

Advanced Practical Genetics

Microarray - Hands-on data analysis

203.305

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Planning

25.9.17 - 9-10am - AHB1.40B

Introduction (lecture)

2.10.17 - 9-10am - AHB1.40B

Paper discussion

3.10.17 - 10-1pm - **C5.15**

From raw data to lists of differentially expressed genes (Step by step analysis of a microarray data set using the R language)

9.10.17 - 9-10am **C5.15**

Lab discussion

10.10.17 - 10-1pm **C5.15**

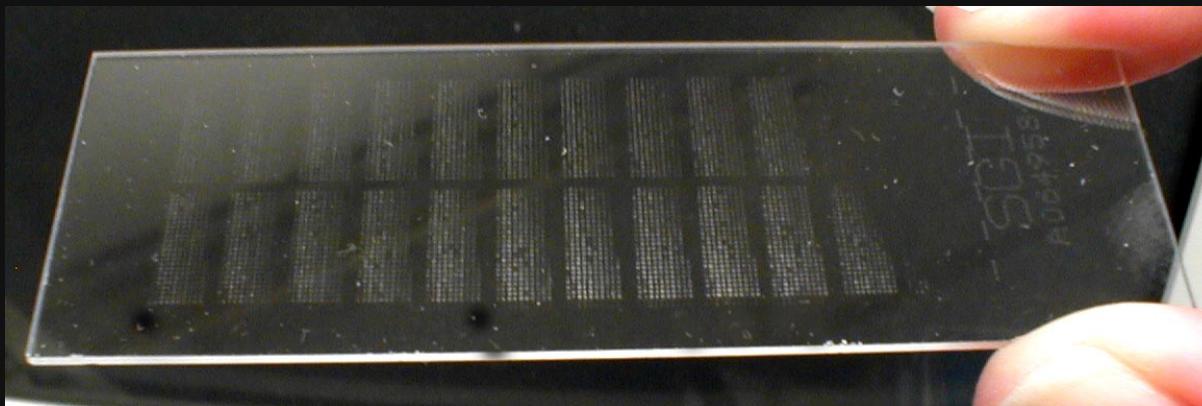
Biological interpretation of microarray data (Gene ontology analysis using the R language + online research of candidate genes)

Microarray studies

1. **Introduction**
2. Microarray technology
3. Analysis
4. MIAME
5. Examples of microarray studies (paper discussion topic and lab topic)

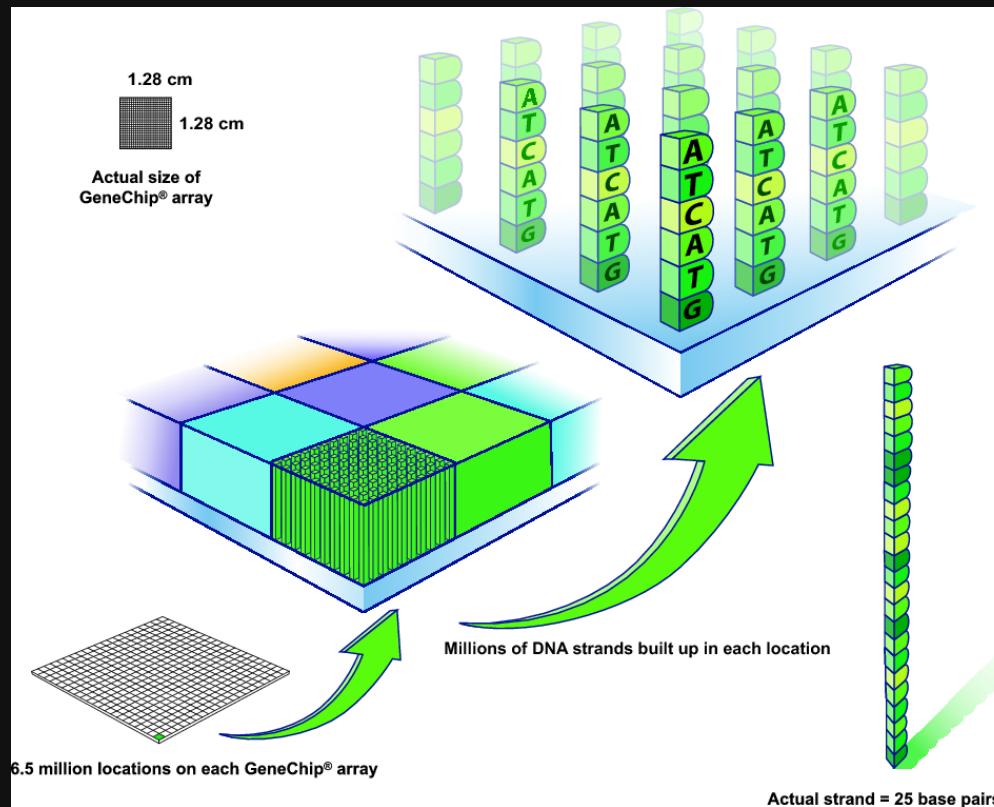
What are microarrays?

A microarray is a **solid support** (such as a membrane or glass microscope slide) on which **DNA of known sequence** is deposited in a **grid-like array**.



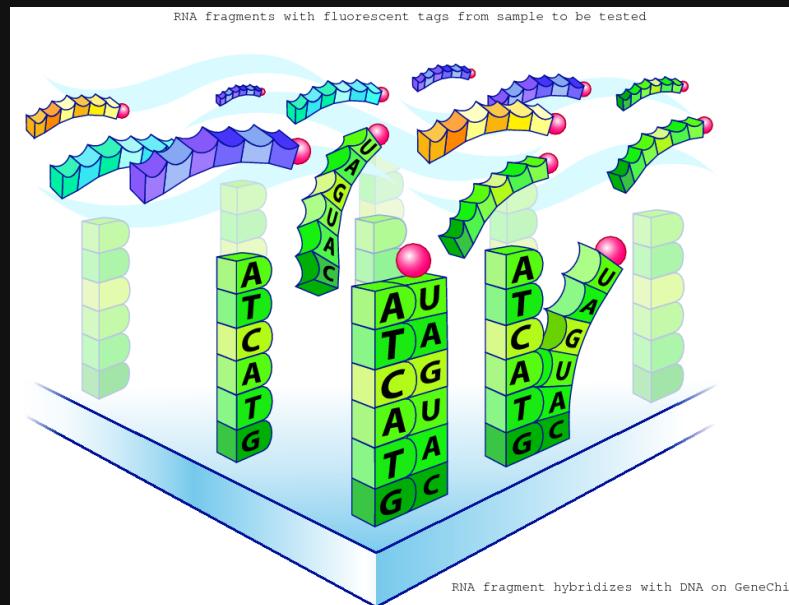
What are microarrays?

DNA microarray



What are microarrays

Hybridisation and transcriptomics?



The amount of RNA hybridised on each grid location can be measured and is a proxy for the gene expression level

Microarray applications

- **Gene expression analysis**
- Re-sequencing
- SNP-analysis
- DNA-Protein interactions

Expression Studies

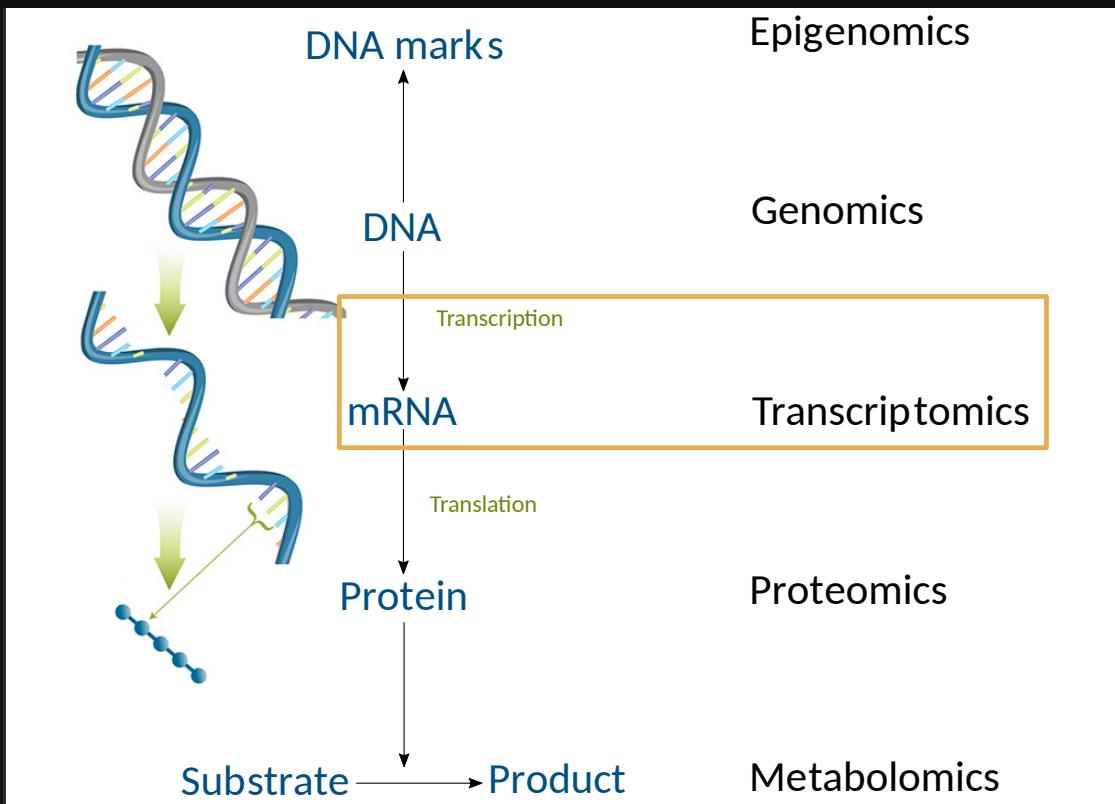


Figure modified from: Katherine Joyce, Woods Hole Oceanographic Institution

Definitions

- **Genome**: entire DNA sequence of an organism
- **Epigenome**: chemical marks of the genome that modify its expression
- **Transcriptome**: all gene transcripts present in a given cell/tissue at a given time (“snapshot”)
- **Transcriptomics**: global analysis of gene expression = genome-wide expression profiling

Definitions

- **cDNA**: complementary DNA made from mRNA by the enzyme reverse transcriptase
- **EST**: Expressed Sequence Tag, small pieces of an expressed gene (cDNA)
- **Hybridisation**: based on complementary molecules, sequences that are able to base-pair with one another. When two complementary sequences find each other, they will lock together, or hybridise (primer annealing, probe-target binding etc).

Genome-wide expression studies - Medical applications

- **Cancer research:** Cell-cycle monitoring, genetic markers detection
- **Drug development and response:** Treatment-induced expression pattern
- **Diagnosis:** Disease-associated expression patterns

Genome-wide expression studies - Biological applications

- **Development biology**: comparison of different developmental stages
- **Ecology**: interactions between organisms (symbiosis, pathogenicity...) or between organisms and environment (temperature, nutrient...)
- **Evolution**: within and between species variation, hybrids vs. parents, diploids vs. polyploids
- **Functional analyses**: wild type vs. mutant

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Microarray analysis principle

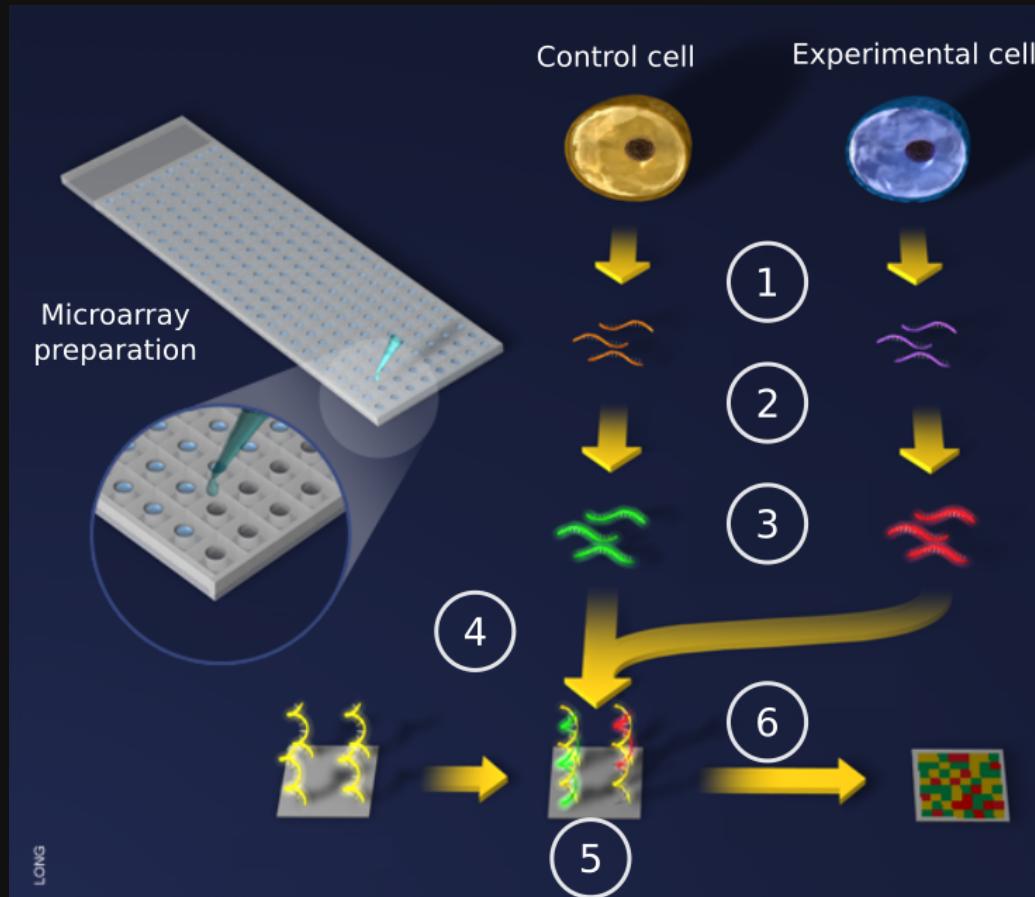


Image from: <http://www.scq.ubc.ca/image-bank/>

Microarray analysis principle

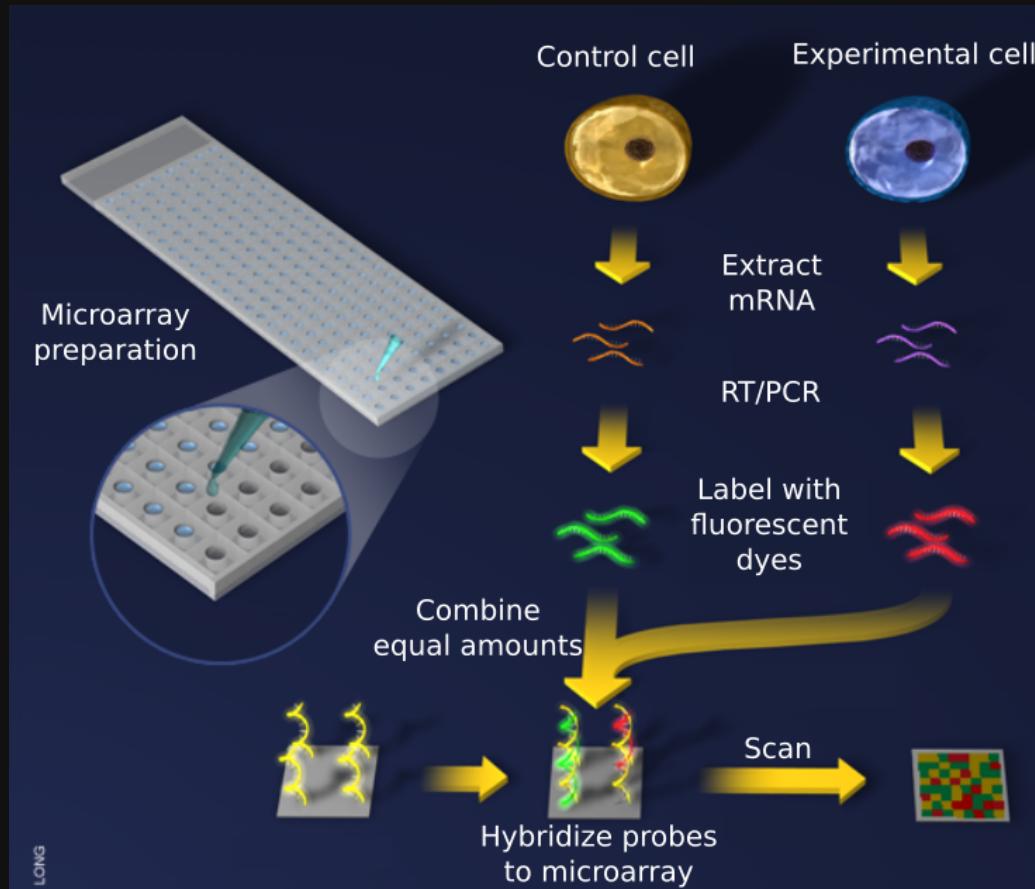


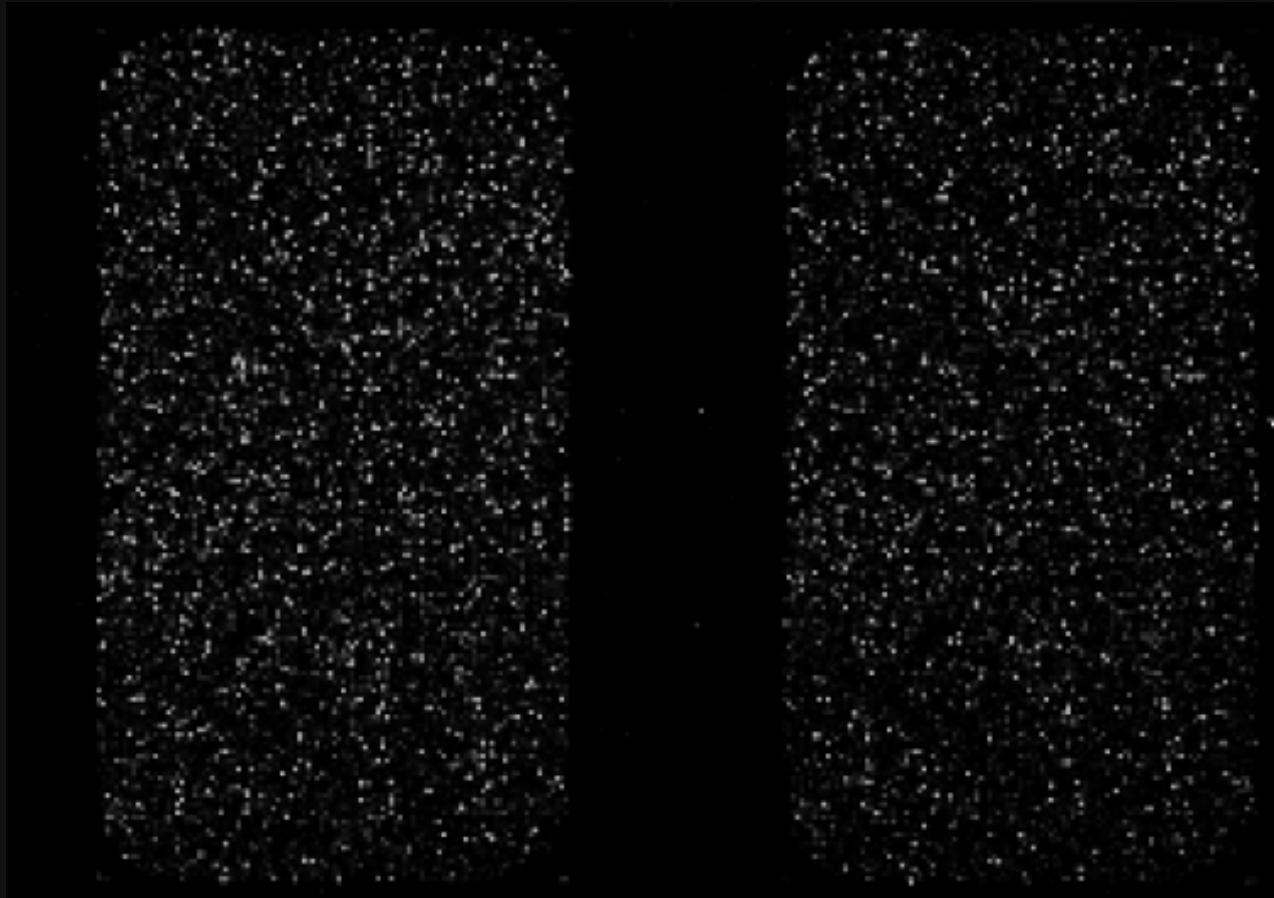
Image from: <http://www.scq.ubc.ca/image-bank/>

Competitive hybridisation

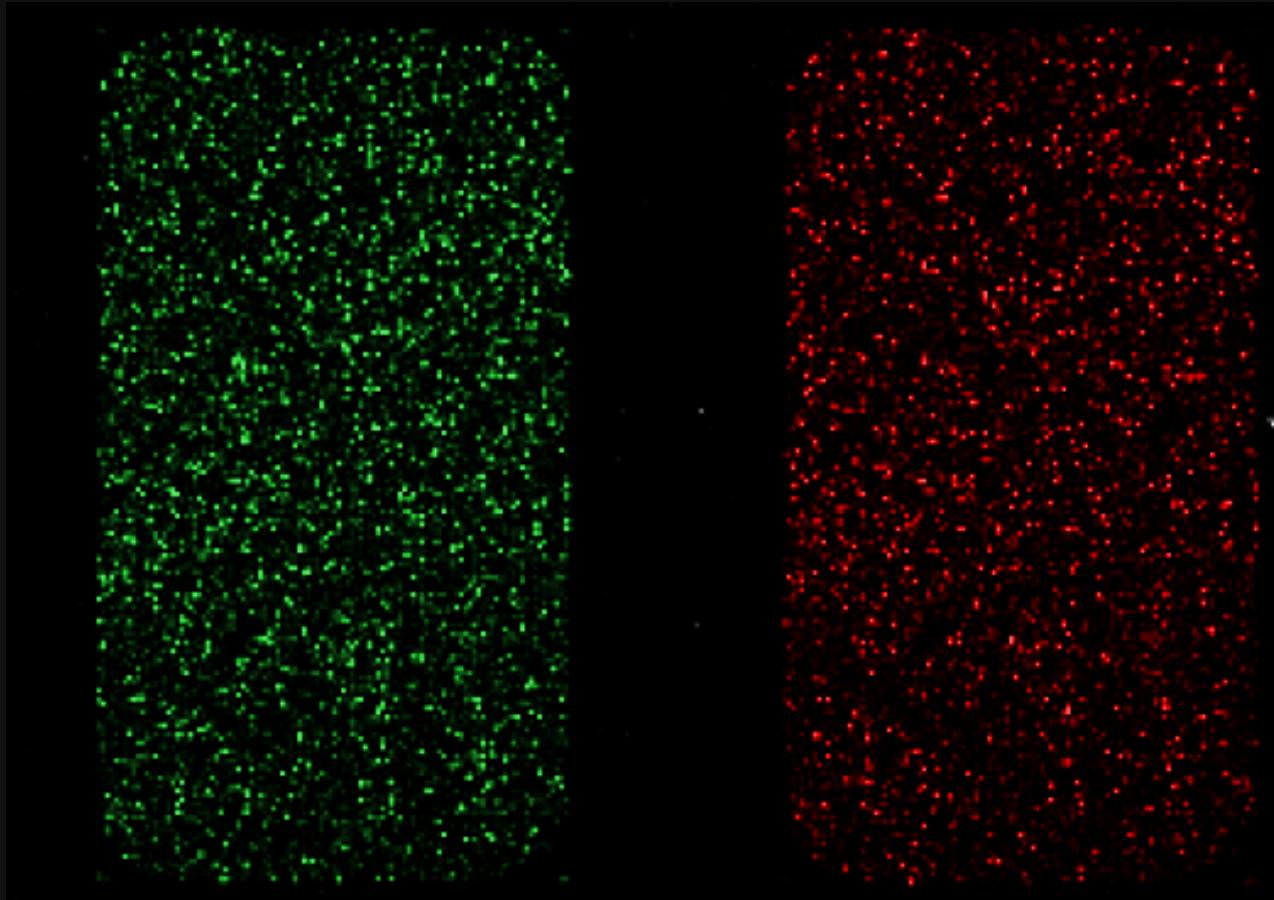
*It is possible to represent **different** samples on **one** microarray using **different fluorescent molecules (fluorophores)***

- **Cyanin 3** (Cy3): green fluorescence (excited at 550nm, emission at 570nm)
- **Cyanin 5** (Cy5): red fluorescence (excited at 650nm, emission at 770nm)

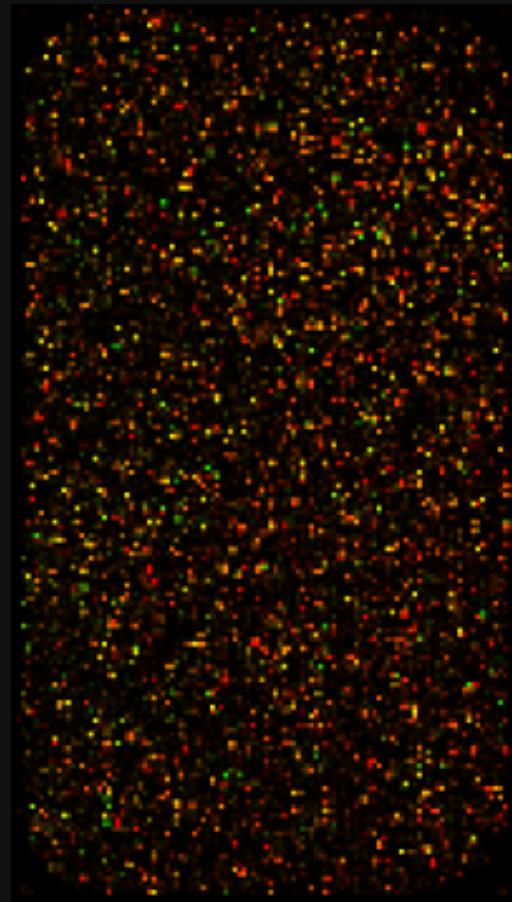
Competitive hybridisation



Competitive hybridisation



Competitive hybridisation



Microarray study pipeline

Question driven

Goals? Hypothesis? Questions?

Microarray study pipeline

- Platform/ design
 - What technology?
 - Source of the gene probes?
 - Cross-species hybridisation?
 - Replication level
 - Hybridisation scheme

Microarray study pipeline

- Platform
- **Laboratory steps**
 - Sample preparation and labelling
 - Hybridisation
 - Washing
 - Image acquisition

Microarray study pipeline

- Platform
- Laboratory steps
- **Bioinformatics steps**
 - Data transformation and normalisation
 - Analysis of differentially expressed genes (**statistical tests**, gene ontology, ...)
 - Visualisation (graphics)
 - Data storage (databases, MIAME standards)

Microarray study pipeline

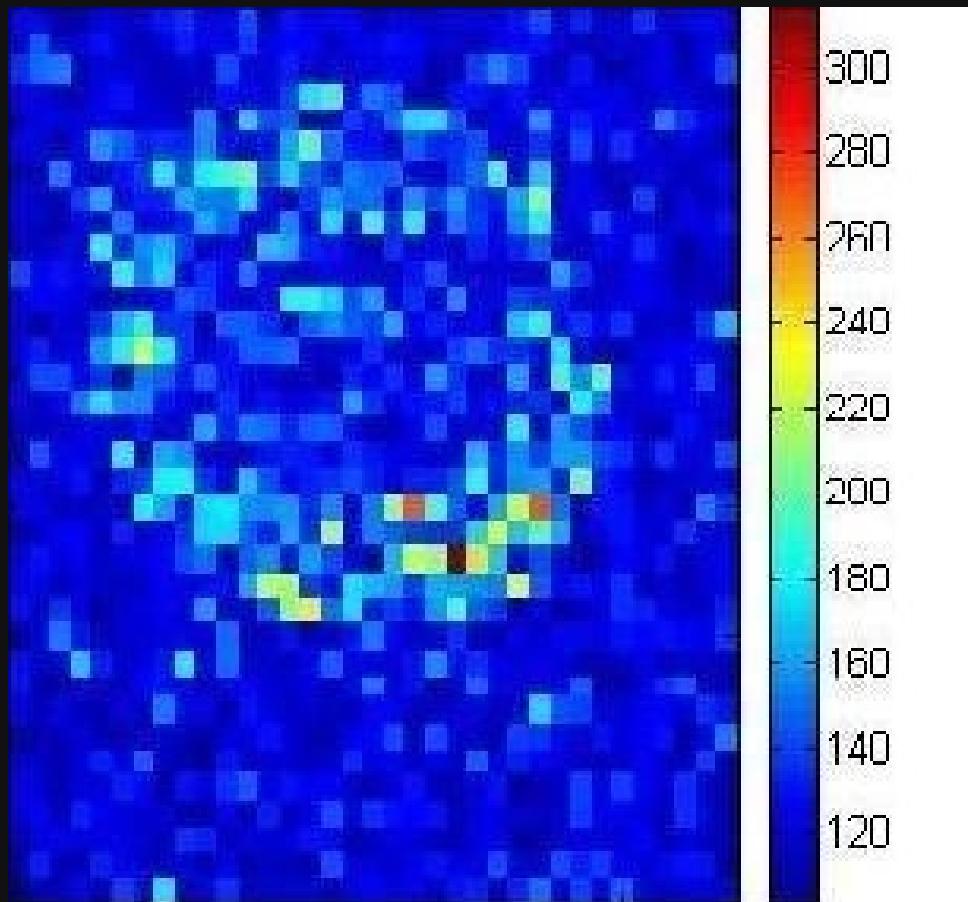
- Platform
- Laboratory steps
- Bioinformatics steps
- **Data interpretation**
 - Answers?
 - New hypotheses?
 - Follow-up experiments?
 - Validation?

Microarray studies

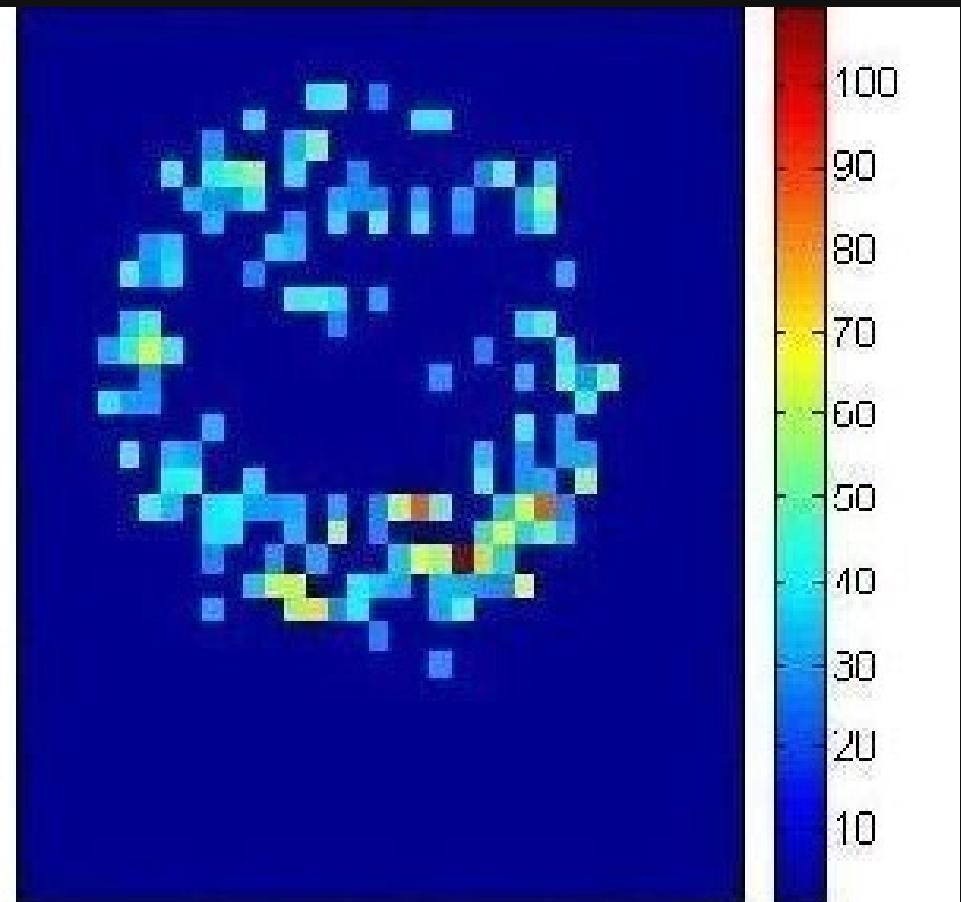
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Noise reduction

Before

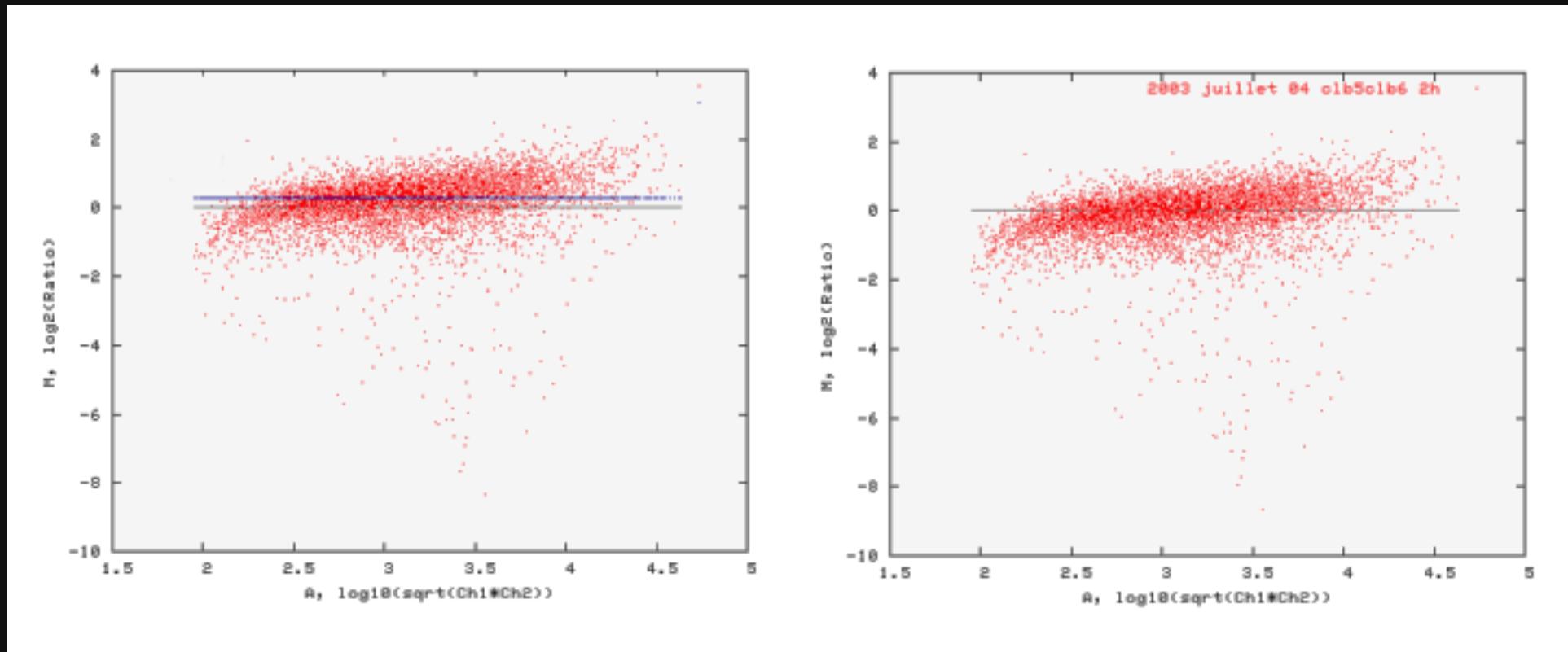


After



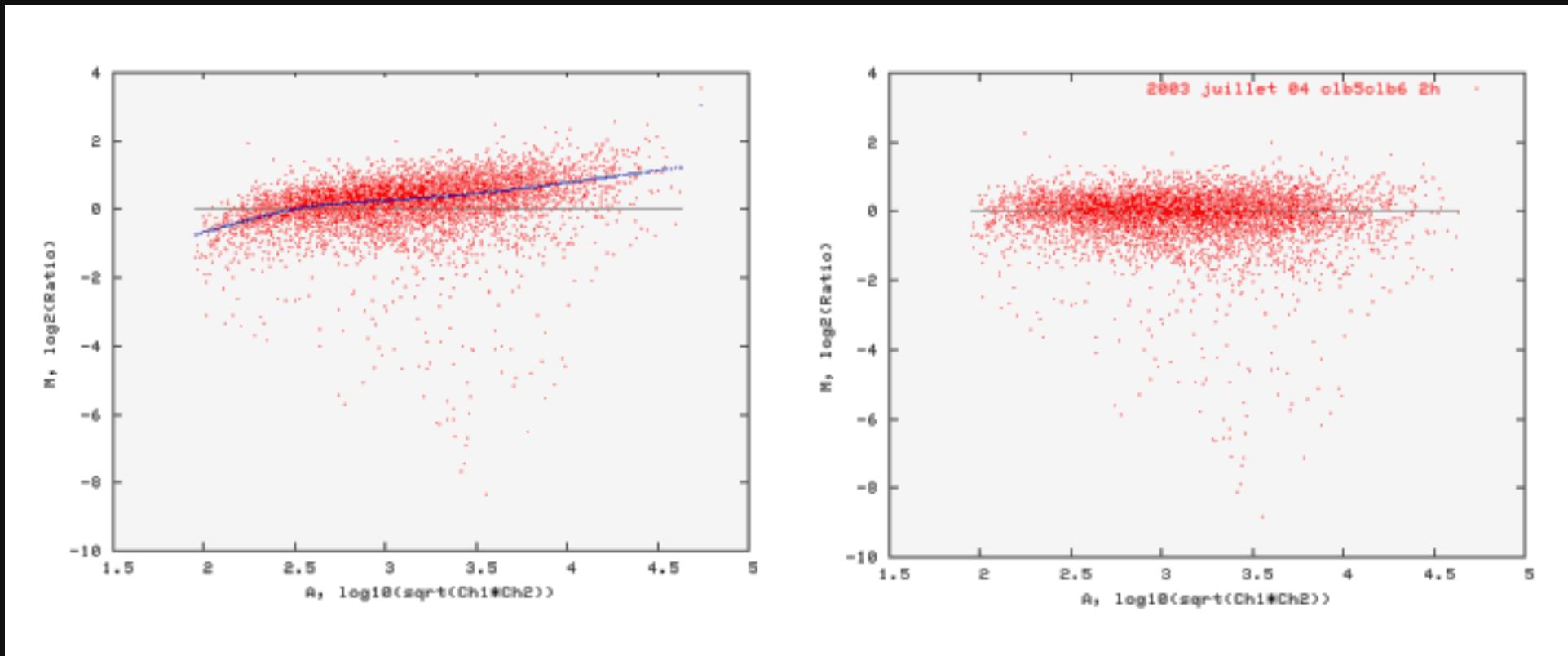
Data normalisation

Global normalisation



Data normalisation

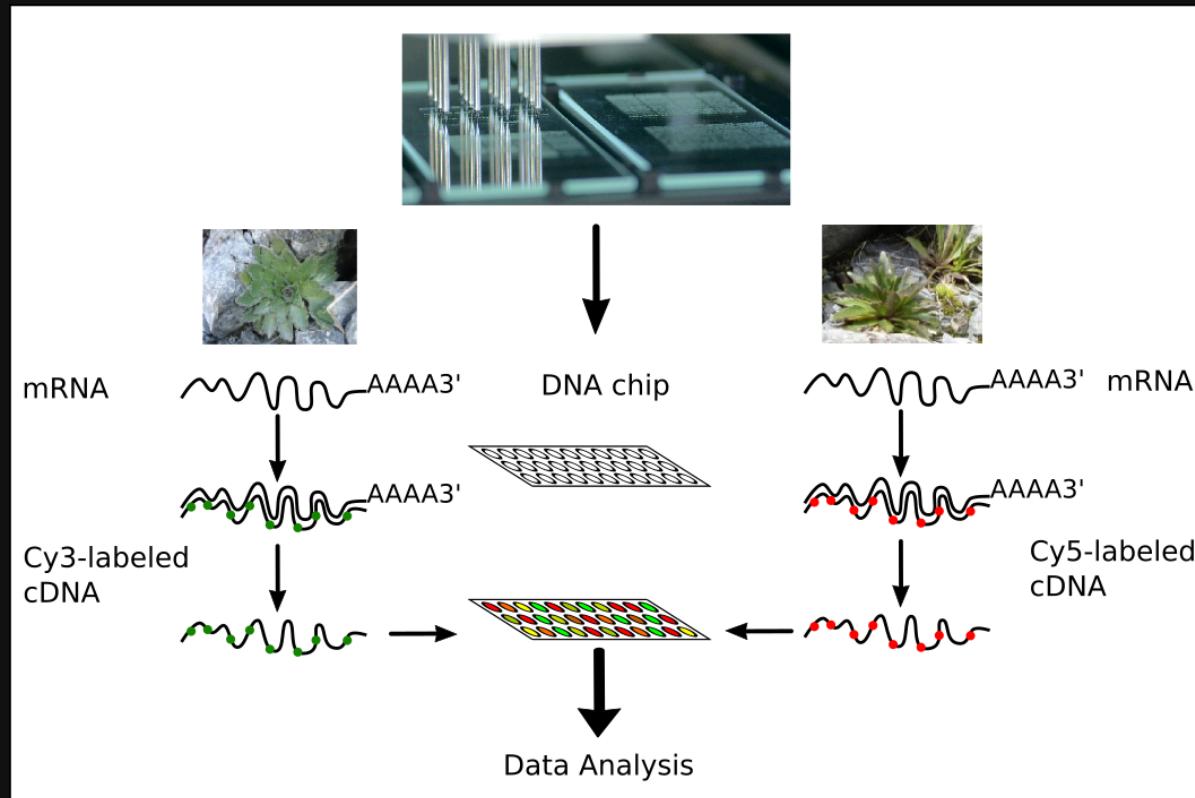
Lowess normalisation (LOcally Weighted Scatterplot Smoothing)



Expression ratios, M & A

- $Cy3 = Sample1$ (Green)
- $Cy5 = Sample2$ (Red)
- $Cy5 > Cy3$: higher expression in sample 2
- $Cy3 > Cy5$: higher expression in sample 1
- Log fold ratio: $M = \log_2\left(\frac{Cy5}{Cy3}\right) = \log_2(Cy5) - \log_2(Cy3)$
- Expression average: $A = \frac{1}{2}(\log_2(Cy5) + \log_2(Cy3)) = \frac{1}{2}\log_2(Cy5Cy3)$

Log Fold Ratio



Expression ratio: $\log\left(\frac{Cy5}{Cy3}\right)$

Log Fold Ratio

Reminder: $\log_2(x)$ is the unique real number y such that:

$$2^y = x.$$

For example: $\log_2(8) = 3$ because $2^3 = 8$

$Cy5/Cy3$	$\log_2(Cy5/Cy3)$
4	2
2	1
1	0
0.5	-1
0.25	-2

Hypothesis testing

T-test

Null hypothesis (H_0): gene x is **not** differentially expressed between two treatments

Mean:

$$\bar{x} = \frac{1}{M} \sum_{i=1}^M x_i; \text{ for gene } x \text{ in } M \text{ replicates}$$

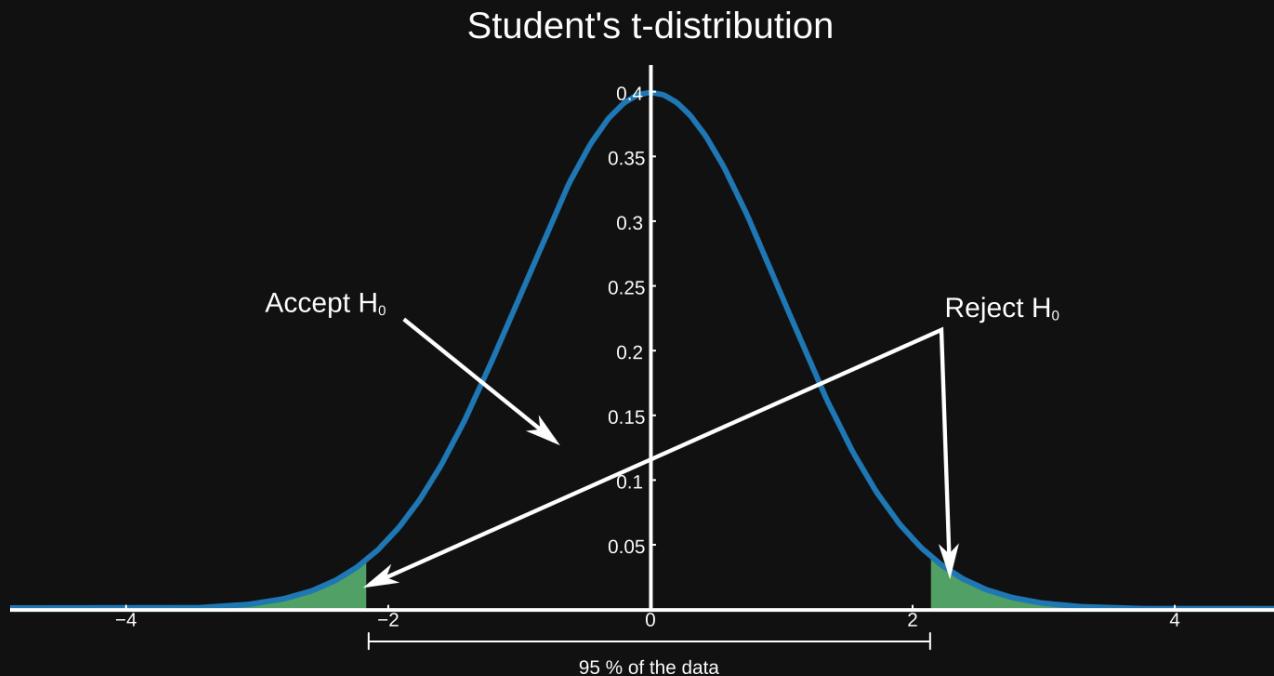
Variance:

$$S_x^2 = \frac{1}{M-1} \sum_{i=1}^M (x_i^2 - \bar{x}^2)$$

T-statistic:

$$T_x = \frac{\overline{x}_{C_1} - \overline{x}_{C_2}}{\sqrt{\frac{s_{x_{C_1}}^2}{M} + \frac{s_{x_{C_2}}^2}{N}}}$$

T-test and P-value



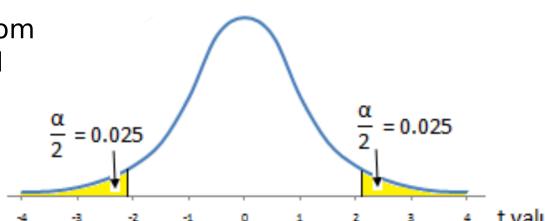
T-test is used only to compare two samples. To compare more than two samples, ANOVA (Analysis Of Variance) is used.

T-test and P-value

Student's t Distribution Table

Critical T value for 2 degrees of freedom
is 4.303 for a 95% confidence interval
(2-tail $\alpha=0.05$)

Degree of freedom = Sample size



	90%	95%	97.5%	99%	99.5%	99.95%	1-Tail Confidence Level
	80%	90%	95%	98%	99%	99.9%	2-Tail Confidence Level
	0.100	0.050	0.025	0.010	0.005	0.0005	1-Tail Alpha
df	0.20	0.10	0.05	0.02	0.01	0.001	2-Tail Alpha
1	3.0777	6.3138	12.7062	31.8205	63.6567	636.6192	
2	1.8856	2.9200	4.3027	6.9646	9.9248	31.5991	
3	1.6377	2.3534	3.1824	4.5407	5.8409	12.9240	
4	1.5332	2.1318	2.7764	3.7469	4.6041	8.6103	
5	1.4759	2.0150	2.5706	3.3649	4.0321	6.8688	
6	1.4398	1.9432	2.4469	3.1427	3.7074	5.9588	

Hypothesis testing

T-test

*Null hypothesis (H_0): gene A is **not** differentially expressed between two treatments*

1. Compute the signal to noise ratio (difference of the means or medians) for each gene
2. Compute the t-statistic for each gene using the replicates
3. Compare t-statistic with the t-distribution
4. If t-statistic is more extreme than the critical t-statistic at a chosen significance level (e.g. $\alpha = 0.05$) reject the null hypothesis, otherwise accept it. **P-value estimation**

Quiz

Usually, a $p < \textbf{0.05}$ is considered small enough to reject the null hypothesis of no biological effect in favour of the alternative hypothesis of a biological effect.

P-values are also known under type **1** error – the probability of rejecting the null hypothesis when it is actually true (= false positive rate).

P-value of 0.01 means a false positive rate of **1** %.

When analysing multidimensional data sets, p-values need to be adjusted for **multiple testing**.

Two common p-value adjustment methods are **Bonferroni** and **False Discovery Rate**.

Bonferroni Correction

- If you hypothesize that **a specific gene** is up-regulated, $p < 0.05$ is fine.
- If you hypothesize that **any of 10,000 genes** is up-regulated, with $p < 0.05$ you can expect to see 5% (**500 genes**) up-regulated by chance alone.
- To account for the thousands of repeated measurements, some researchers apply a Bonferroni correction.

$$p < (0.05) / 10,000 \\ p < 5e^{-6}$$

*The Bonferroni correction is generally considered to be **too** conservative and **False Discovery Rate (FDR)** should be used.*

False Discovery Rate

Benjamini-Hochberg method

Imagine an array with 6400 genes and an experiment where 184 genes are differentially expressed at $P = 0.01$: 64 genes would be expected to appear differentially expressed by chance alone.

$$\text{FDR} = \text{false discovery rate} = \frac{64}{184} * 100 = 35\%$$

False Discovery Rate

Benjamini-Hochberg method

P-value	Observed number of genes	Expected number of false positives	FDR
10^{-2}	184	64	35
10^{-3}	35	6	18
10^{-4}	15	0.6	4

With decreasing p-value, FDR also decreases, but so does the number of differentially expressed genes – choose a p-value which balances both!

Microarray studies

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MIAME Standard

Minimum Information About a Microarray Experiment that
is needed to enable the interpretation of the results of the
experiment unambiguously and potentially to reproduce the
experiment

<http://fged.org/Workgroups/MIAME/miame.html/>

MIAME Standard

1. **Raw data** for each hybridisation (CEL or GPR files)
2. **Processed** (normalised) **data** (used to draw the conclusions from the study)
3. Essential **sample annotation** including experimental factors and their values
4. **Experimental design** including sample data relationships (e.g. which hybridisations are technical and biological replicates)
5. Sufficient **array annotation** (e.g. gene identifiers, probe sequences)
6. Essential **laboratory and data processing protocols** (e.g. normalisation method used to obtain the final data)

Gene expression databases

Gene Expression Omnibus (GEO) @NCBI (<http://www.ncbi.nlm.nih.gov/geo/>)

NCBI Resources How To

GEO Home Documentation Query & Browse Email GEO pydupont My NCBI Sign Out

Gene Expression Omnibus

GEO is a public functional genomics data repository supporting MIAME-compliant data submissions. Array- and sequence-based data are accepted. Tools are provided to help users query and download experiments and curated gene expression profiles.

 Gene Expression Omnibus

Keyword or GEO Accession

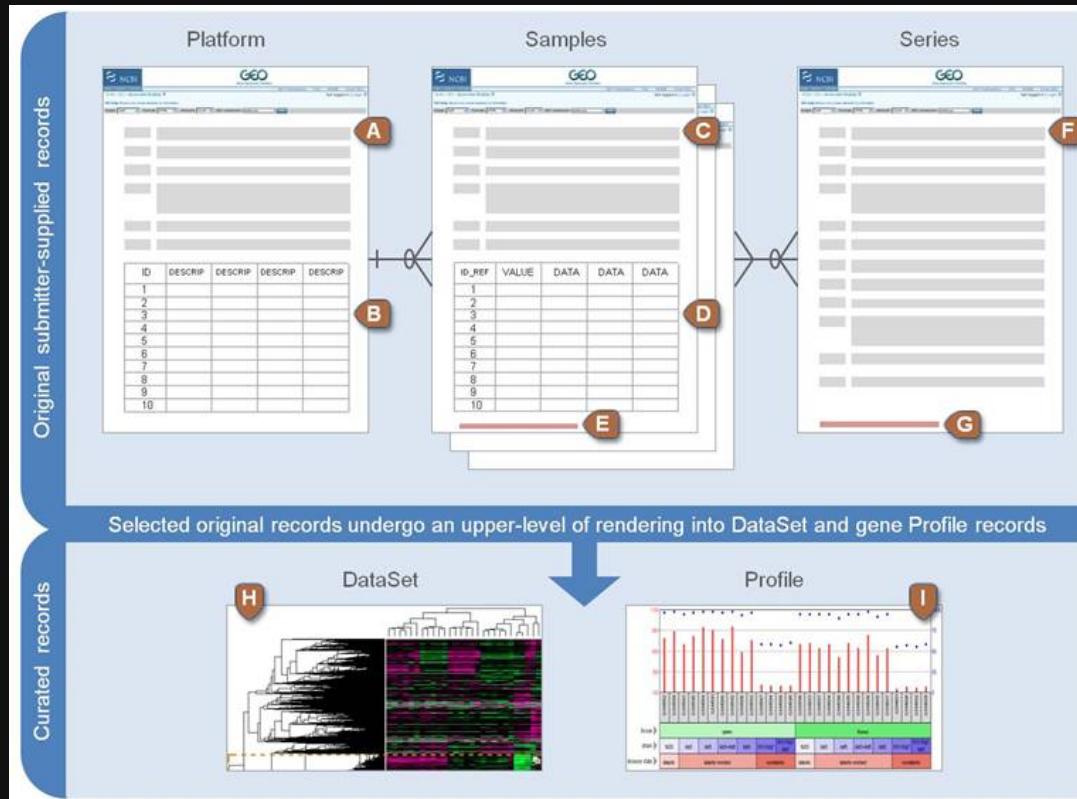
Getting Started	Tools	Browse Content
Overview	Search for Studies at GEO DataSets	Repository Browser
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About GEO DataSets	Search GEO Documentation	Series: 59282
About GEO Profiles	Analyze a Study with GEO2R	Platforms: 14769
About GEO2R Analysis	GEO BLAST	Samples: 1539231
How to Construct a Query	Programmatic Access	
How to Download Data	FTP Site	

Information for Submitters		
Login to Submit	Submission Guidelines	MIAME Standards
	Update Guidelines	Citing and Linking to GEO
		Guidelines for Reviewers
		GEO Publications

Gene expression databases

Geo Datasets @NCBI (<http://www.ncbi.nlm.nih.gov/gds/>)

Geo Profiles @NCBI (<http://www.ncbi.nlm.nih.gov/geoprofiles/>)



Gene expression databases

ArrayExpress @ EBI (<http://www.ebi.ac.uk/arrayexpress/>)

The screenshot shows the homepage of the ArrayExpress functional genomics data archive. At the top, there's a navigation bar with links for Services, Research, Training, and About us. Below the navigation is a search bar with examples like "E-MEXP-31, cancer, p53, Geuvadis". The main content area features a large title "ArrayExpress – functional genomics data" and a sub-section "ArrayExpress Archive of Functional Genomics Data stores data from high-throughput functional genomics experiments, and provides these data for reuse to the research community." A "Browse ArrayExpress" button is present. To the right, there's a "Data Content" section showing statistics: Updated today at 04:00, 58541 experiments, 1729714 assays, and 34.89 TB of archived data. Below this, there's a "Latest News" section with a recent entry about a revamped submission tool. The footer contains links for EMBL-EBI services, research, training, industry, and about us, along with copyright information.

EMBL-EBI

ArrayExpress

Services | Research | Training | About us

Search Examples: E-MEXP-31, cancer, p53, Geuvadis Advanced

Feedback | Login

ArrayExpress – functional genomics data

ArrayExpress Archive of Functional Genomics Data stores data from high-throughput functional genomics experiments, and provides these data for reuse to the research community.

Browse ArrayExpress

Latest News

7 July 2015 - Revamped guide for ArrayExpress submission tool "Annotate"

It's been almost a year since we launched Annotate. We have listened to our submitters and rolled out a revamped guide covering many frequently asked questions. Spare a few minutes to pick up some bite-size hints to make your submission experience smoother. For example, did you know we have introduced a few time-saving features in Annotate?

Links

Information about how to search ArrayExpress, understand search results, how to submit data and FAQ can be found in our [Help section](#).

Find out more about the [Functional Genomics group](#).

Tools and Access

[ArrayExpress Bioconductor package](#): an R package to access ArrayExpress and build data structures.

[Programmatic access](#): query and download data using web services or JSON.

[FTP access](#): data can be downloaded directly from our FTP site.

Related Projects

Discover up and down regulated genes in numerous experimental conditions in the [Expression Atlas](#).

Explore the [Experimental Factor Ontology](#) used to support queries and annotation of ArrayExpress data.

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Gene expression databases

Expression Atlas @ EBI (<http://www.ebi.ac.uk/gxa/>)

The screenshot shows the homepage of the Expression Atlas at EMBL-EBI. At the top, there's a navigation bar with links for Services, Research, Training, and About us. Below the navigation is a search bar with placeholder text "Enter gene query..." and a "Search" button. To the right of the search bar are examples of queries: ASPM, REACT_284558, ENSMUS000000021789, and "zinc finger".

The main content area has a title "Expression Atlas: Differential and Baseline Expression". Below it, a sub-section titled "iRAP: RNA-seq analysis tool" provides a brief description of the tool and its purpose. There are also sections for "Publications" (listing two academic papers), "Browse..." (with categories for Baseline Experiments, Plant Experiments, and All Experiments), and a "Search..." section with fields for Gene query, Organism (set to Homo sapiens), and Sample properties.

At the bottom of the page, there's a footer with links to various EMBL-EBI services like News, Brochures, and Intranet, as well as research, training, industry, and about us sections. The footer also includes copyright information: "EMBL-EBI, Wellcome Trust Genome Campus, Hinxton, Cambridgeshire, CB10 1SD, UK +44 (0)1223 49 44 44" and "Copyright © EMBL-EBI 2015 | EBI is an outstation of the European Molecular Biology Laboratory | Privacy | Cookies | Terms of use".

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Microarray paper discussion

MOLECULAR ECOLOGY

Molecular Ecology (2009) 18, 3227–3239

doi: 10.1111/j.1365-294X.2009.04261.x

Adaptive differences in gene expression associated with heavy metal tolerance in the soil arthropod *Orchesella cincta*

DICK ROELOFS,* THIERRY K. S. JANSSENS,* MARTIJN J. T. N. TIMMERMANS,*
BENJAMIN NOTA,* JANINE MARIËN,* ZOLTÁN BOCHDANOVITS,† BAUKE YLSTRA‡ and
NICO M. VAN STRAALEN*

*Institute of Ecological Science, VU University Amsterdam, De Boelelaan 1085, 1081 HV Amsterdam, The Netherlands,

†Department of Clinical Genetics, Section Medical Genomics, VU Medical Center, Van der Boechorststraat 7, 1081 BT Amsterdam, The Netherlands, ‡Microarray Facility CCA, VU Medical Center, De Boelelaan 1117, 1081 HV Amsterdam, The Netherlands

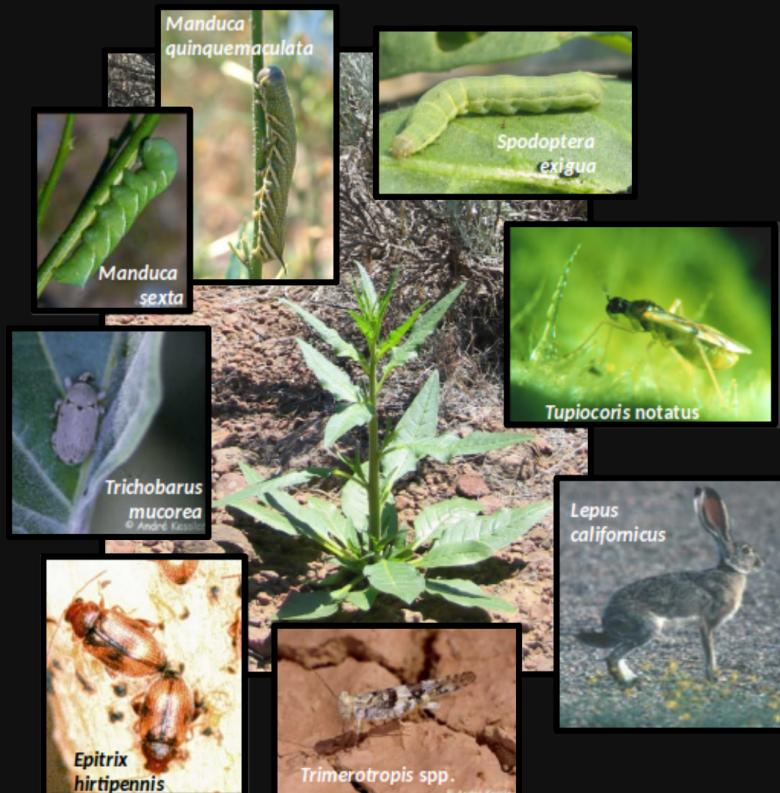
Lab case study: Herbivory in *Nicotiana attenuata* (Solanaceae)



What type of research?

- Which genes and metabolites defend plants against insects?
- Costs and benefits of defense
- Genetic engineering of defense traits
- Plant pollination

Lab case study: Herbivory in *Nicotiana attenuata* (Solanaceae)



Why *N. attenuata*?

- Diverse herbivore community
- High plasticity (direct and indirect defense)
- Easily cultivated annual species

Case study - Chips, veggies & vegetarians

Specificity in Ecological Interactions. Attack from the Same Lepidopteran Herbivore Results in Species-Specific Transcriptional Responses in Two Solanaceous Host Plants^{1[w]}

Dominik D. Schmidt^{2,3}, Claudia Voelckel², Markus Hartl, Silvia Schmidt, and Ian T. Baldwin*

Department of Molecular Ecology, Max Planck Institute for Chemical Ecology, Beutenberg Campus,
07745 Jena, Germany

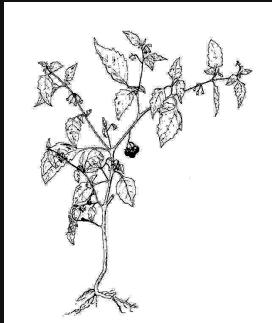
Case study - Chips, veggies & vegetarians

The chip: cDNA array with 15,264 potato genes from TIGR (The Institute for Genomic Research)



The veggies

Solanum nigrum
Black nightshade



Nicotiana attenuata
Coyote tobacco



The vegetarian

Manduca sexta



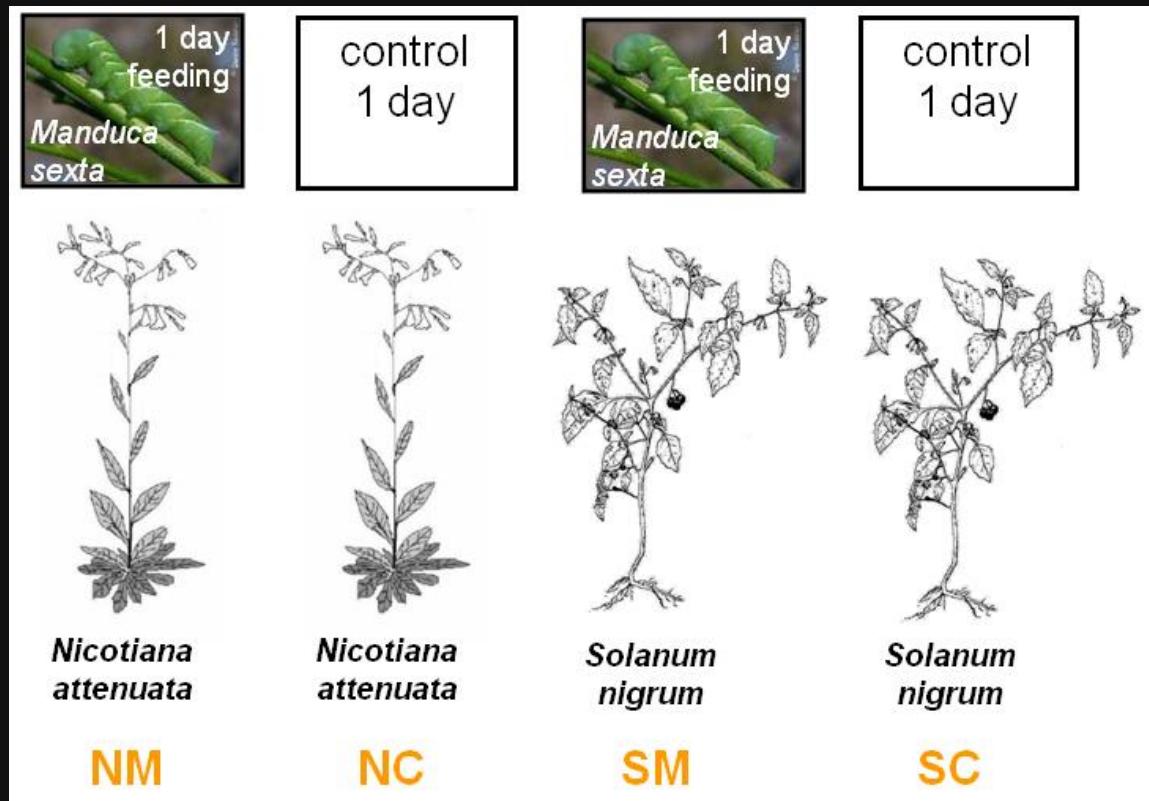
Question:

Do tobacco and black nightshade plants respond differently to caterpillar attack?

Microarray Case Study

RNA source

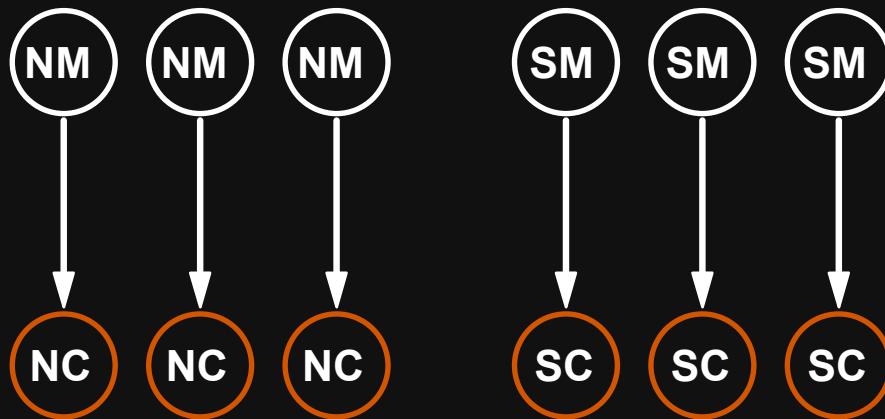
2 herbivore treatments and 2 controls



Microarray Case Study

6 arrays

Each arrow represents one array. Herbivore-induced tissue (Cy3) was co-hybridised control tissue (Cy5). Each comparison was replicated three times.



What will you do in the lab?

Lab 1

R warm-up exercise. Identification of **differentially expressed genes**

Lab 2

Identification of **differentially expressed biological processes**

Thanks

Slides available here:

http://bit.ly/massey_203305