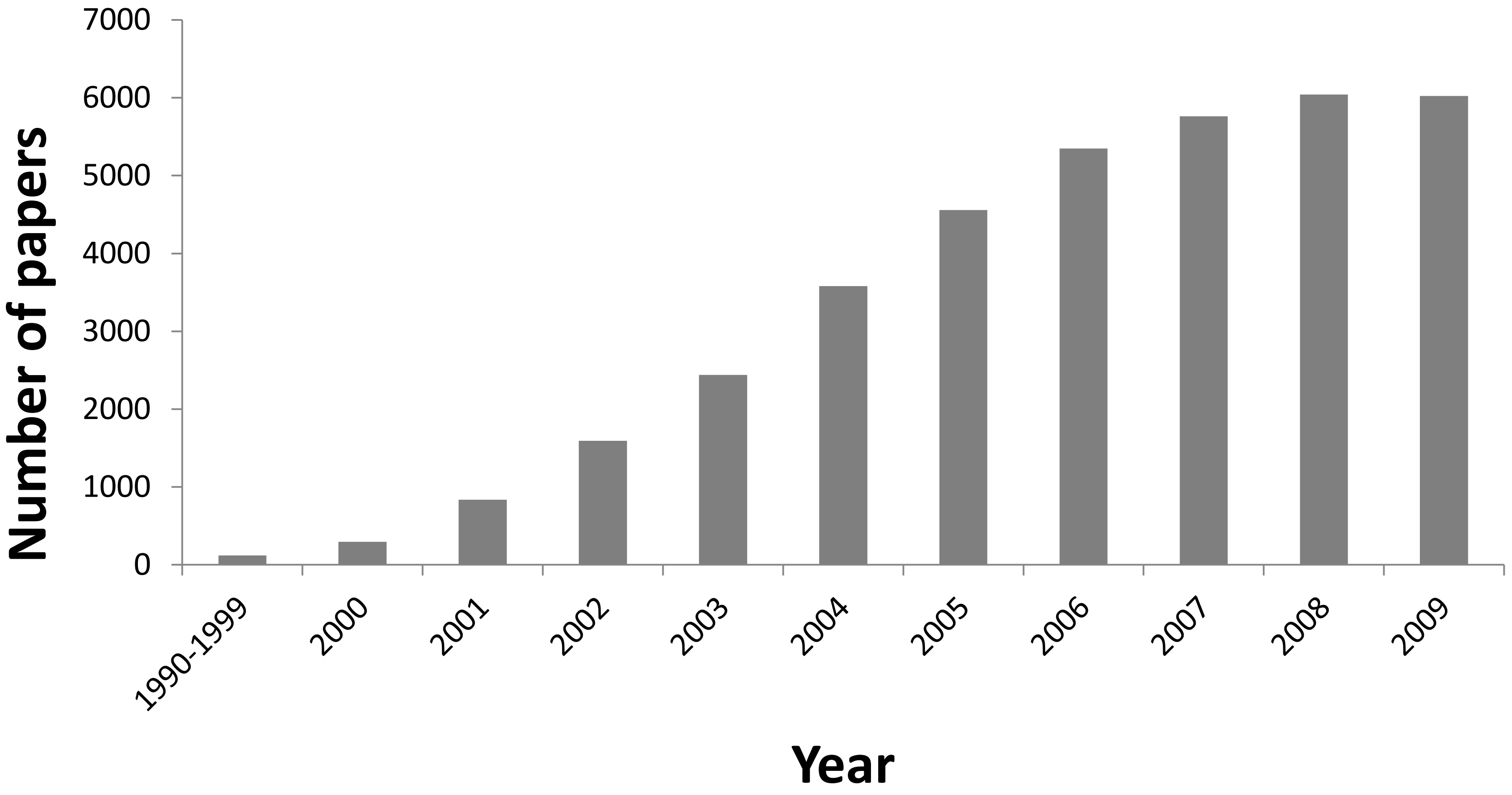
Complementary hybridisation
early 1990s onwards

RNA-Seq


RNA-Seq reads
Align reads to genome
Assemble transcripts *de novo*
Genome
Assemble transcripts from spliced alignments
Align transcripts to genome
More abundant
Less abundant

Next-generation sequencing
2007 onwards

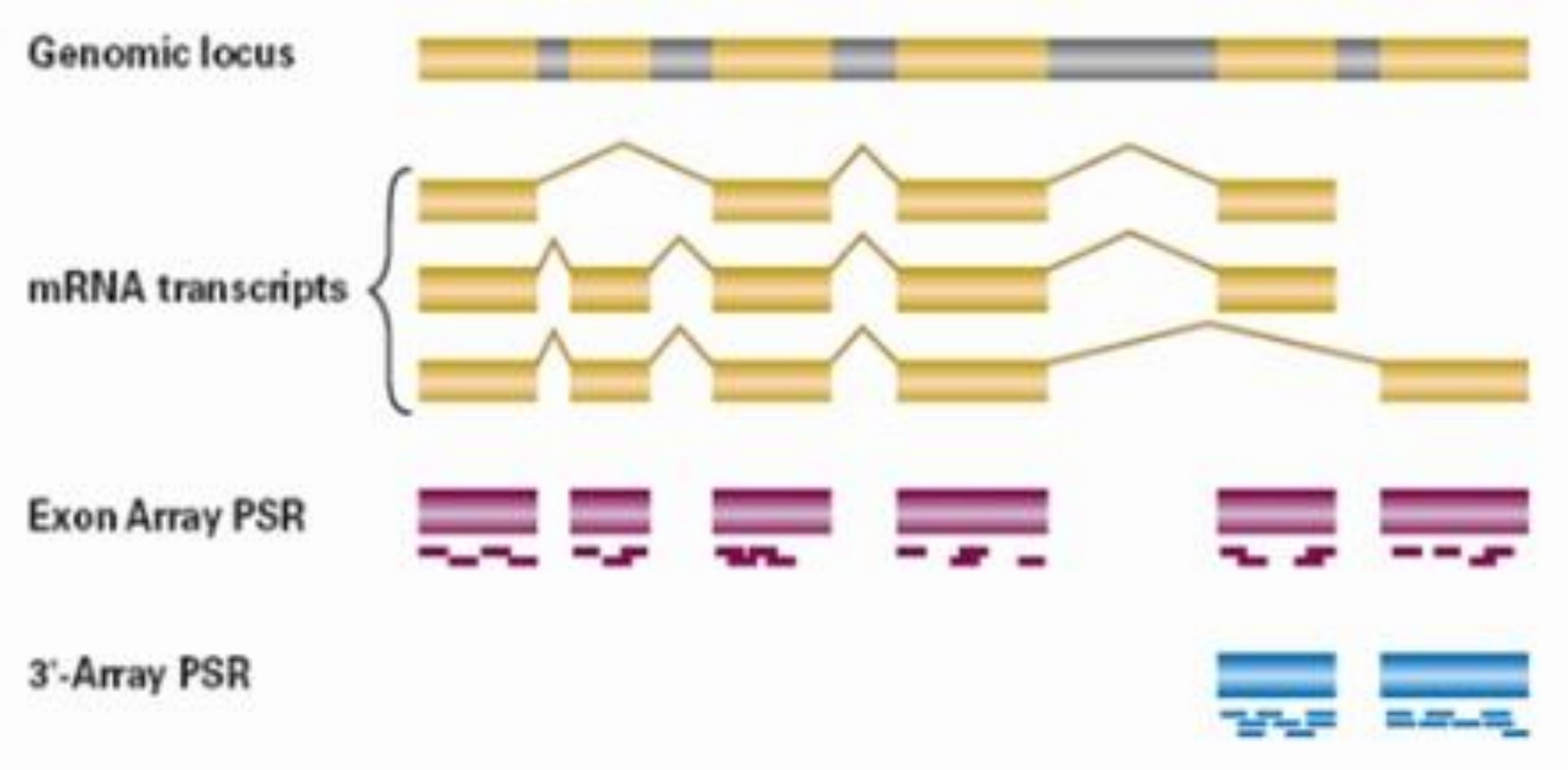
Microarrays in the Literature



Which technology to use?

- Microarrays and RNA-Seq are complementary technologies (despite common perception that RNA-Seq superior)
- Choice usually depends how detailed a characterisation of the transcriptome is required
  - Gene level changes => microarrays sufficient
  - Isoform structure, splicing, novel transcripts => RNA-Seq
- Many low expressed genes in a given sample type in both technologies

Exon Array Design



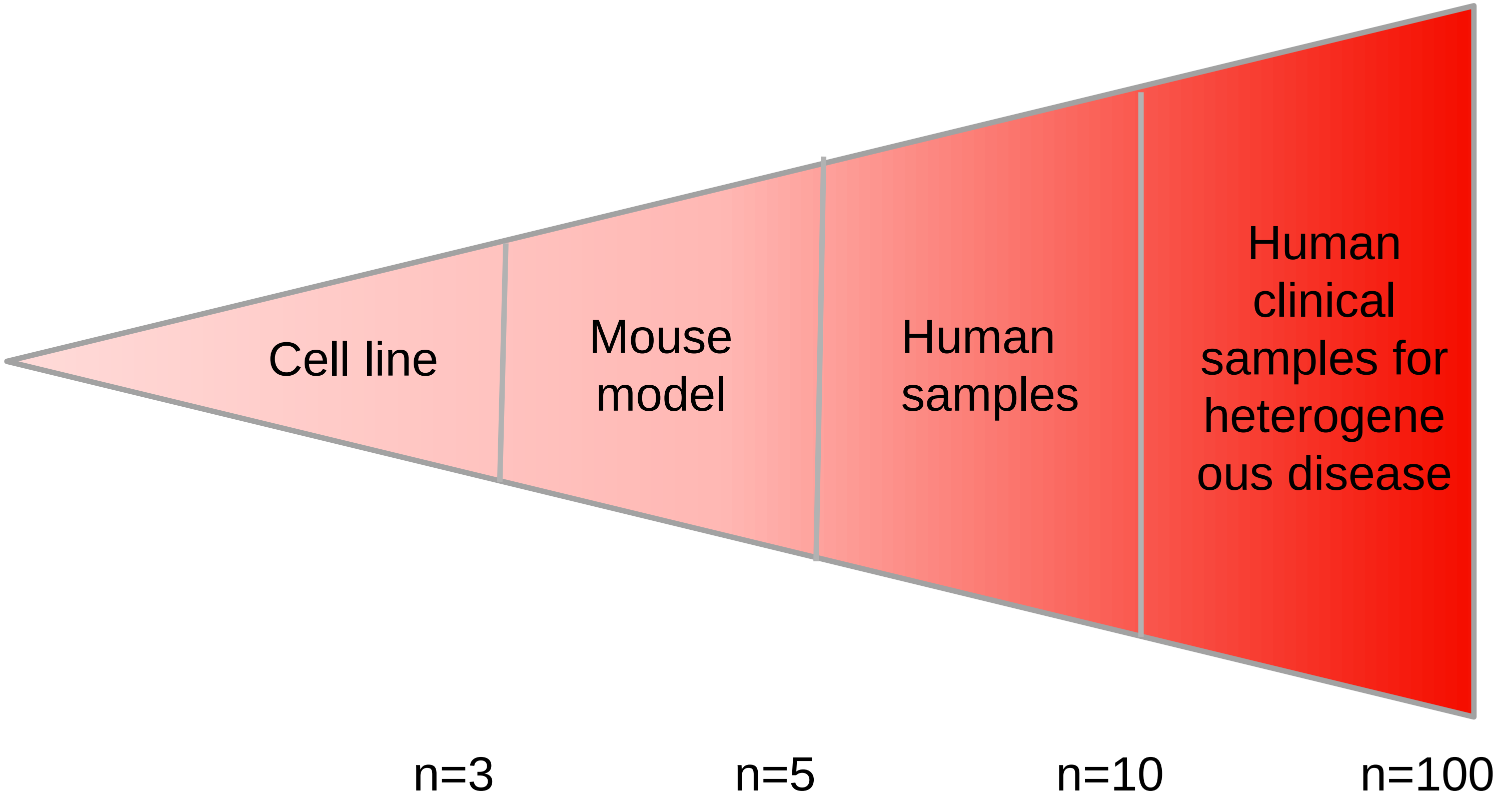
Picture from Affymetrix

Experimental Considerations

- Number of replicates
- Sequencing depth (number of reads per sample)
- Good experimental design principles

Replicates

- Depends on context – type of sample, size of effect, heterogeneity within conditions



Sequencing Depth

- Number of reads required per sample depends on experimental question

- HiSeq4000 – one lane = 250 million reads
- Multiplexing e.g. 10-plex human samples gives ~25m reads for each, plenty for quantifying gene expression (except for very low/unexpressed genes)

- Higher depth required in some situations e.g. for splicing analysis, certain library prep methods (Ribo-depletion)

Single or Paired-end?

- Increased mapped reads with PE data – possibly one read maps to non-unique regions, while second read helps anchor to a specific gene/location

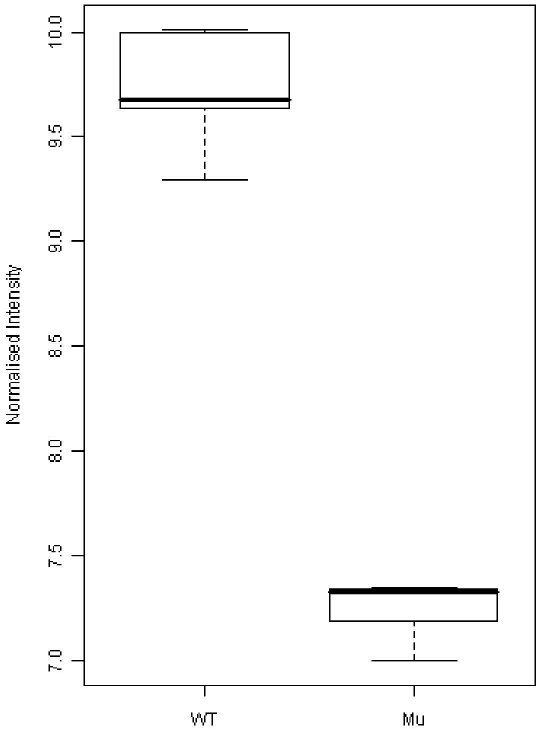
Other experimental considerations

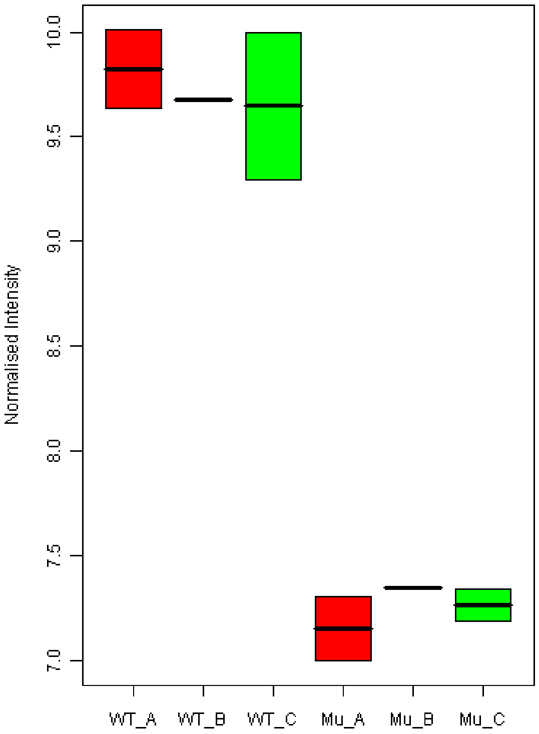
- Sufficient replication/depth for purpose

- Avoid confounding factors when obtaining/preparing samples – gene expression data highly sensitive to many factors

- Be aware of potential effects of unrelated factors on the data, which may need to be accounted for to optimise analysis

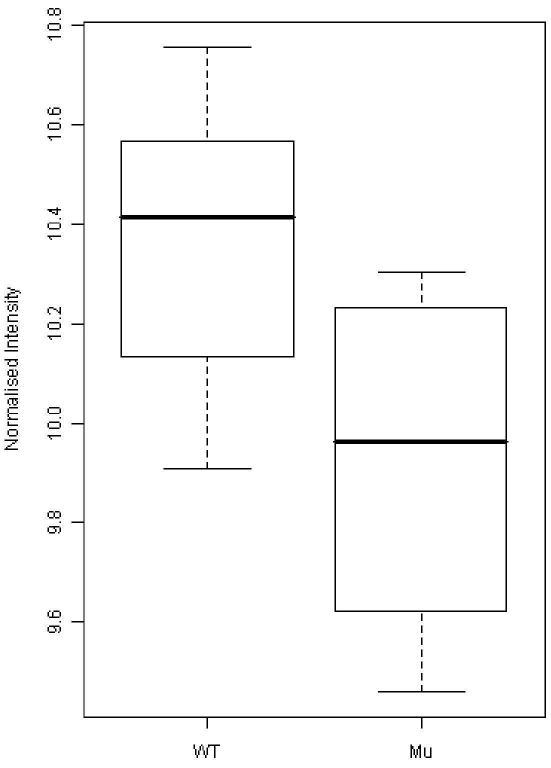
Effect of other variables

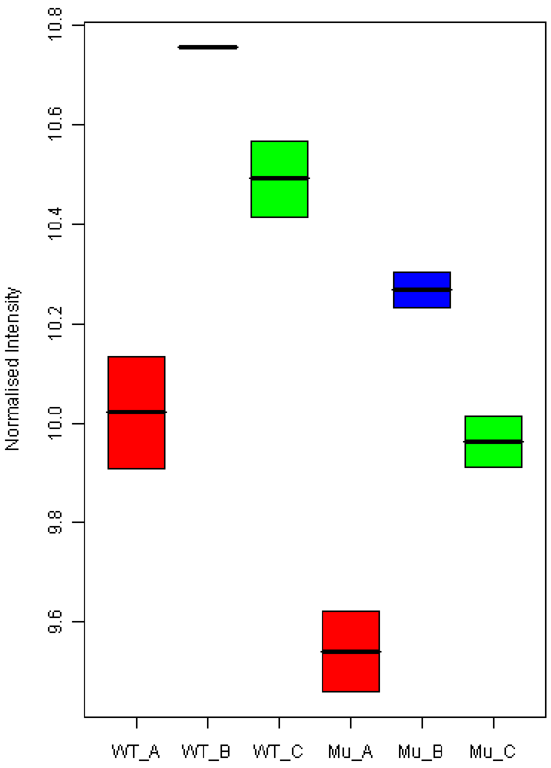




- Wt and Mut groups
- Three different litters
- Top gene ~ 5x higher expression in Wt compared to Mut
- Similarly expressed across litters in both genotypes

Strong litter effect





- Overlap between groups
- Within litters, consistent pattern of higher expression in WT vs Mut
- Within genotypes, B>C>A – expression depends on litter
- Accounting for this variance increases power