Biochem 3BP3

Gene Expression Analysis

Week of Nov 8, 2021

Gene Expression Analysis

- Gene Expression Analysis used primarily for two purposes:
  - Determine genetic underpinnings of observed phenotype – experimental
  - Annotation of genomes – development of gene models (intron/exon)
- The complete set of messenger RNAs in a cell is called the “Transcriptome”
- Transcriptomes are dynamic while genomes are static
  - the transcriptome will vary among cells, organs, life-stages, over time
  - many transcriptome libraries are needed for a full sampling
  - cell sorting technologies + NGS = single cell transcriptomics
- Transcriptome analysis is both genomic and statistical in methodology

Gene Expression Analysis

- For most studies, transcriptome = mRNA
  - rRNA is ignored as it dominates the transcriptome and would overwhelm library construction & sequencing
  - mRNA is sampled exclusively via isolation of polyadenylated RNA during library construction
  - transcriptome = mRNA = protein-coding portion of the genome
- transcriptome ≠ proteome
- Increasingly, studies sample:
  - total mRNA
  - ribosome-bound mRNA (i.e. actively translated)
  - miRNA (i.e. post-transcriptional regulation)

Transcriptomes can be sampled and measured in two ways:

INDIRECT = probe based technologies = microarrays

DIRECT = sequencing technologies = RNA-Seq

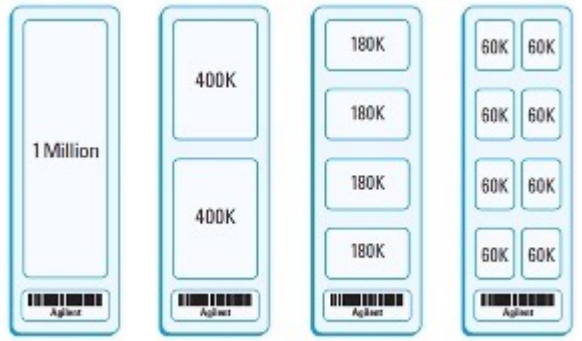
Direct Sampling

1. Construction and Sanger sequencing of cDNA libraries
   - long reads but only a small number of them (1,000s)
   - cDNA libraries normalized so all mRNA were equally abundant – sequencing was about sampling a broad diversity of mRNA but not about relative abundance
2. 1990s – Serial Analysis of Gene Expression (SAGE)
   - Higher volume isolation of 14 bp or 21 bp tags from mRNAs
   - Tags mapped to a known genome sequence (required!)
   - 10,000s
   - Relative abundance
3. RNA-Seq (focus of next week)
   - NGS of mRNA libraries
   - 1,000,000s
   - Gene identification and relative abundance data

Indirect Sampling

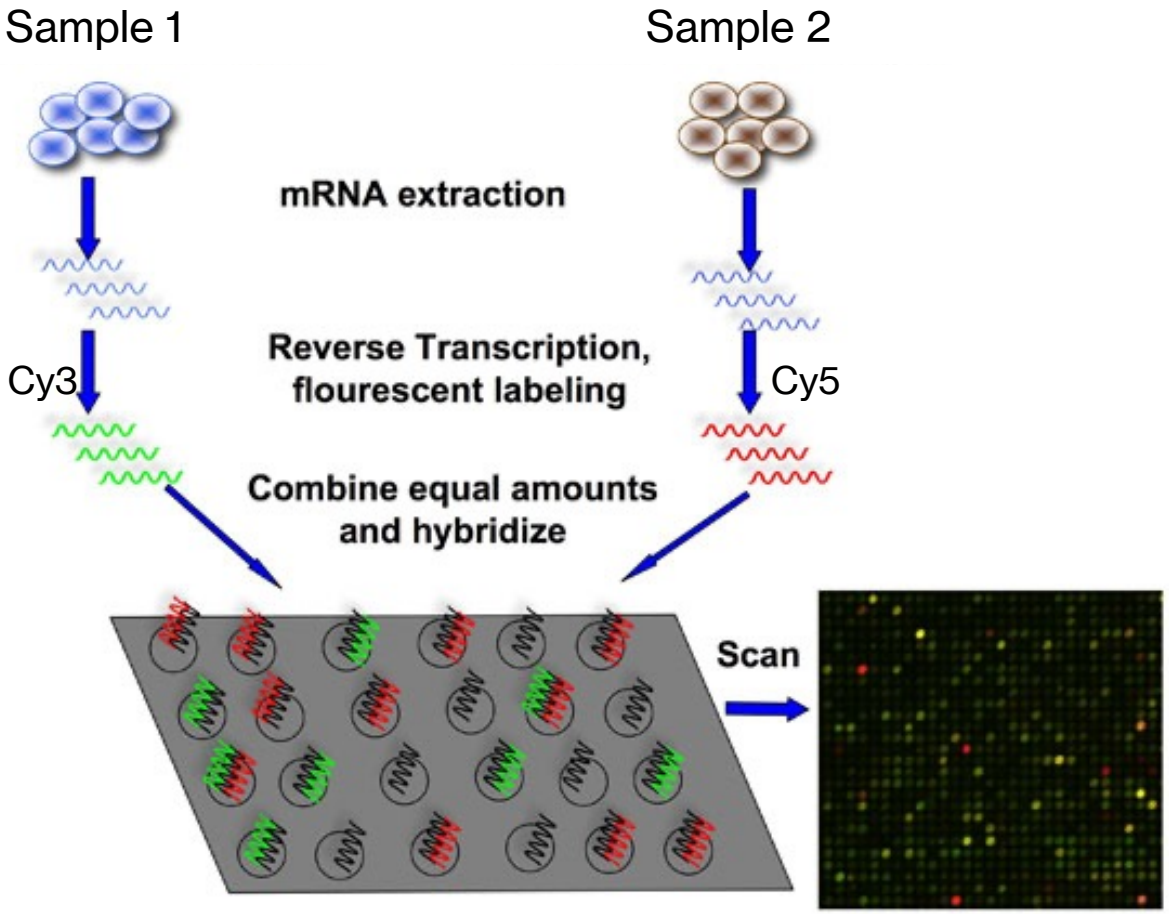
- Hybridization of mRNA samples to a set of pre-defined DNA probes
- **Probes designed based on known gene sequences – not a complete sampling of the transcriptome**
- More abundant mRNA molecules will bind more frequently to probes = higher signal (usually fluorescent); signal is used a proxy for relative abundance
- Original “microarrays” involved robotic spotting or *in situ* synthesis of probes on glass slides
- NGS was expected to make microarray technology obsolete but new BeadChip approaches have improved accuracy and very competitive cost
- For example, human muscle biopsy transcriptomics @ McMaster:
  - RNA-Seq – $540 / sample – 25 million mRNA sequenced
  - HT-12 BeadChip – $178 / sample – 47,000 probes

Glass Slide Microarrays



- High density probe arrays – many formats

Microarrays – Two-Dye Methods



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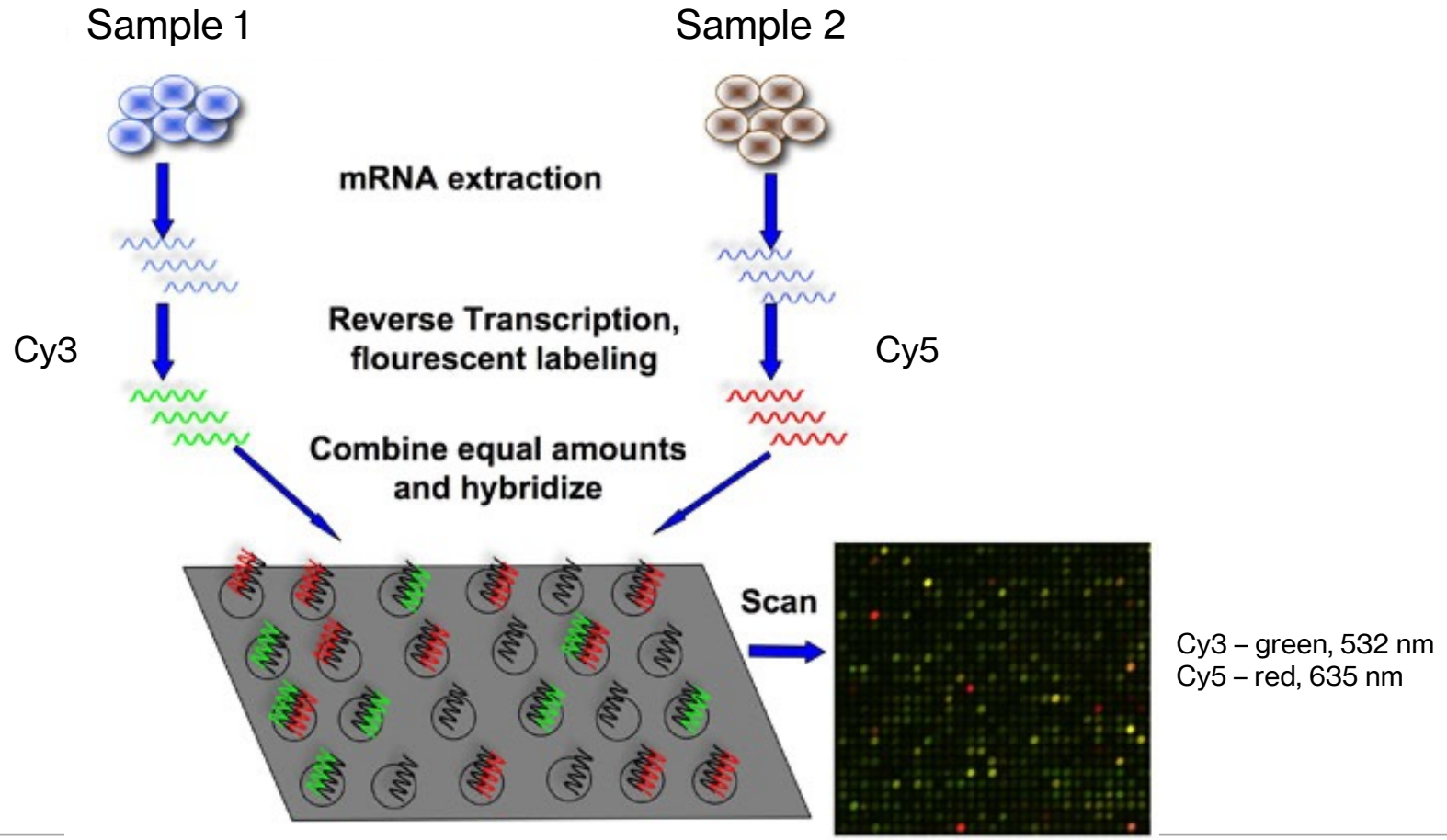

Cy3 – green, 532 nm
Cy5 – red, 635 nm

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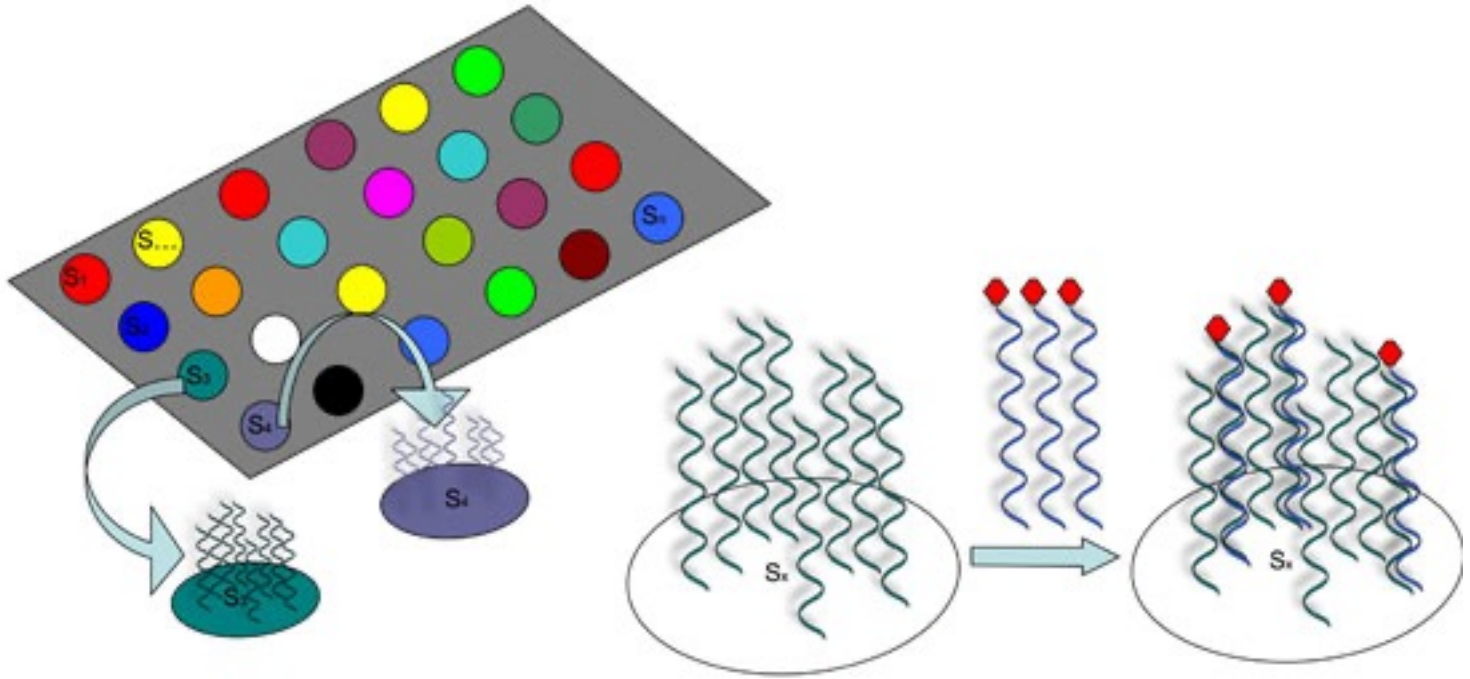
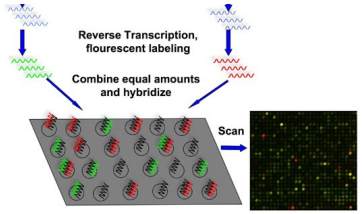
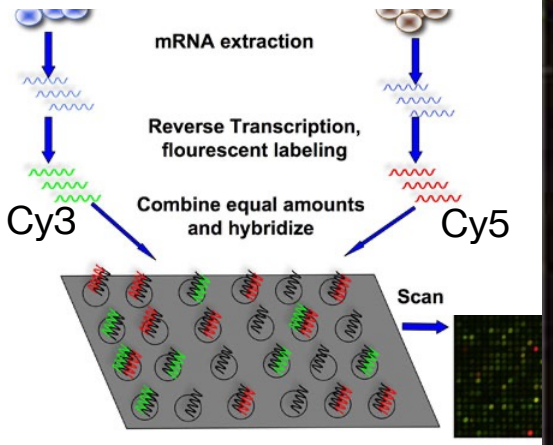




- Each spot is a collection of thousands of identical probes
- The more mRNA that bind to a probe within a spot, the brighter the overall signal for that spot

Microarrays – Two-Dye Methods



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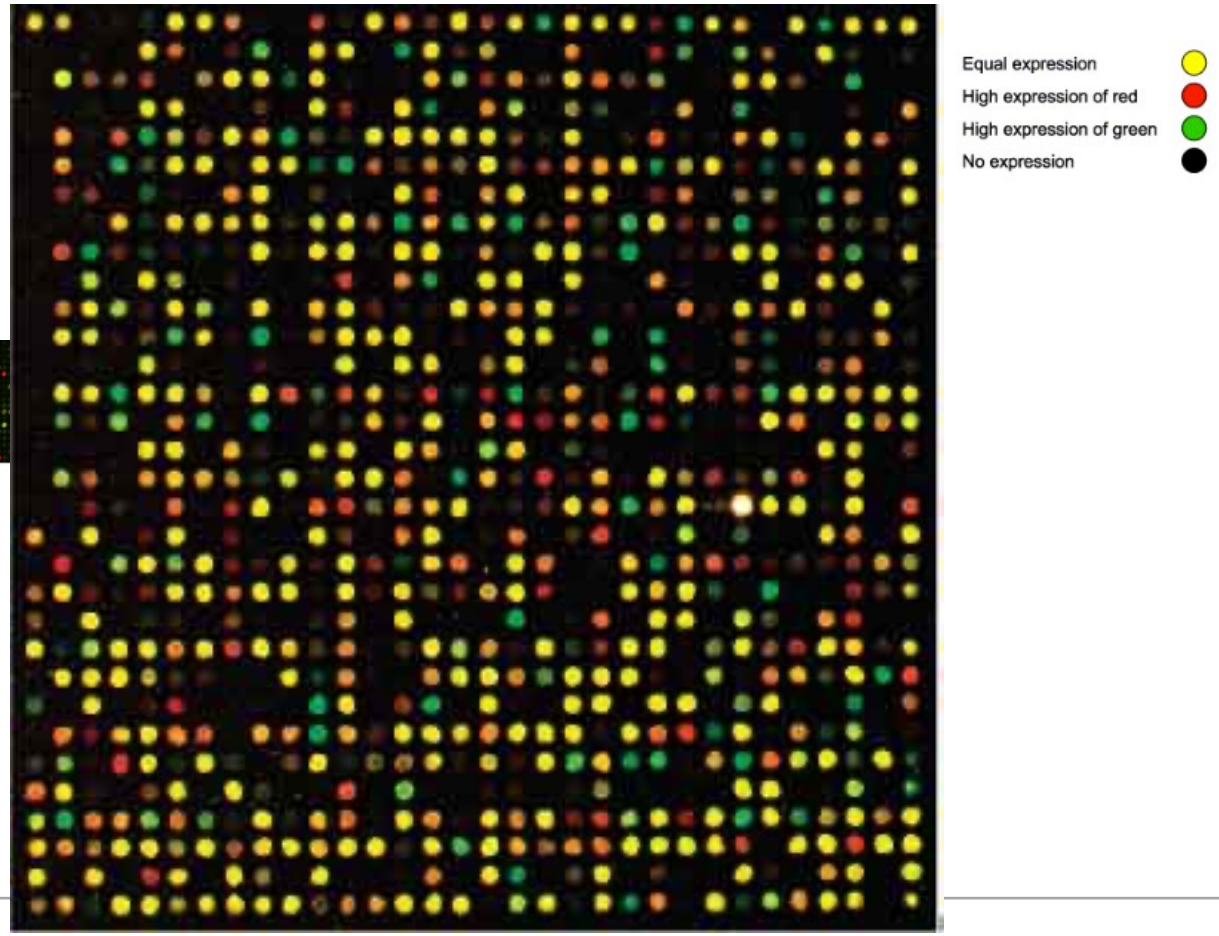

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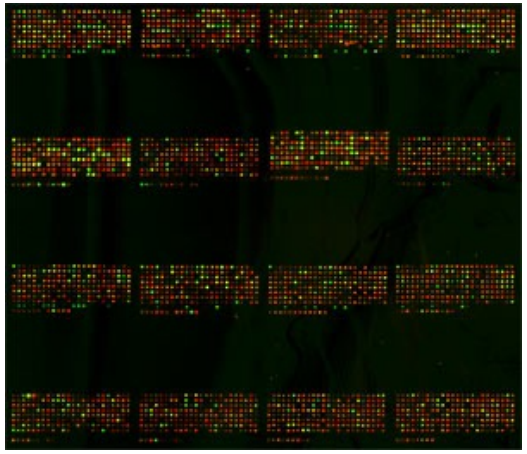
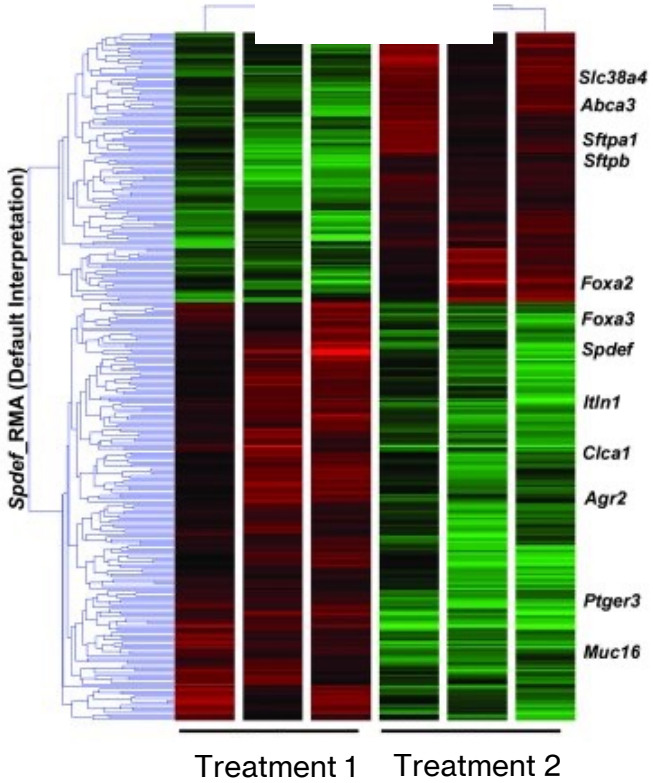
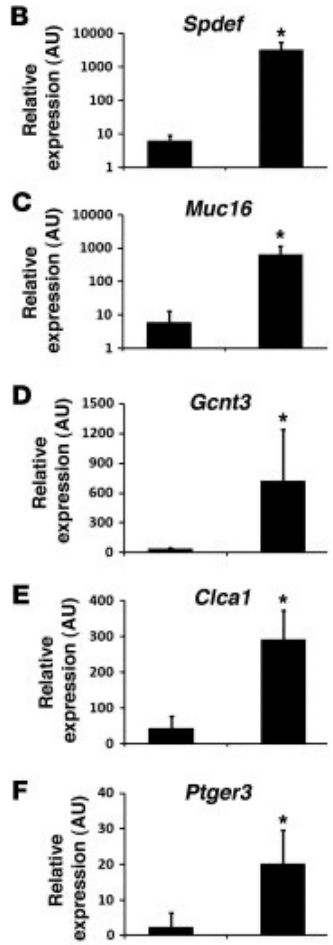


Fold change between the two samples is calculated

GREEN – downregulated

RED - upregulated





Bench work

RNA extraction

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cDNA synthesis and Cy3/5 labeling

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Hybridize to Microarray

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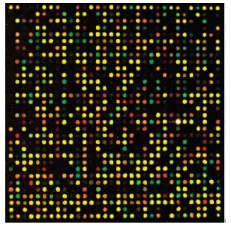
Dry & Scan

Data cleanup



Feature Extraction

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Normalization

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Data Filtering

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Data Transformations & Adjustments

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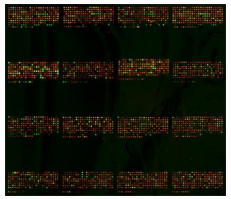

Statistical Tests

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Post-hoc Tests

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Visualization

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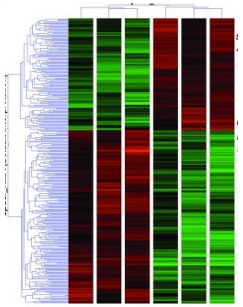

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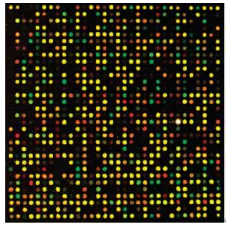

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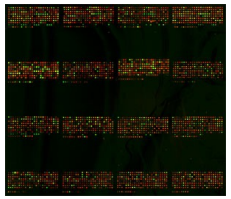

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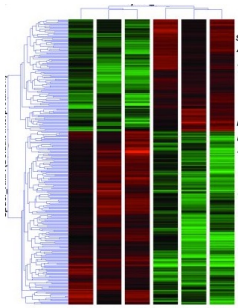


Feature Extraction

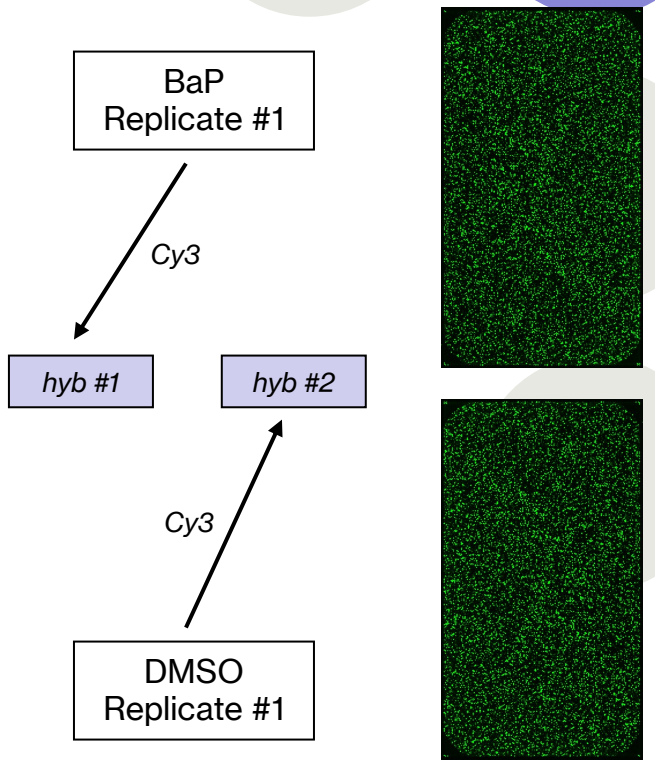
- Vendor Software
- Spot finding
- Outlier detection
- Measure feature intensity (Cy3 and/or Cy5)
- Background Subtraction or Detrending
- Commercial microarrays make extensive use of internal control probes

Two-Dye Approaches - Normalization

- Human error and imprecision of tools leads to slightly different loading of Cy3 labeled cDNA and the Cy5 labeled cDNA on the microarray
- Normalization attempts to factor out this technical variation
  - Normalize within microarrays (Cy3 versus Cy5 load)
  - Normalize among microarrays (each microarray will have slightly different loadings)
- Lowess normalization most commonly used (see Flash Update)

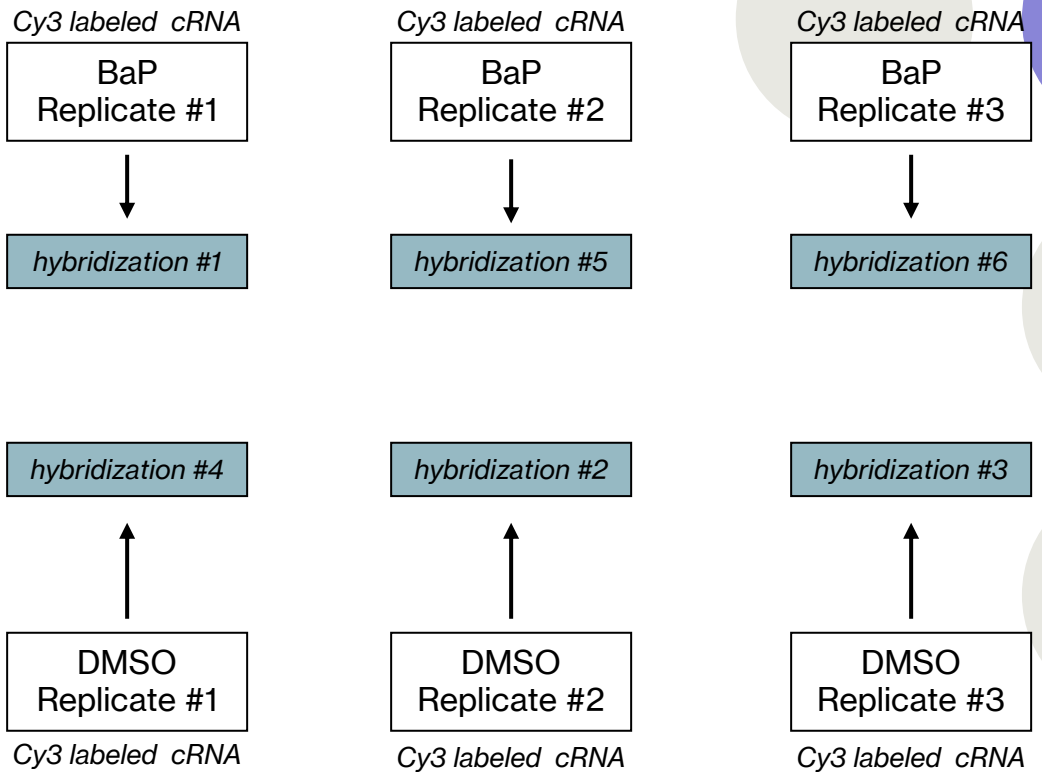
Single Sample Approaches

- Single sample approaches best for high quality microarrays & BeadChip technologies
- Each sample is hybridized to its own microarray
- You rarely see two-dye approaches anymore
- Only uses Cy3 and thus saves money on dye usage
- Cy3 is also more stable to laboratory ozone
- Avoids difficult statistical properties of fold change estimates
- Measurement is the intensity of Cy3 for each sample – how bright is the green?



Normalization

- Each sample is hybridized to its own microarray
- Dye normalization is needed to factor out microarray loading error
- Non-linear scaling method based on rank invariant probes (see lab)
- Result is reliable estimates of relative transcript abundance not fold change estimates



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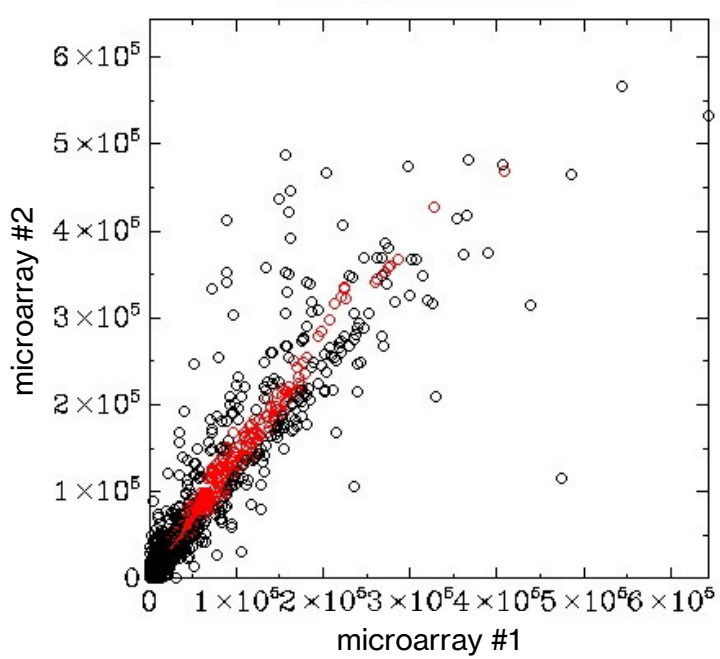

Cy3 intensity between two samples. Putative housekeeping genes in red – expected to be in equal abundance in both samples. Non-linear normalization needed based on plot above.

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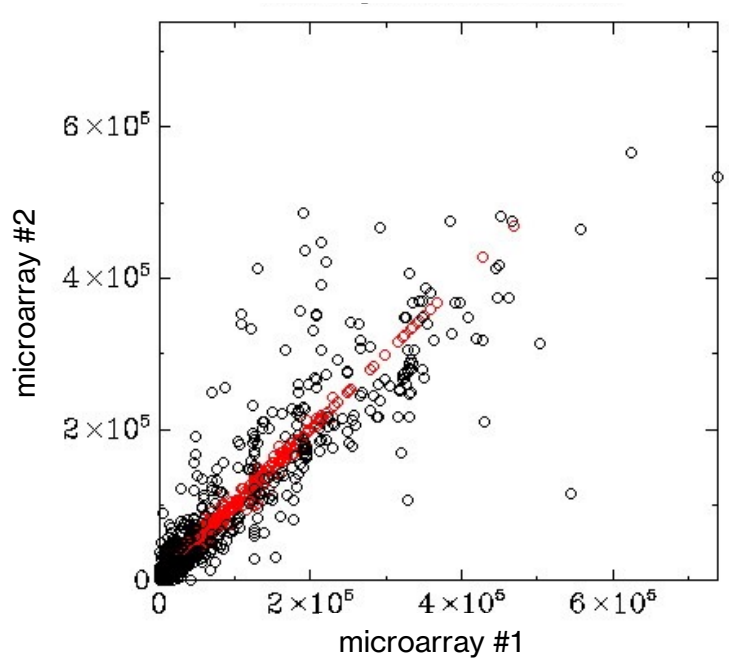

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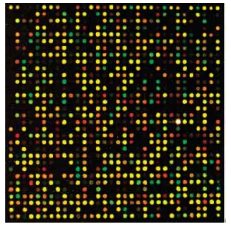

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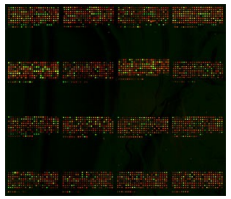

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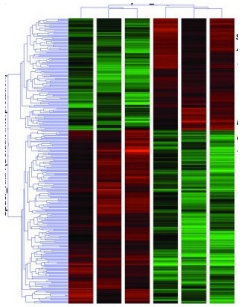

Filtering

- Saturated probes – Cy3 signal at it's maximum – all probes hybridized
- Probes not above background
  - within 2.6 x standard error of background
- Non-uniform probes
- Poorly replicated probes

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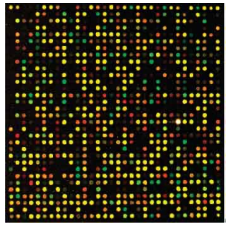

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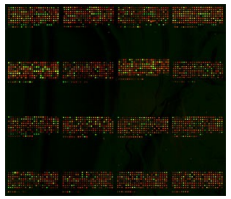

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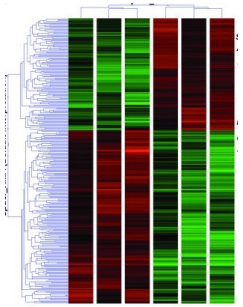
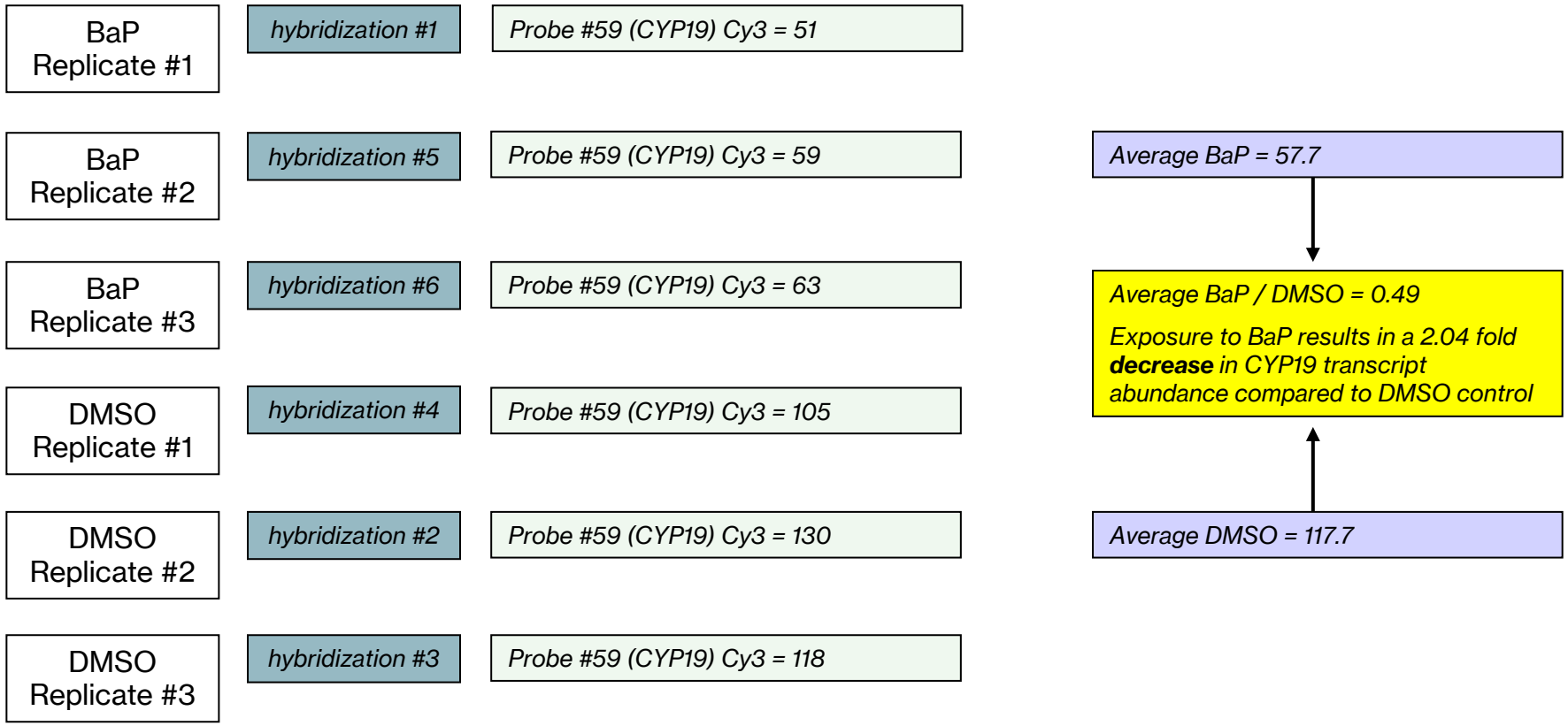


Experimental Design



- Normalized values are quantitative and have normal statistical properties
- Mean, standard error, and ANOVA calculations follow normal formulas

Data Transformations

- Log transformation
  - Correction for fold change data in two dye experiments
    - 2 fold increase = 2.0
    - no change = 1.0
    - 2 fold decrease = 0.5
  - Reduce mean and variance relationships in one dye experiments, reduce Type I and Type II error
- Median centering
  - Focus analysis upon variation in the data, not magnitude
  - Important so analysis is not biased toward most abundant transcripts

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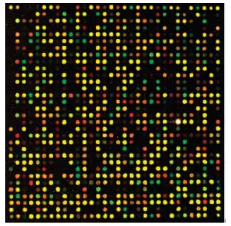

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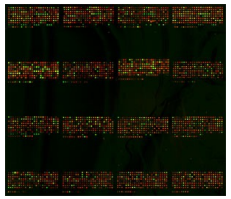

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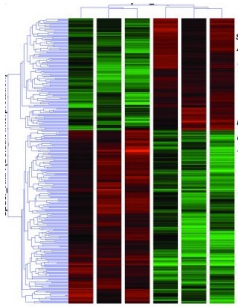


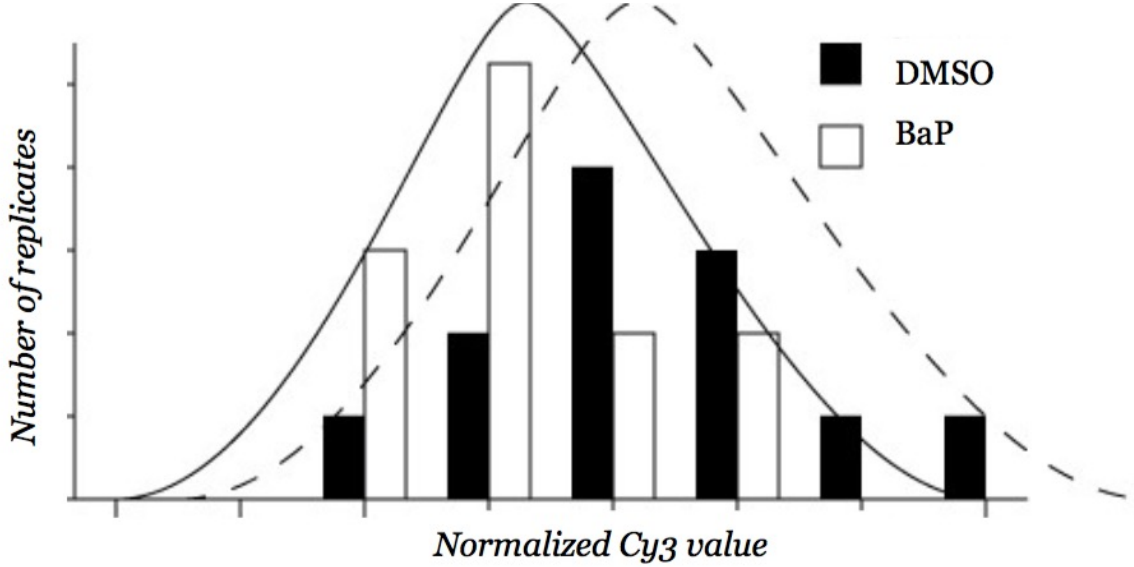
Statistical Significance - Replication

- Microarrays use familiar statistical tests such at t-test and ANOVA
- Increased replication within treatments increases statistical power among treatments
- Glass slide microarrays are expensive so many experiments only use triplication
- BeadChip microarrays are cheaper and often use higher replication

Average BaP / DMSO = 0.49

*Exposure to BaP results in a 2.04 fold **decrease** in CYP19 transcript abundance compared to DMSO control*

t-test with $p<0.05$



Statistical Significance - Replication

- Type I error
  - false positive or false discovery
  - controlled by the significance level of the test (α), e.g. α=0.05
- Type II error
  - false negative
  - has value β but often actual value is unknown
  - related to Power of the test (1 – β) and experimental replication

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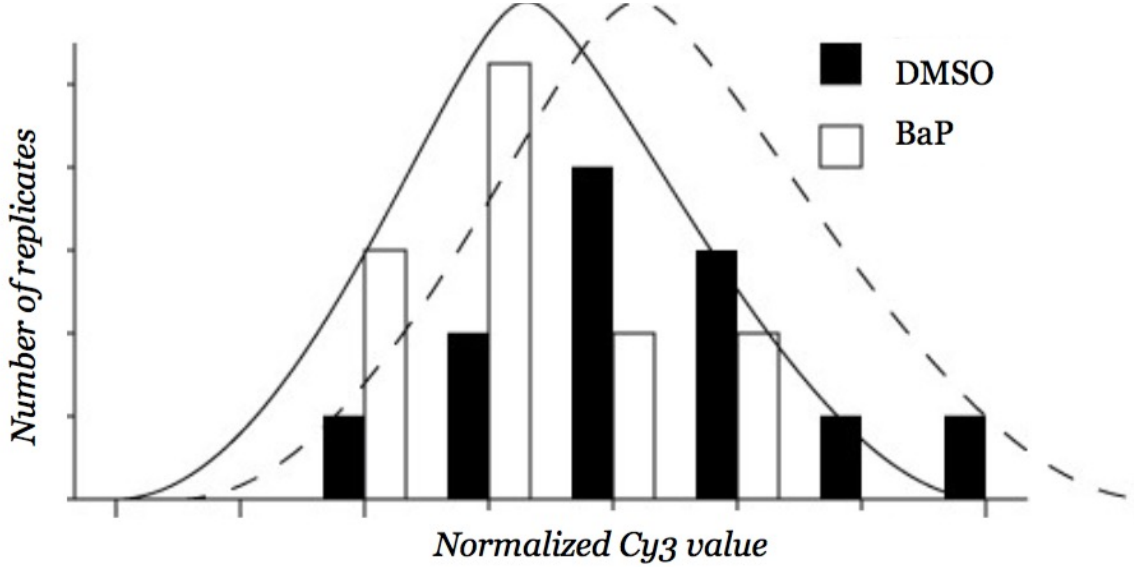
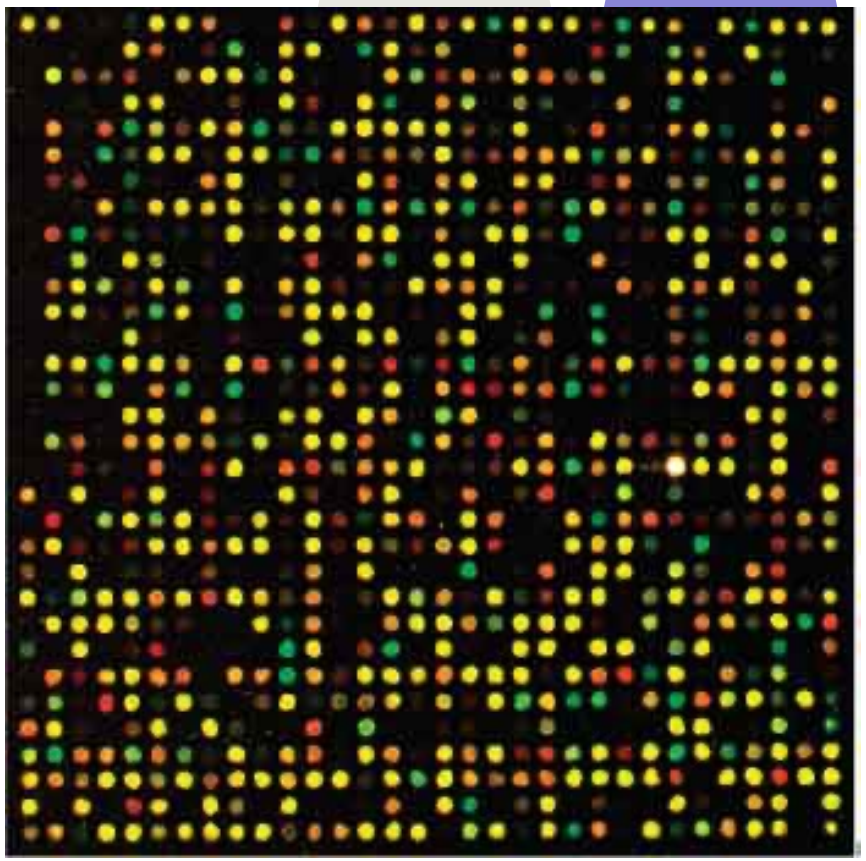


Statistical Significance - Error

- Microarrays perform multiple tests, inflating false positives
- Traditional statistics use the Bonferroni correction α/n where n = # tests, but this is often not appropriate for microarrays
- Microarrays methods often instead estimate the false discovery rate (FDR) and use arbitrary cut-offs
- Microarray methods often permutate appropriate FDR test distributions

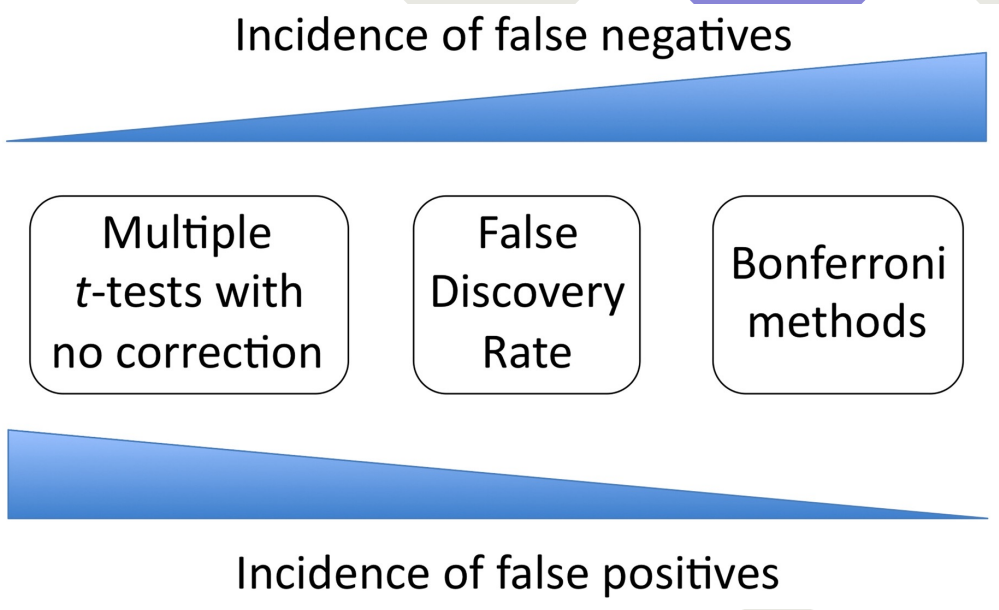


Agilent zebrafish microarray has 43,803 probes

Each spot measures Cy3 for a single probe (i.e. gene) and each spot undergoes it's own significance test. Thus there are 43,803 tests performed on the same mRNA sample – the tests are not independent!

Statistical Significance - Error

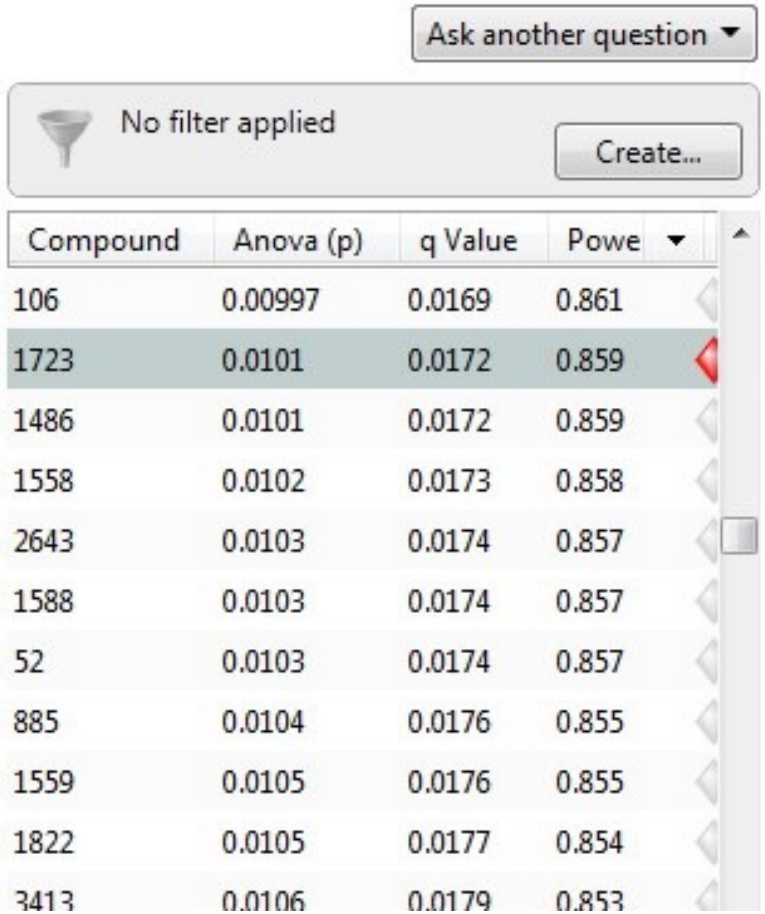
- The more t-tests you run on the same mRNA sample, the greater the chance of obtaining a statistically significant result through chance sampling
- Microarrays thus have the unavoidable presence of false-positive findings (Type I errors)
- Traditional corrections (i.e. Bonferroni) only work for small number of multiple tests; FDR permutations are a compromise for microarrays to use reasonable Type I and Type II error
- **Q-values are the name given to the adjusted p-values found using an optimized FDR approach,** http://www.nonlinear.com/support/progenesis/comet/faq/v2.0/pq-values.aspx



http://www.wormbook.org/chapters/www_statisticalanalysis/statisticalanalysis.html

Statistical Significance – Metabolomics Example

- Order the results by q-value, Compound #1723 is the 800th compound in the list of 3516 total compounds
- Compound #1723 has p=0.0101 and q=0.0172
- A p-value of 0.0101 implies a 1.01% chance of false positives in the experiment. 0.0101 x 3516 = 35.51 false positives in the top 800 hits
- A q-value of 0.0172 implies only 1.72% of the top 800 compounds are false positives = 800 * 0.0172 = 13.76 false positives
- p-values are biased by 3516 multiple tests, q-values correct for this effect



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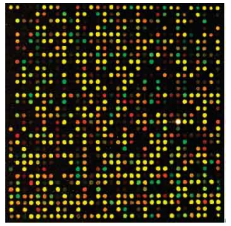
Dry & Scan

Data cleanup



Feature Extraction

↓

Normalization

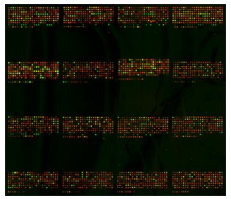
↓

Data Filtering

↓

Data Transformations & Adjustments

Analysis



Statistical Tests

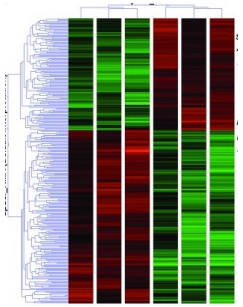
↓

Post-hoc Tests

↓

Visualization

↓

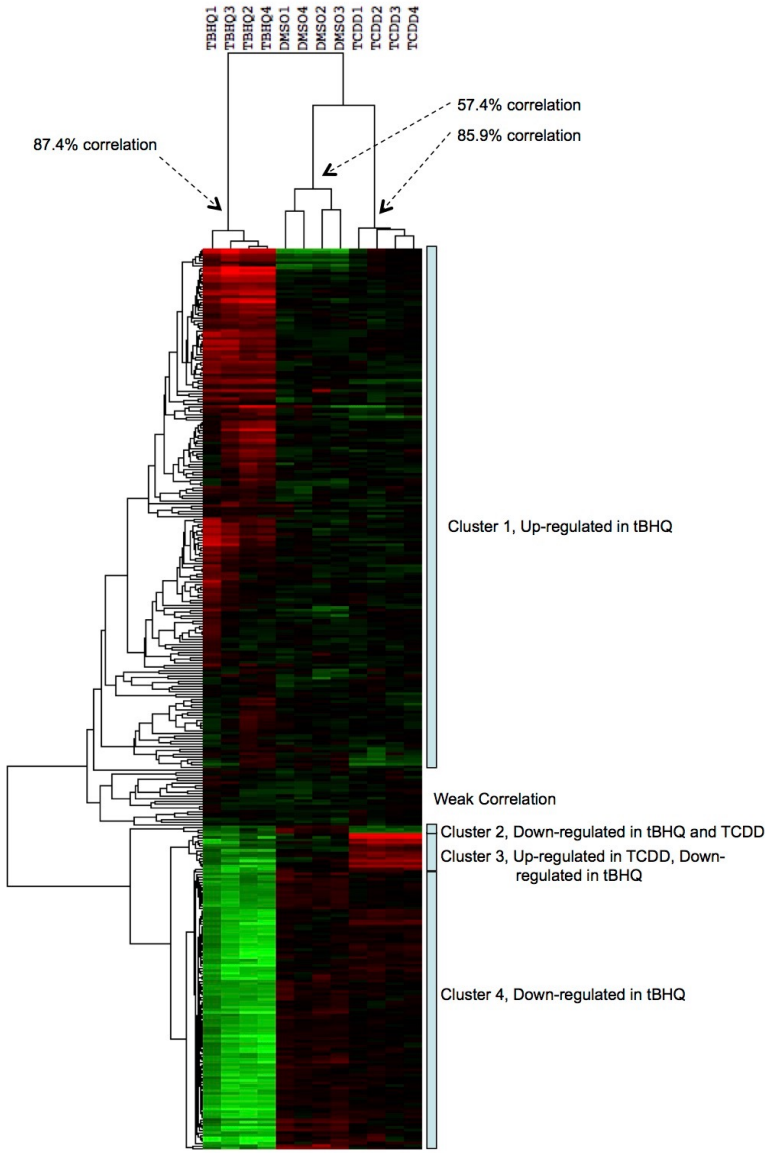


Visualization

- Heat Maps
  - variety of clustering algorithms
  - clusters of co-variation may indicate common regulatory pathways
  - particularly good for time series data

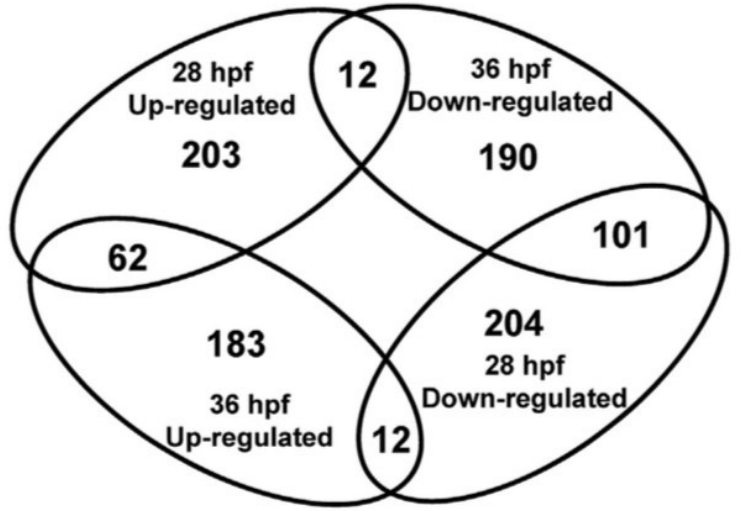
Visualization

- Cluster by genes (rows) and / or treatments (columns)
- Clusters are hierarchical
- Smaller clusters have higher correlation in expression patterns
- Median centre to downplay abundance and focus analysis on patterns



Visualization

- Heat Maps
  - variety of clustering algorithms
  - clusters of co-variation may indicate common regulatory pathways
  - particularly good for time series data
- VENN diagrams
  - excellent for visualizing shared response and designing follow-up experiments

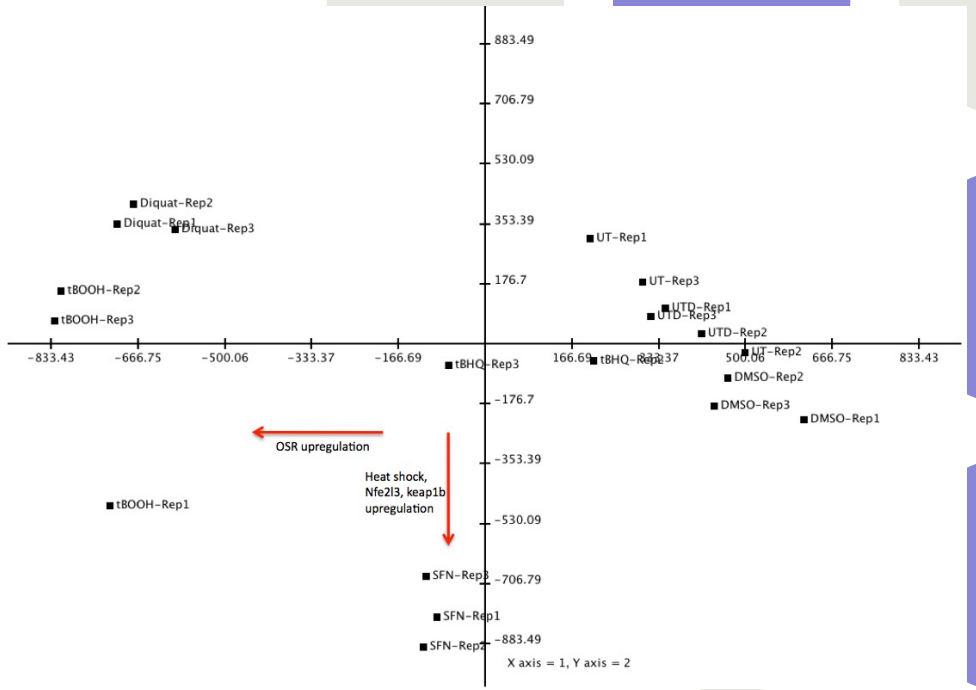


28 hpf (eGFP → dnMTF-1)
277 probes up-regulated and 317 probes down-regulated

36 hpf (eGFP → dnMTF-1)
257 probes up-regulated and 303 probes down-regulated

Visualization

- Heat Maps
  - variety of clustering algorithms
  - clusters of co-variation may indicate common regulatory pathways
  - particularly good for time series data
- VENN diagrams
  - excellent for visualizing shared response and designing follow-up experiments
- Principal Component Analysis (PCA)
  - powerful tool for trend analysis
  - look at the genes involved in the trends – what are they?
  - use DAVID for Gene Ontology term enrichment analysis for genes involved in trends



Conclusions

- Design of the experiment and the microarray probes critical
  - How many probes?
  - Quality of probes for each gene?
  - Coverage of the transcriptome and target genes?
  - Coverage of the genome and hypothetical genes?
  - Multiple probes for genes?
- Design and analysis involve trade-offs
  - Level of replication, experiment cost, **statistical power**
  - Replication costs: BeadChip < microarray < RNA-Seq
  - Subtle data may require permissive analyses
    - Allow higher false discovery rate?
    - Downplay variance (e.g. rank product methods)?
- Visualization and post-hoc tests needed when large numbers of probes are significant

Conclusions

- Independent verification often required
  - Quantitative PCR verification of selected results
  - Gene knockdown or over-expression experiments
- Trust and Reliability
  - Robust experimental design, microarray probes, and statistical analyses engender trust of overall results
  - Subtle results, high variation, or downplaying of error require more extensive independent verification of overall results
- Interpretation of overall results can be difficult
  - Fold change versus biological relevance
  - Poorly understood genes
  - Prediction of underlying biological processes
    - GO enrichment? Interactome analyses? KEGG?

WEEK 10 (NOVEMBER 8 and 10) - GENE EXPRESSION ANALYSIS

LIVE lecture in class Wednesday 12:30pm,

Recorded Content

1. Overview of Laboratory #8 - Microarray Analysis, https://web.microsoftstream.com/video/63a2a60c-5784-497f-a627-076e2cff7206

Tutorial

- **LIVE** session with Teaching Assistants and Flash Updates
    - Monday, https://web.microsoftstream.com/video/4fb86131-c358-48eb-bacb-d863b4e78a79
    - Wednesday,

Flash Updates

- **Microarrays**. Review microarray technology for measurement of absolute or relative gene expression levels. Highlight the key difference between microarrays and RNA sequencing approaches. See Nature Education 1:195 (http://www.nature.com/scitable/topicpage/transcriptome-connecting-the-genome-to-gene-function-605) and http://www.nature.com/scitable/topicpage/scientists-can-study-an-organism-s-entire-6526266
- **Normalization**. Introduce the concept of normalization and why it is needed in microarray analysis. Review the major normalization approaches. See Quackenbush. 2002. Microarray data normalization and transformation. Nat Genet. 32 Suppl:496-501. [PMID 12454644]
- **False Discovery**. Introduce the concept of the false discovery rate and how it is handled in genomic analyses. See Storey & Tibshirani. 2003. Statistical significance for genomewide studies. Proc Natl Acad Sci U S A. 100: 9440-5. [PMID 12883005] and http://www.nonlinear.com/support/progenesis/comet/faq/v2.0/pq-values.aspx