Microarray Technology

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Black Ochre Data Labs
The Kids Research Institute Australia

Acknowledgement Of Country

I'd like to acknowledge the Kaurna people as the traditional owners and custodians of the land we know today as the Adelaide Plains, where I live & work.

I also acknowledge the deep feelings of attachment and relationship of the Kaurna people to their place.

I pay my respects to the cultural authority of Aboriginal and Torres Strait Islander peoples from other areas of Australia, and pay my respects to Elders past, present and emerging, and acknowledge any Aboriginal Australians who may be with us today

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Technology Development

Microarray Technology

- Marked the birth of the modern transcriptomics era (~1996)
  - Thousands of genes analysed simultaneously
  - Microarrays are still in use!
- Developed alongside Human Genome Project (+ other organisms)
  - Reference genome was nearing completion
  - Gene/Transcript sequences available at scale
- Focus was quantitative analysis $\implies$ *differential gene expression*

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Microarray Technology

- Compute resources were increasing rapidly
  - CPU multi-threading becoming commonplace
  - `python 2.0` released in 2000
  - `R` v1.0.0 released in 2000
  - Bioconductor release 1.0 in 2002
  - High Performance Computing (HPC) becoming accessible

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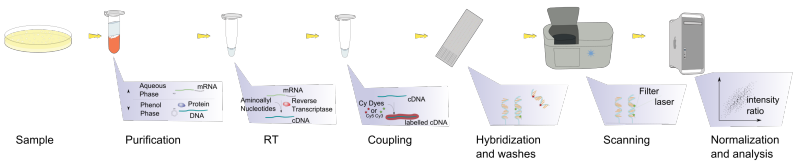
Microarray Technology

- Probes complementary to target sequence positioned on array
- RNA $\rightarrow$ fluorescently labelled cDNA

1. Labelled cDNA binds to probe



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Microarray Technology

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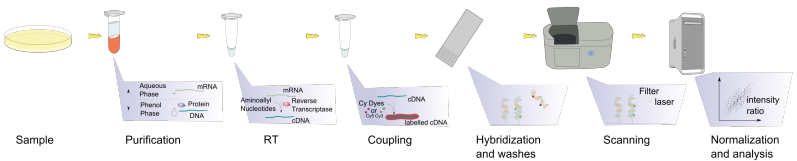
1. Labelled cDNA binds to probe
2. Microarray excited with laser



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Microarray Technology

- Probes complementary to target sequence positioned on array
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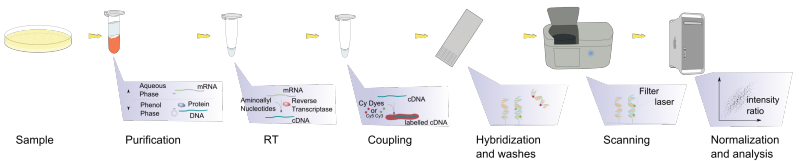
1. Labelled cDNA binds to probe
2. Microarray excited with laser
3. Fluorescence at a given probe $\propto$ target RNA abundance



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Two Colour Arrays

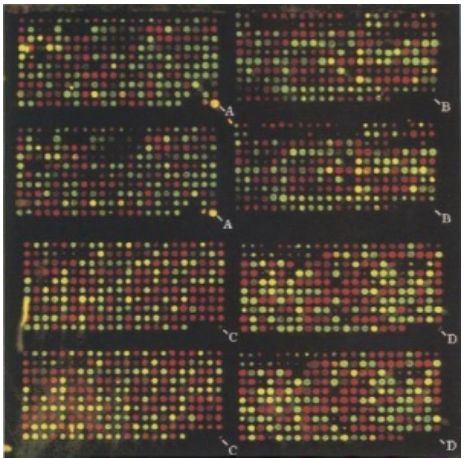

Figure 2: Section of two-colour array taken from Shalon, Smith, and Brown (1996)

- Two colour microarrays were printed microscope slides
- Known probe sequences were *printed* to the surface in defined locations
  - 60-75mer oligonucleotide probes
  - Highly customisable by project
- Two samples per array
  - Samples labelled with Cy5 (Red) or Cy3 (Green)
- Scanned at 570nm (Cy3) and 670nm (Cy5)

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MA Plots

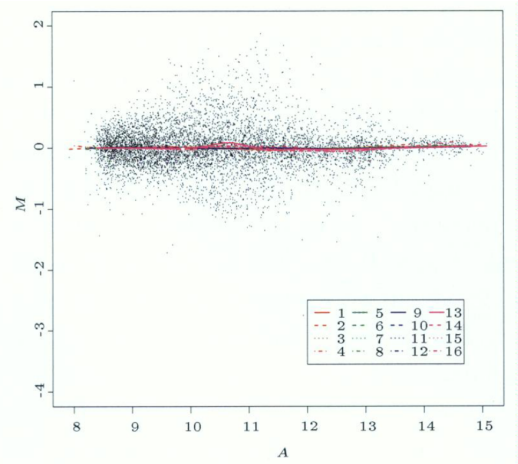

Figure 3: Image from Dudoit et al. (2002)

- Mean of Differences $M = \log_2(\frac{R}{G}) = \log_2(R) - \log_2(G)$
- Average Signal $A = \frac{1}{2}\log_2(RG) = \frac{\log_2(R)+\log_2(G)}{2}$
- Assess bias within and between arrays
- Also to show DE genes
- Term "MA Plot" still used in RNA-Seq despite no connection to formula

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Single Channel Arrays

Figure 4: No author known. Schutz assumed based on copyright claims. CC BY-SA 3.0, via Wikimedia Commons

- Affymetrix Arrays became dominant
  - Factory manufactured
- Standardised layout for each organism
- Single sample per array
  - Only scanned at one frequency $\implies$ no dye bias
- More genes/array
- 25mer probes targeting 3' end of transcript
  - Captured only intact transcripts

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Single Channel Arrays

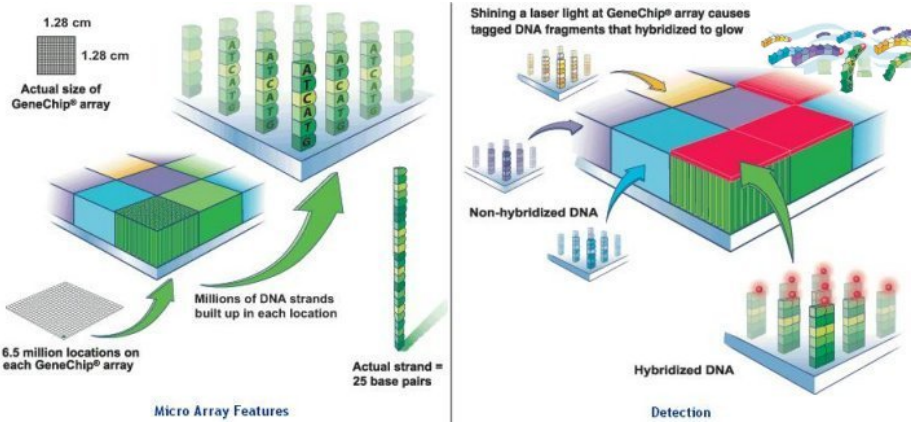

Figure 5: The basic methodology underpinning Affymetrix array design. Source Affymetrix

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3' Arrays

- Each 3' exon targeted by 11 unique 25mer probes $\implies$ *a probeset*
- Possible to detect different transcripts only if 3' exons differ
- *Perfect Match* (PM) probes $\implies$ exactly matches target sequence

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  - Literally half the array
  - Intended to quantify NSB properties of each probe
  - Sometimes returned more signal than PM probes

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Other Array Types

- 3' Arrays replaced by whole transcript (WT) arrays
  - Exon/Gene Arrays
  - Maximum of 4 probes/exon
  - Multiple probes target missing exons
- Illumina Bead arrays
  - 65-mer probes
  - Used barcoded beads instead of set probe locations

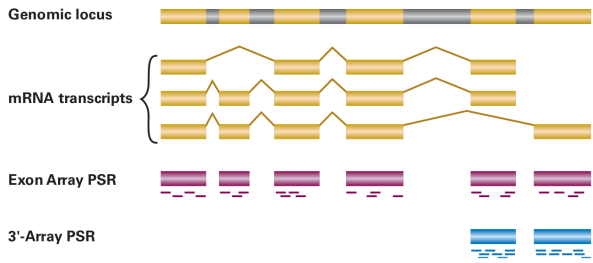

Figure 6: Source: Affymetrix Technical Note

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Differential Gene Expression

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Motivation

- The aim is to detect *differentially expressed* (DE) genes
- Classic design is Control samples vs Treated samples e.g.
  - Resting T cells vs Stimulated T cells
  - T47D + Estrogen vs T47D + Estrogen + Testosterone
- What genes are DE between conditions
- Need replicates for each condition $\implies$ t-test for each gene
  - Anything with $n < 3$ per condition is unacceptable
  - What values do we use for t-test?

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Quantile Normalisation

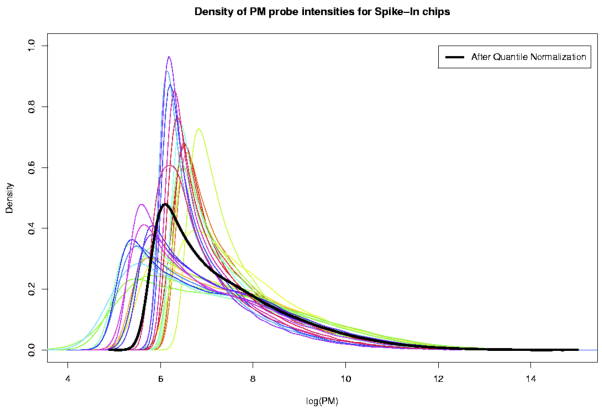

Figure 7: Example of raw $\log_2$PM probe intensities. Taken from Bolstad et al. (2003)

- Need to summarise probes to a probeset
  - Probes can be very noisy
  - BG signal + inconsistent binding
- Individual arrays give different fluorescence distributions $\Longrightarrow$ technical noise
  - Normalisation between arrays

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Quantile Normalisation

- Quantile normalisation is perfect for arrays with probes and probesets
  - Normalise probe-level signal, but estimate gene expression at the probeset level
  - Smooths out any normalisation artefacts

1. Select the lowest signal probe on each array$\longrightarrow$ Likely to be a different probe on each array

- Effectively randomises noise
- Leads to arrays with identical distributions

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2. Calculate the average signal across all arrays

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1. Select the lowest signal probe on each array$\longrightarrow$ Likely to be a different probe on each array
2. Calculate the average signal across all arrays
3. Give each of the selected probes the average signal
4. Move to the next lowest signal probe until finished

- Effectively randomises noise
- Leads to arrays with identical distributions

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Quantile Normalisation

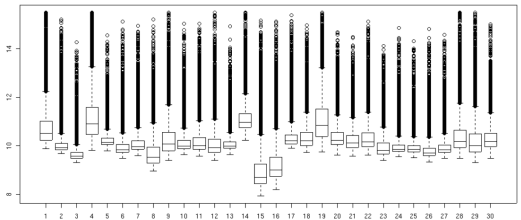

Figure 8: Before quantile normalisation

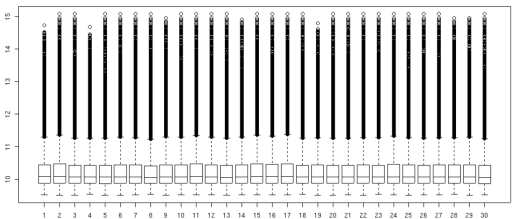

Figure 9: After quantile normalisation[1]

- Now we have identical distributions of signal across all arrays
- Equivalent to having identical amounts of source material (mRNA)
- Reduces technical noise across dataset $\implies$ more statistical power

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Background Correction

- Background Correction performed *simultaneously with estimation of signal*
- Robust Multichip Average (RMA) (Irizarry et al. 2003)
  - Estimates signal for each array (μ_i) $\implies$ used for differential expression
  - Model includes probe affinities (α_j)
  - Doesn't include MM probes
  - Fitted using robust statistics to reduce impact of outlier probes

$$\log_2 PM_{ij} = \mu_i + \alpha_j + \epsilon_{ij}$$

- Extended to GC-RMA (Wu et al. 2004) to include GC content of probes

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Linear Regression

$$\log_2 PM_{ij} = \mu_i + \alpha_j + \epsilon_{ij}$$

- This formula is a linear regression equation:
  - Fitted values for expression and probe affinity
  - An error term $\epsilon_{ij} \sim \mathcal{N}(0, \sigma)$
- The expression estimate μ_i is the array-specific intercept

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- The expression estimate μ_i is the array-specific intercept
- Probe affinities are common and consistent across arrays

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Differential Gene Expression

- For each gene $\rightarrow$ take the array-level estimates of gene expression (μ_i)
- Perform a t-test grouped by treatment group
  - Estimates variance (σ_g^2)
  - Estimate change in expression: $logFC = \beta_g$
- logFC is on $\log_2$ scale:
  - logFC $= 0 \implies$ unchanged expression
  - logFC $= 1 \implies$ doubling in RNA abundance

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Differential Gene Expression

- logFC estimates can also be from fitting *linear regression models*
  - Might use a covariate (e.g. age) alongside key predictor (i.e. diagnosis)

$$\mu_{ij} = \beta_0 + \beta_1 x_j + \epsilon_{ij}$$

- Commonly β_0 is the average expression
- The group variable x_j would be $x_1 = 0$ for control ($j = 1$) and $x_2 = 1$ for treated
  - β_0 becomes the *expected expression in control samples*
  - β_1 becomes the *expected difference in expression after treatment* $\implies$ logFC
- The variability around the predicted values is $\epsilon_{ij} \sim \mathcal{N}(0, \sigma)$

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Differential Gene Expression

$$T = \frac{\beta_1}{\sigma/\sqrt{n}}$$

- Some estimates of variance will be over/under-estimates
  - $\hat{\sigma}_g$ too low $\implies T \uparrow \implies$ significant result where no change
  - $\hat{\sigma}_g$ too high $\implies T \downarrow \implies$ no significant result where there is change
- Variance estimates moderated by *taking distribution of σ across all genes*
  - Bayesian posterior estimate of variance $\implies$ moderated t-statistic (Smyth 2004)

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Differential Gene Expression

- t-test for each gene $\implies$ p-value for each gene
- Use False Discovery Rate (Benjamini and Hochberg 1995) to adjust p-values
  - Allows an expected α as a proportion of false discoveries
  - Usually $\alpha = 0.05 \implies \leq 5\%$ of genes accepted as DE are *false discoveries*
  - Assumes a bit of noise will be drowned out by true discoveries

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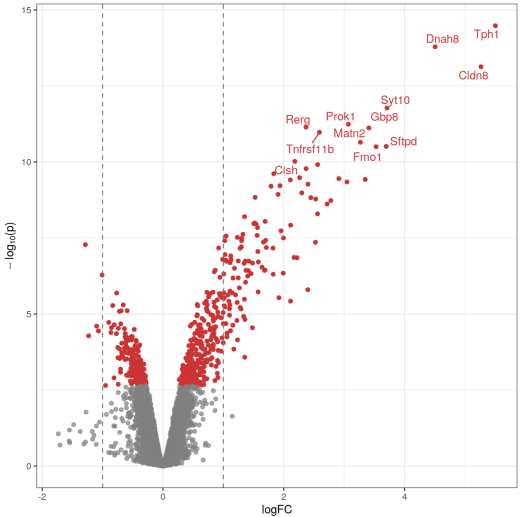
Presentation Of Results From DGE Analysis



- MA plots
  - Often used to check bias
  - Show logFC vs Average signal
- Volcano plots:
  - logFC vs Significance (e.g. -$\log_{10}$p)
  - Label genes as chosen

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Closing Comments

- The microarray era defined the analytic approaches still taken
  - FDR-adjustment, statistical modelling, enrichment testing etc
  - Most methods use $\log_2$ transformed + normally-distributed data (t-tests)
  - Variance moderation
  - Bioconductor packages (e.g. `limma`) (Ritchie et al. 2015)

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Additional Reading

- StatQuest: Linear Regression, Clearly Explained!!!
- A guide to creating design matrices for gene expression experiments

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