Designing Complex Omics Experiments

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Some slides are from previous lectures given by Grier Page

The Myth That Metabolomics does not need a Hypothesis

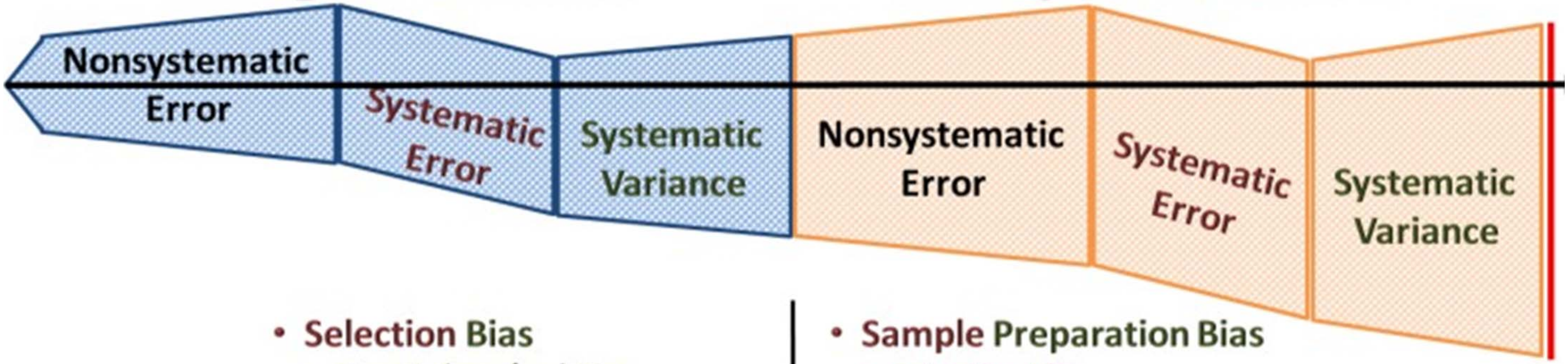
- There always needs to be a biological question in the experiment.
- The question could be nebulous: What happens to the metabolome of this tissue when I apply Drug A.
- The purpose of the question drives the experimental design.
- Make sure the samples answer the question

Experimental design

- ***Experimental design:*** is a term used about efficient methods for planning the collection of data, in order to obtain the maximum amount of information for the least amount of work. Anyone collecting and analyzing data, be it in the lab, the field or the production plant, can benefit from knowledge about experimental design.
  http://www.stat.sdu.dk/matstat/Design/index.html

Biological Variance

Analytical Variance



- Selection Bias
  - Genetic (race/sex) Bias
  - Epigenetic Bias
    - Tissue/Cell Selection Bias
  - Temporal Selection Bias
- Biological Conditions Bias

Biological Biases

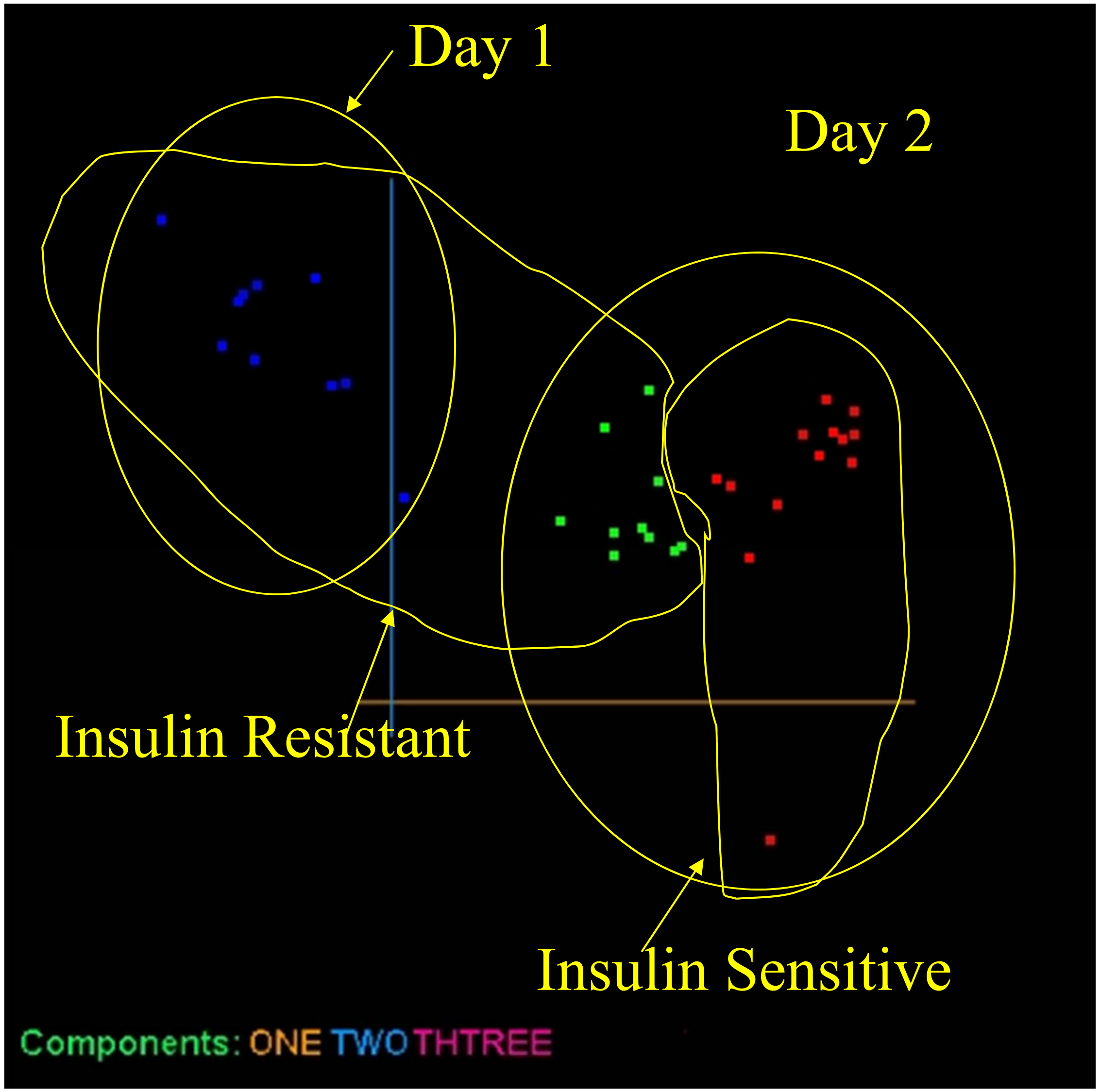
- Sample Preparation Bias
  - Extraction Bias
  - Procedural Bias
  - Storage Bias
- Standards Bias
- Sample Complexity Bias
- Analytical Conditions Bias

Analytical Biases

- Methodological Bias
  - Statistical Assumptions
  - Lack of Statistical Power
  - Multiple Testing
- Assignment Error
  - Metabolite Assignment Error
  - Class (Group) Assignment Error
- Confirmation Bias

Interpretive Biases and Errors

UMSA Analysis



Experimental design general principals

- Randomization
- **Replication**
- **Blocking**
- Use of factorial experiments instead of the one-factor-at-a-time methods.
- Orthogonality

Randomization

- The experimental treatments are assigned to the experimental units (subjects) in a random fashion. It helps to eliminate effect of "lurking variables", *uncontrolled factors* which might vary over the length of the experiment.

Commonly used randomization method

- Number the objects to be randomized and then randomly draw the numbers using paper pieces in a hat or computer random number generator.

Example: Assign two treatments, Hormone and control, to 6 plants

1

2

3

4

5

6

Hormone treatment:	**(1,3,4)**	**;**	**(1,2,6)**
Control :	**(2,5,6)**	**;**	**(3,4,5)**

Design Issues in Omics Exp

- Known sources of non-biological error (not exhaustive) that must be addressed
  - Technician / post-doc
  - Reagent lot
  - Temperature
  - Protocol
  - Date
  - Location
  - Cage/ Field positions

Randomization in Omics Experiments

- Randomize samples in respect to treatments
- Randomize the order of handling samples.
- Randomize arrays/runs/gels/days in respect to samples

How to Randomize

- **Not** "covering your eyes and pick a subject"
- Number your subjects and use random generator on your computer to pick random numbers.
- Write numbers on pieces of paper and do a random pick.

Replication

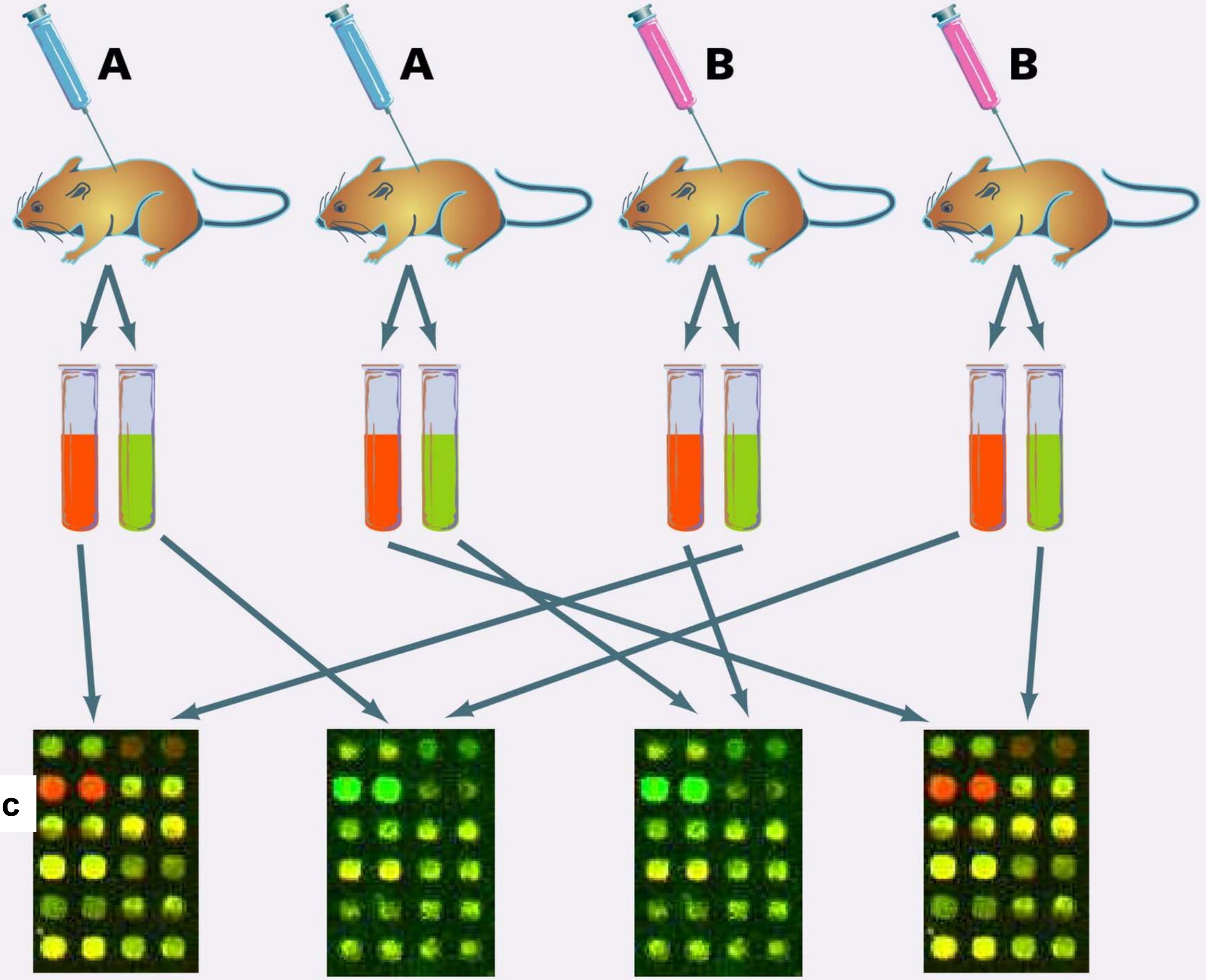
- **Replication** is repeating the creation of a phenomenon, so that the variability associated with the phenomenon can be estimated.

  Replications should not be confused with repeated measurements which refer to literally taking several measurements of a single occurrence of a phenomenon.

Replication in omics experiments

- What to replicate?
  - Biological replicates (replicates at the experimental unit level, e.g. mouse, plant, pot of plants…)
    - Experimental unit is the unit that the experiment treatment or condition is directly applied to, e.g. a plant if hormone is sprayed to individual plants; a pot of seedlings if different fertilizers are applied to different pots.
  - Technical replicates
    - Any replicates below the experimental unit, e.g. different leaves from the same plant sprayed with one hormone level; different seedlings from the same pot; Different aliquots of the same RNA extraction; multiple arrays hybridized to the same RNA; multiple spots on the same array.

- Treatment
- Biological replicate
- Blood
- Technical replicate
- Array or Spec
- Duplicate spot /run



Replication in Omics experiments

- Biological replicates are typically more important than technical replicates unless estimating the variation at different levels is the purpose of the experiment in evaluating the technology.
- Biological replicates are often more effective in increasing the power for detecting differential metabolites/genes.
- Technical replicates are useful when technical variability is large and technical replicates are cheap.

How Many to Replicate?
---Sample Size

- **Replication** is repeating the creation of a phenomenon, so that the variability associated with the phenomenon can be estimated.
- The accuracy of the estimation of the variability depends on the degree of freedom for estimating the variability.

  Degree of freedom (df) is a measure of the number of independent pieces of information on which the precision of a parameter estimate (e.g. variance) is based. The degrees of freedom for an estimate equals the number of observations (values) minus the number of additional parameters estimated for that calculation.

How many replicates?
---- Sample Size

Example: degree of freedom (df) for estimating the variance.

Using a 2x2 factorial design to examine the effects of two factors, A and B. Each factor has two levels.

ANOVA model:

$$y = \mu + A + B + A * B + \varepsilon$$

Factorial 2 x 2

	A1(m)	A2(f)
B1 (Trt)	r	r
B2 (Ctr)	r	r

S.V.	(r=1)	(r=2)	(r=3)
μ	1	1	1
A	1	1	1
B	1	1	1
A*B	1	1	1
Var	0	4	8
Total	4	8	12

Sample Size and Power

- Sample size for a general two sample comparison

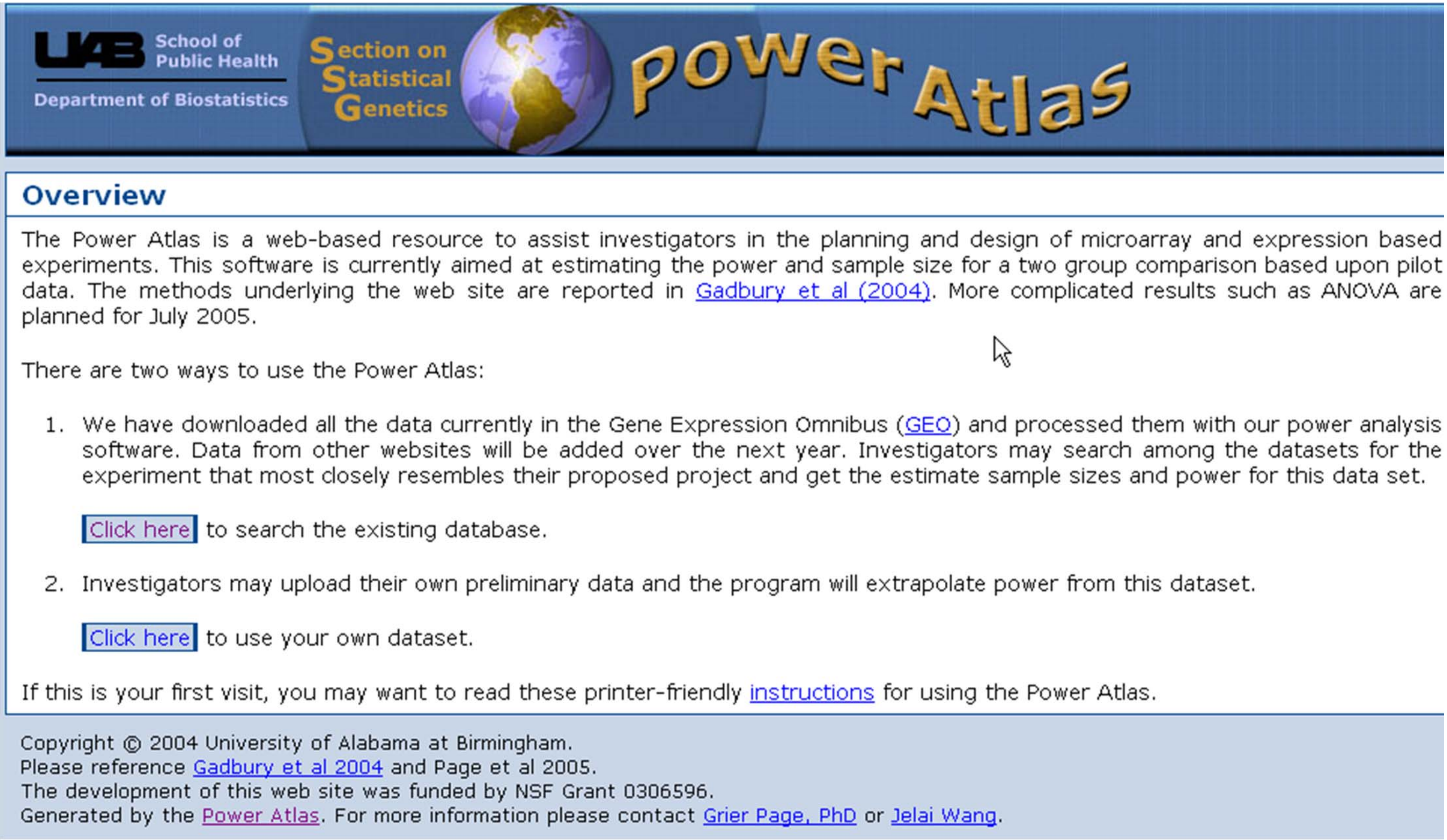
$$n = \frac{2\left(z_{(1-\alpha/2)} + z_{(1-\beta)}\right)^2}{(\delta/\sigma)^2}$$

n increases as error, σ, increases.

n increases as the difference between two means, δ,decreases.

n increases as the significant level of the test, α, decreases.

n increases as the power of the test, 1-β , increases.


UAB School of Public Health
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Section on Statistical Genetics
power Atlas

Overview

The Power Atlas is a web-based resource to assist investigators in the planning and design of microarray and expression based experiments. This software is currently aimed at estimating the power and sample size for a two group comparison based upon pilot data. The methods underlying the web site are reported in Gadbury et al (2004). More complicated results such as ANOVA are planned for July 2005.

There are two ways to use the Power Atlas:

1. We have downloaded all the data currently in the Gene Expression Omnibus (GEO) and processed them with our power analysis software. Data from other websites will be added over the next year. Investigators may search among the datasets for the experiment that most closely resembles their proposed project and get the estimate sample sizes and power for this data set.

   Click here to search the existing database.

2. Investigators may upload their own preliminary data and the program will extrapolate power from this dataset.

   Click here to use your own dataset.

If this is your first visit, you may want to read these printer-friendly instructions for using the Power Atlas.

Please reference Gadbury et al 2004 and Page et al 2005.
The development of this web site was funded by NSF Grant 0306596.
Generated by the Power Atlas. For more information please contact Grier Page, PhD or Jelai Wang.

Some R Power Packages in Bioconductor

- RNASeqPower
- Sizepower
- SSPA
- CSSP

Multilevel Replication and Resource allocation:

When there are both biological replications and technical replications.

Example:

Biological variation

Technical variation

$$EV = \frac{\sigma_M^2}{m} + \frac{\sigma_e^2}{mn}$$

Error variance of the fold change

m	**mouse / trt (biorep)**
n	**runs / mouse**
C_M	**cost / mouse**
C_A	**cost / run**

Note: to reduce EV increasing m (number of biological replicates) is more efficient.

Resource Allocation

Considering the error variance and the cost equations, we can obtain how many biological replicates and how many technical replicates to best allocate the money.

$$EV = \frac{\sigma_M^2}{m} + \frac{\sigma_e^2}{mn}$$ (reference design with dye-swaps)

$$Cost = mC_M + m \cdot nC_A$$

The optimum number of runs per biological replicate:

$$n = \sqrt{\frac{\sigma_e^2}{\sigma_M^2} \cdot \frac{C_M}{C_A}}$$

Examples for resource allocation in early microarray experiments

- Using variance components estimated from kidney in Project Normal data.
- No replicated spots on array
- Reference design

Mouse price	Array price/pair	# of array pairs per mouse
$15	$600	1
$300	$600	1
$1500	$600	2

More efficient array level designs, such as direct comparisons and loop designs, can reduce the optimum number of arrays per mouse.

Pooling Biological Samples

Theoretically, pooling can reduce the biological variance but not the technical variances. The biological variance will be replaced by:

$$\sigma^2_{pool} = \frac{1}{k}\sigma^2_M$$

k :	# of samples per pool
σ^2_M	individual biorep variance
σ^2_{pool}	pool variance

Note: It is often assumed that pooling will reduce the biological variance , therefore, be more efficient.

Potential problems of Pooling

- Reduced ability to estimate individual variability
- Prevent from identifying proper transformation and removing outliers.
- Not valid for classification studies (important for biomarker identification)
- Pooling samples is averaging at the raw level while the average of multiple samples is often after transformation (e.g. log2).
- The biological variability reduction is often smaller than 1/k.

$$\sigma_{pool}^{2} = \frac{1}{k^{\alpha}}\sigma_{M}^{2}$$

α *:* constant for the effect of pooling. $0 \le \alpha \le 1$

α = 1, pooling has maximum effect.

α = 0, pooling has no effect.

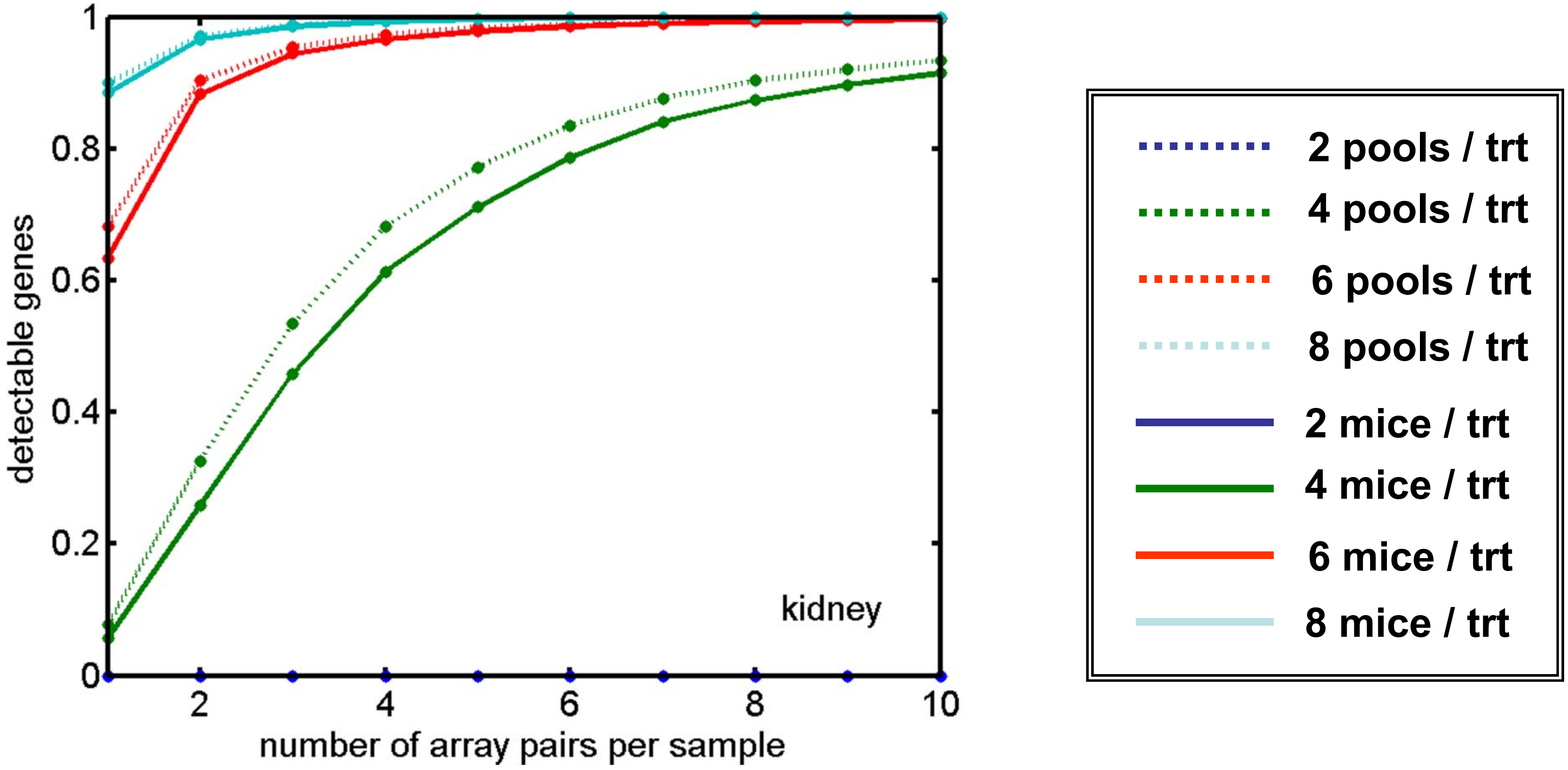
α < 0, pooling has negative effect.

Potential Advantage of Pooling

- When individual sample quantity is limited or technology is extremely expensive, pooling samples can increase the accuracy of the Fold Change estimation between two groups.
- Pooling has the potential to reduce the overall variance.

Example: Power Increase to Detect 2 fold change by Pooling in a mouse experiment (CAMDA 2002)

(Pool size *k* = 3, α = 1)



Significance level:
0.05 after Bonferroni correction

General Design Principles -- Continues

- Use of factorial experiments instead of the one-factor-at-a-time methods.
- Orthogonality: Factors are perpendicular to each other. Otherwise, the factors are called confounded or even nested.

To compare two treatments (T1, T2) and two strains (S1, S2)

	T1	T2
S1	T1S1	T2S1
S2	T1S2	T2S2

Example

- If we want to compare cases vs controls and male vs female. This two-factor design is more efficient than two experiments focusing on one factor in each.

	male	female
case	m/case	f/case
ctrl	m/ctrl	f/ctrl

Blocking

- Some identified uninteresting but varying factors can be controlled through blocking.
  - COMPLETELY RANDOMIZED DESIGN
  - COMPLETE BLOCK DESIGN
  - INCOMPLETELY BLOCK DESIGNS

Completely Randomized Design

There is no blocking

- Example
  - Compare two hormone treatments (trt and control) using 6 Arabidopsis plants (or mice or human).

1

2

3

4

5

6

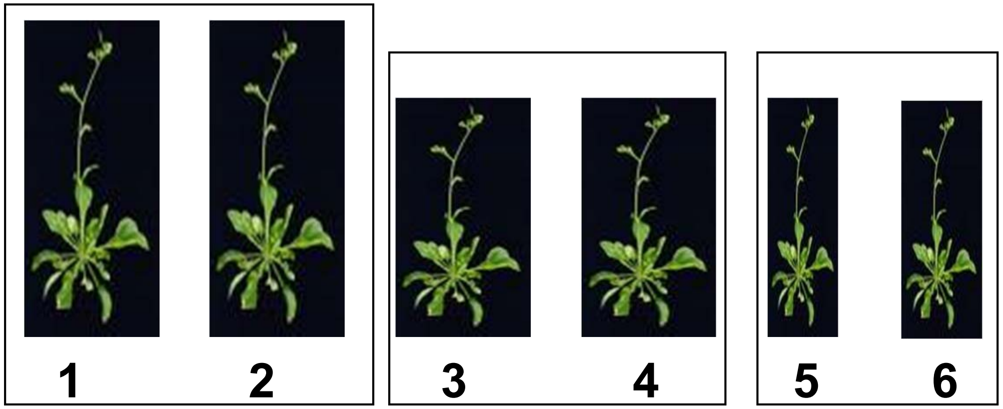
Hormone trt:	**(1,3,4);**	**(1,2,6)**
Control :	**(2,5,6);**	**(3,4,5)**

Complete Block Design

- There is blocking and the block size is equal to the number of treatments.

Example:

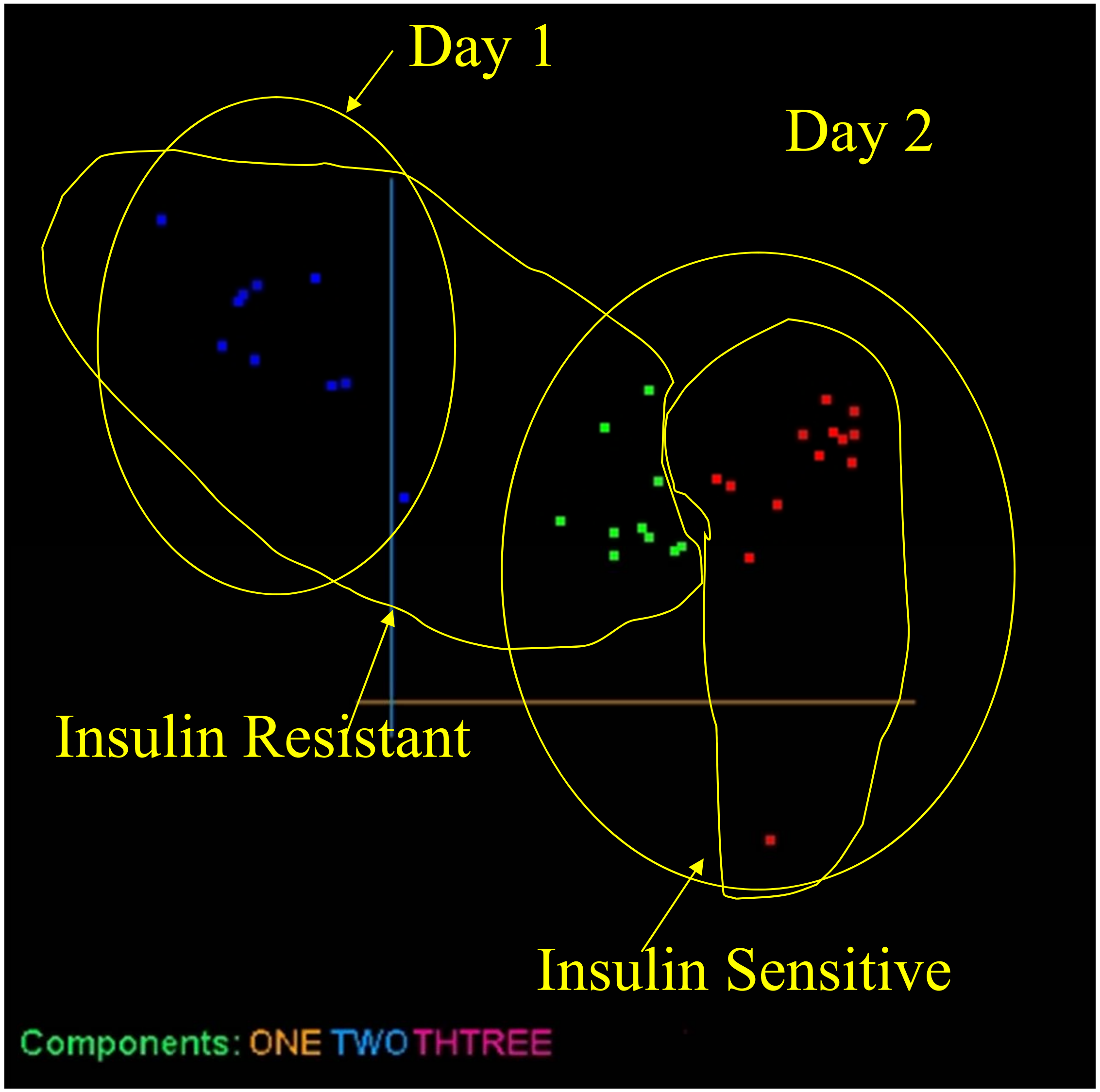
- **Compare two hormone treatments (trt and control) using 6 Arabidopsis plants. For some reason plant 1 and 2 are taller, plant 5 and 6 are thinner.**



Hormone treatment: **(1,4,5) ; (1,3,6)**
Control : **(2,3,6) ; (2,4,5)**

⇨ Randomization within blocks

UMSA Analysis

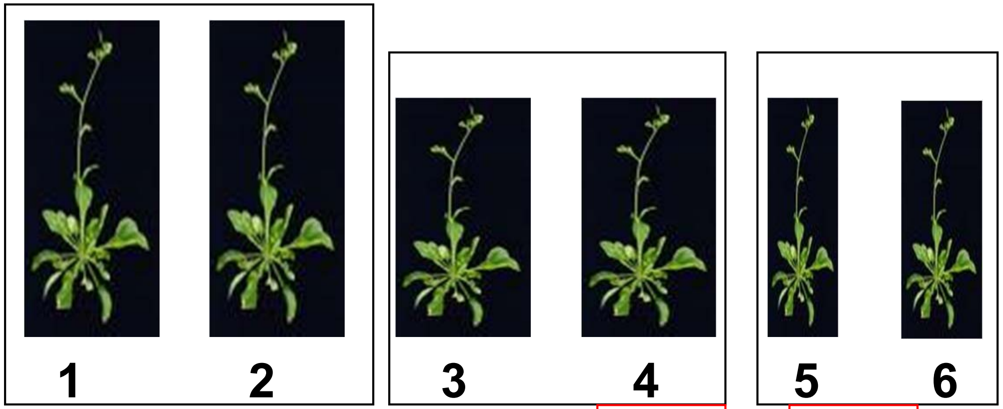


Incomplete Block Design

- There is blocking and the block size is smaller than the number of treatments.

Example:

- **Compare three hormone treatments (hormone level 1, hormone level 2, and control) using 6 Arabidopsis plants. For some reason plant 1 and 2 are taller, plant 5 and 6 are thinner.**



Hormone level1: **(1,4)** ; **(2,4)**
Hormone level2: **(2,5)** ; **(1,6)**
Control : **(3,6)** ; **(3.5)**

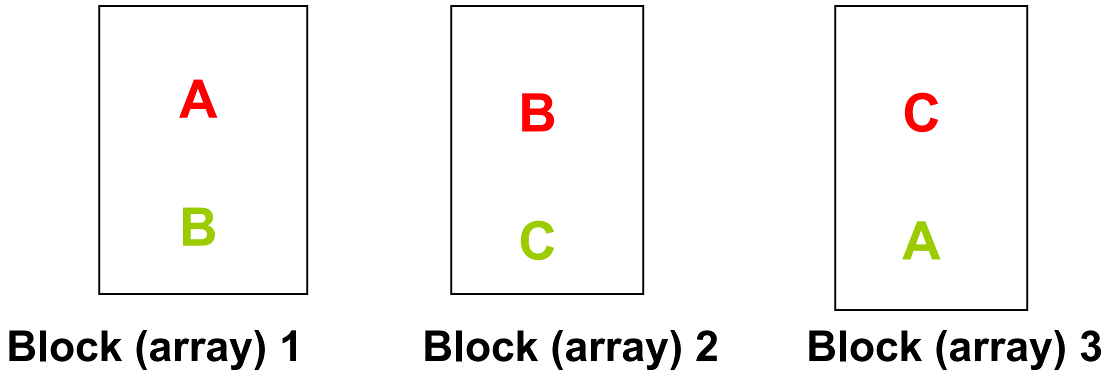
⇨ Randomization within blocks

Example: Incomplete Blocking in 2-color Microarray Experiments

- There are a smaller number of unit in each block than number of treatments/conditions to be compared.

- **Example:** In two-color microarrays, each array is a block of size 2, the samples are compared within each array.

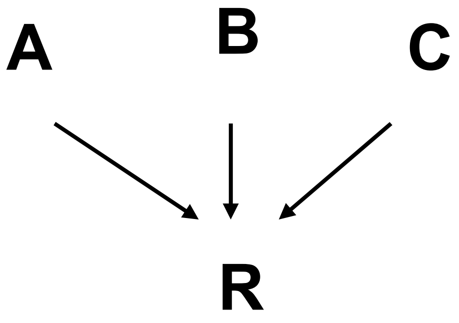
Example: compare three samples: A, B, C



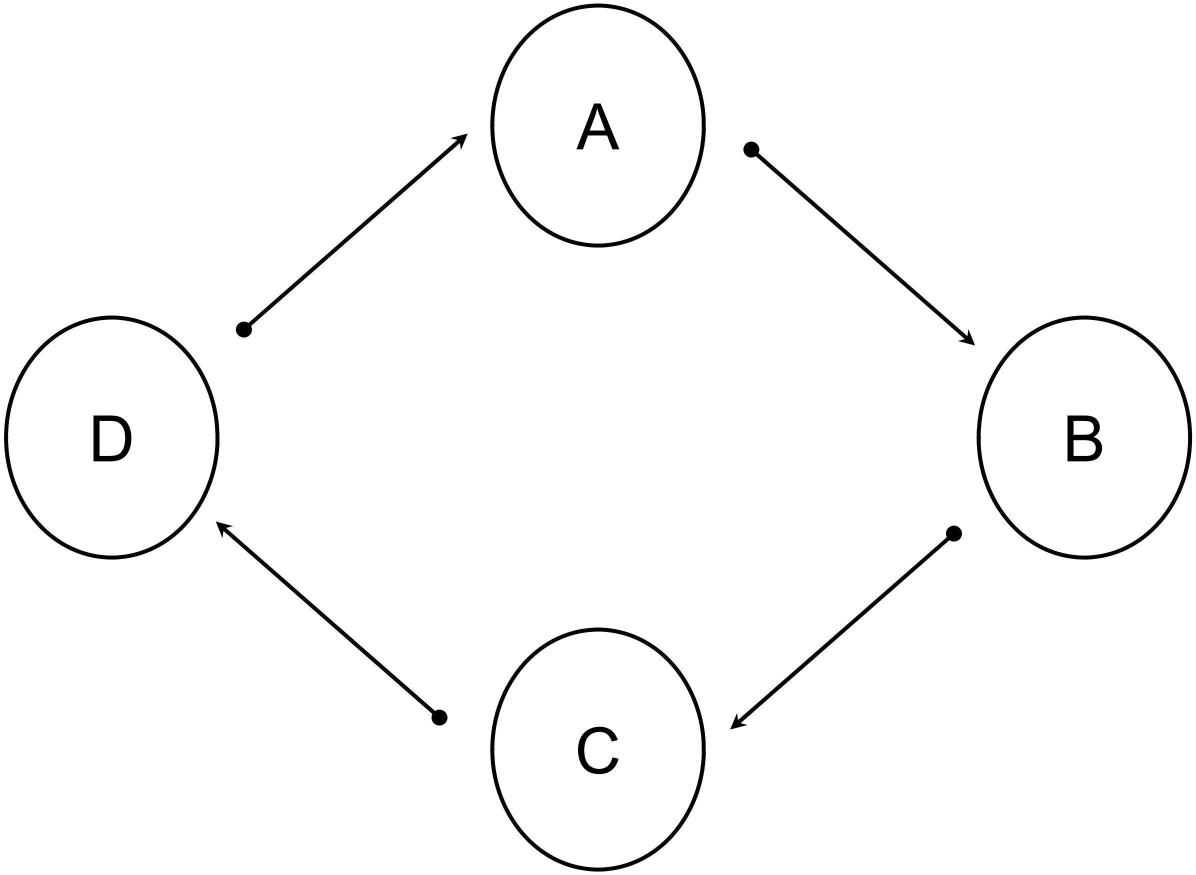
Reference design

All samples are compared to a single reference sample.
The reference sample is of no interest to the investigator.

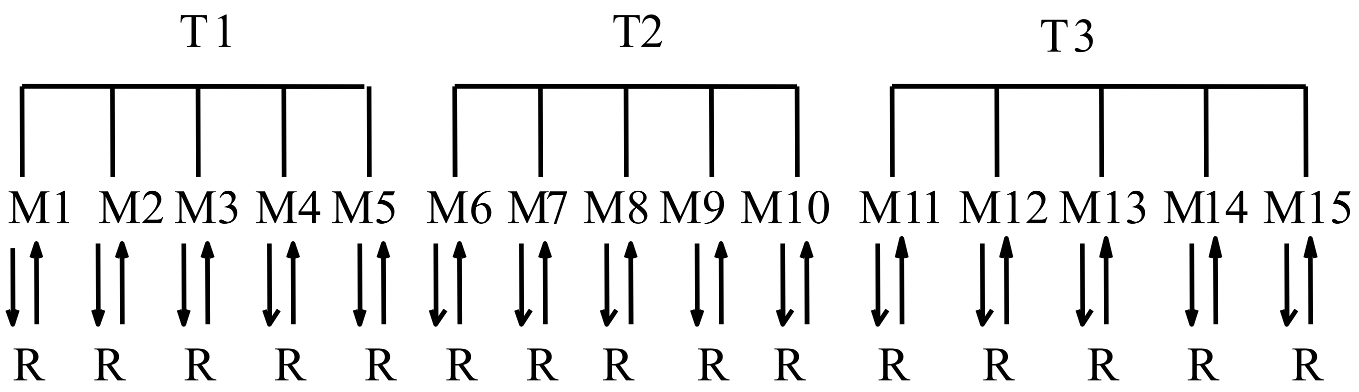
common reference



Loop Design



Can Get Complicated



T1 T2 T3

M1 M2 M3 M4 M5 M6 M7 M8 M9 M10 M11 M12 M13 M14 M15

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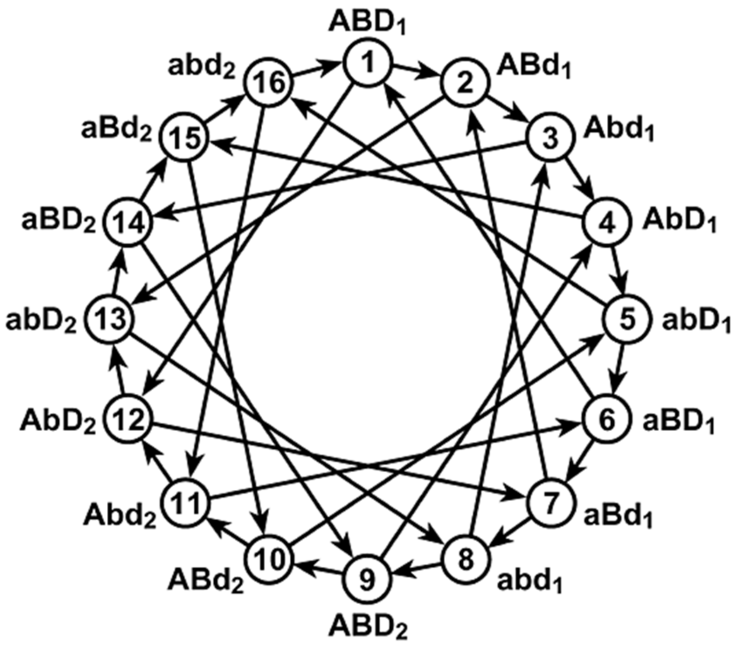
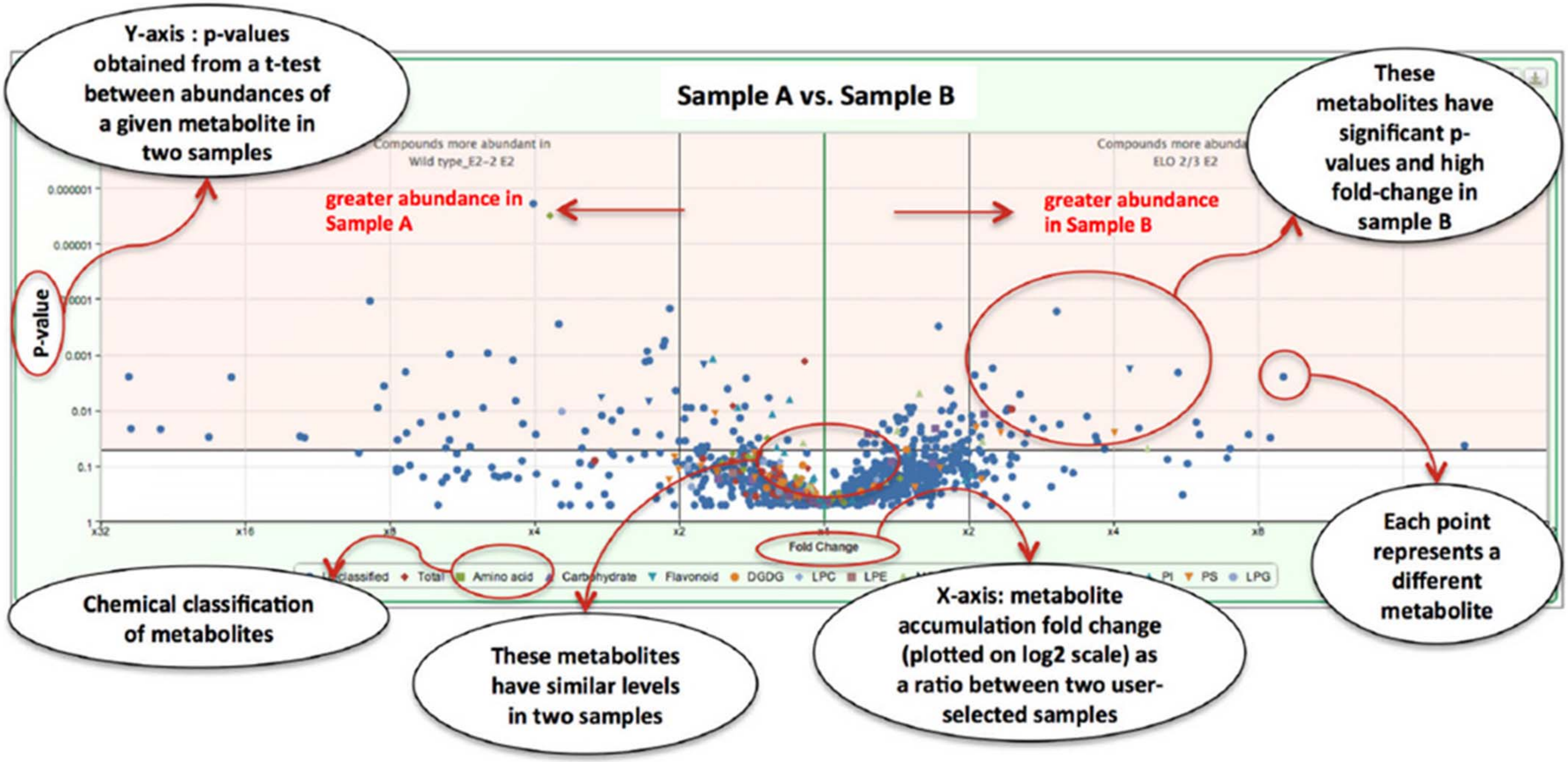


(Churchill and Oliver, 2001)

Statistical analyses

- Supervised analyses – linear models etc
  - Assume IID (independently identically distibuted)
  - Normality
  - Sometimes can rely on central limit
  - ‘Weird’ variances
  - Using fold change alone as a statistic alone is not valid.
  - ‘Shrinkage’ and or use of Bayes can be a good thing.
- False-discovery rate is a good alternative to conventional multiple-testing approaches.
- Pathway testing is desirable.

Volcano Plot for Two Group Comparison



Y-axis : p-values obtained from a t-test between abundances of a given metabolite in two samples

These metabolites have significant p-values and high fold-change in sample B

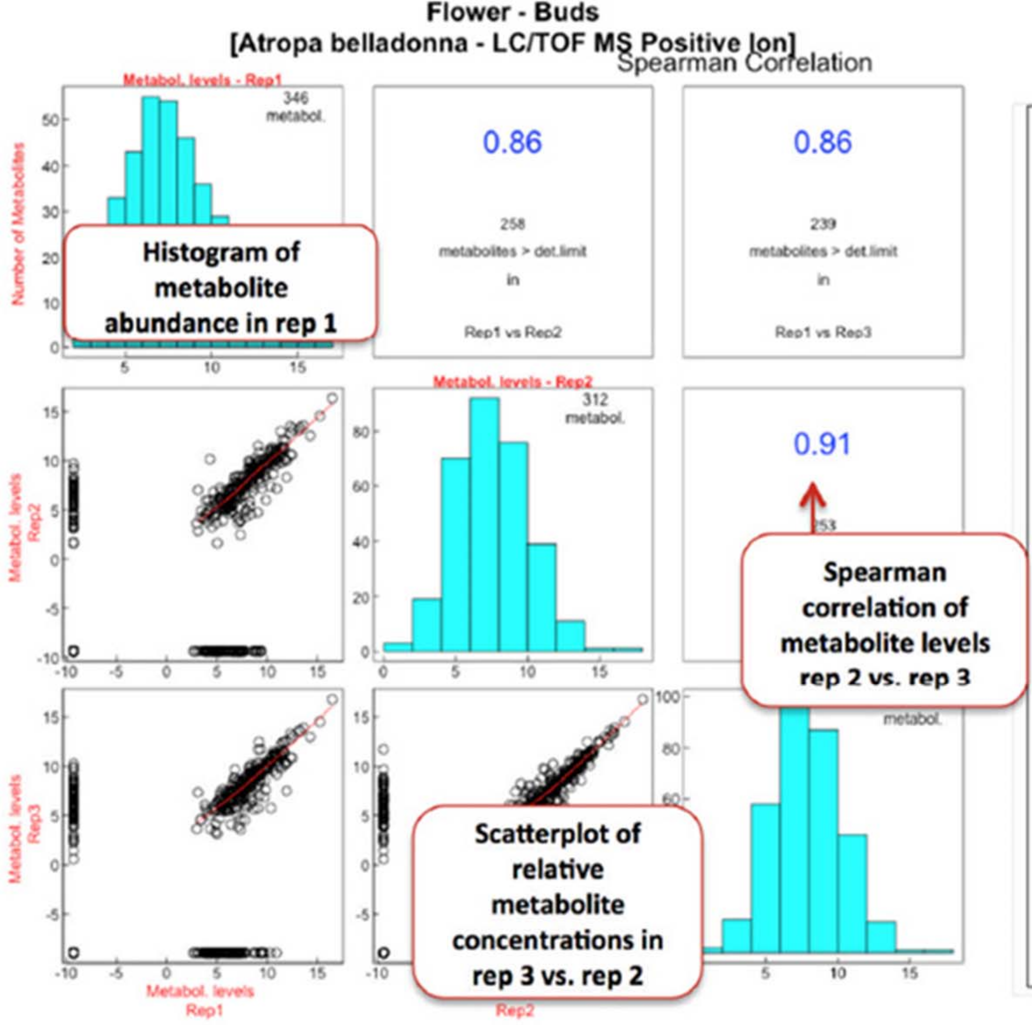
Chemical classification of metabolites

These metabolites have similar levels in two samples

X-axis: metabolite accumulation fold change (plotted on log2 scale) as a ratio between two user-selected samples

Each point represents a different metabolite

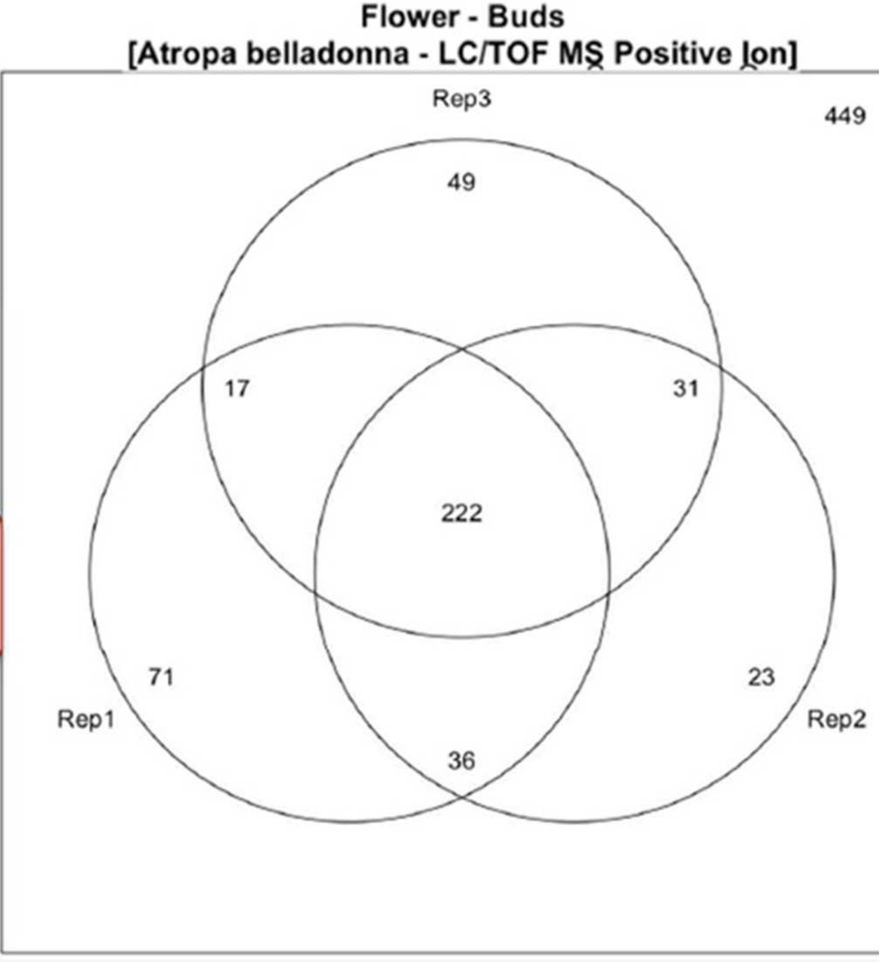
Other Plots



Histogram of metabolite abundance in rep 1

Spearman correlation of metabolite levels rep 2 vs. rep 3

Scatterplot of relative metabolite concentrations in rep 3 vs. rep 2



Classification

- Supervised classification
  - Supervised-classification procedures require independent cross-validation.
  - See MAQC-II recommendations Nat Biotechnol. 2010 August ; 28(8): 827–838. doi:10.1038/nbt.1665.
    - Wholly separate model building and validation stages. Can be 3 stage with multiple models tested
- Unsupervised classification
  - Unsupervised classification should be validated using resampling-based procedures.

Unsupervised classification - continued

- Unsupervised analysis methods
  - Cluster analysis
  - Principle components
- All have assumptions and input parameters and changing them results in very different answers

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