Advanced genetics - 203.305

Microarray - Hands-on data analysis

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Planning

- **15.9.14** – Introduction (lecture), *AHB2.38*
- **22.9.14** – Paper discussion, *AHB2.38* - **Discussion worksheet due**
- **23.9.14** – From raw data to lists of differentially expressed genes (Step by step analysis of a microarray data set using the R language, **3h lab**, *SC5.10*)
- **29.09.14** – **Lab discussion** (feedback!) and new developments in global gene expression analysis, *AHB2.38*
- **30.09.14** – Biological interpretation of microarray data (Gene ontology analysis using the R language + online research of candidate genes, **3h lab**, *SC5.10*)

Microarray studies

1. **Indroduction**
2. Microarray technology
3. Statistics
4. Gene expression databases and MIAME
5. Examples of microarray studies (paper discussion topic and lab topic)

Microarray applications

- **Gene expression analysis**
- Re-sequencing
- SNP-analysis
- DNA-Protein interactions
- Discovery of new transcripts/alternative splice variants

Expression Studies

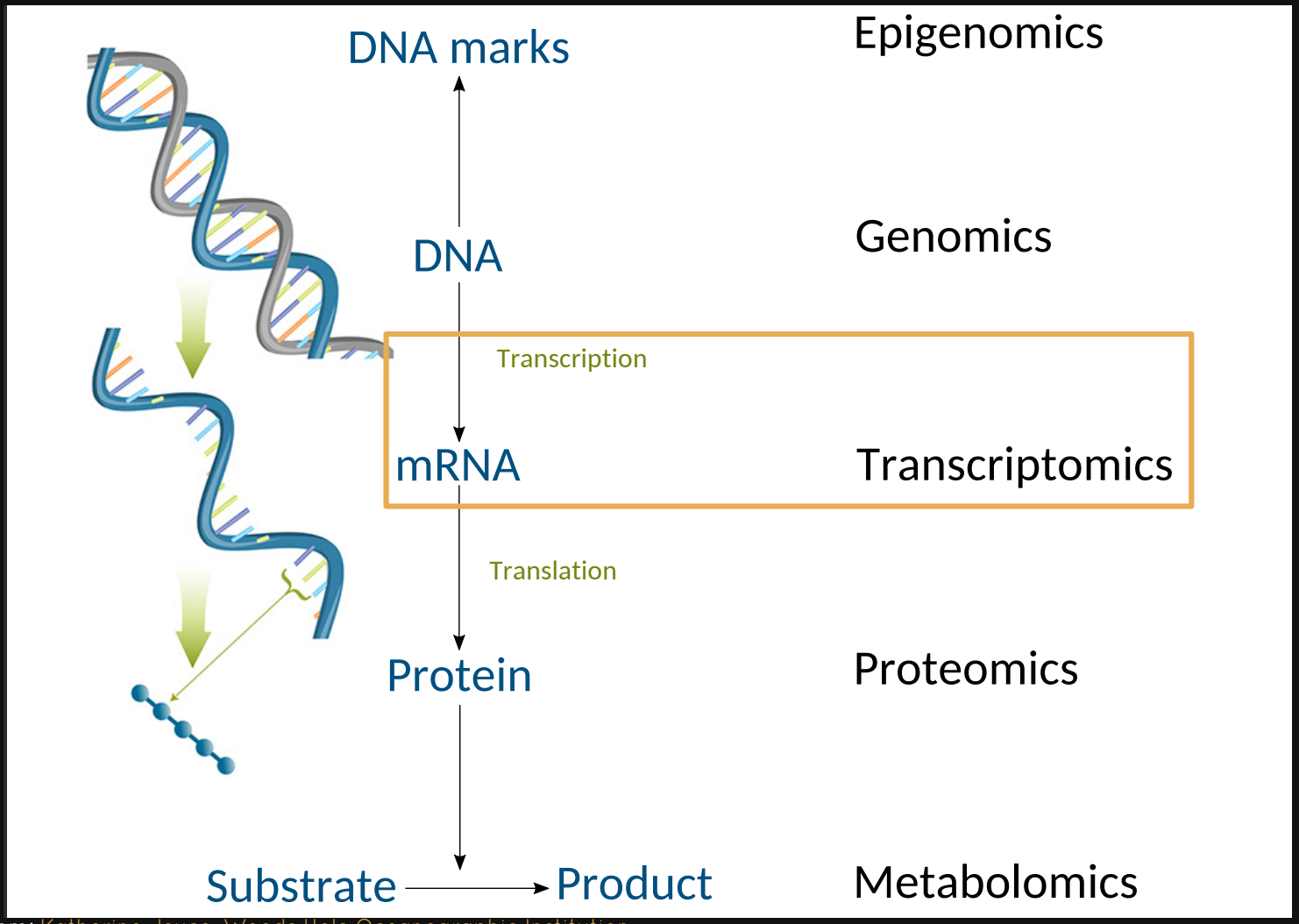

Figure modified from: Katherine Joyce, Woods Hole Oceanographic Institution

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)
- **Hybridization**: 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 hybridize (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

Hypothesis generating tool

Microarray studies

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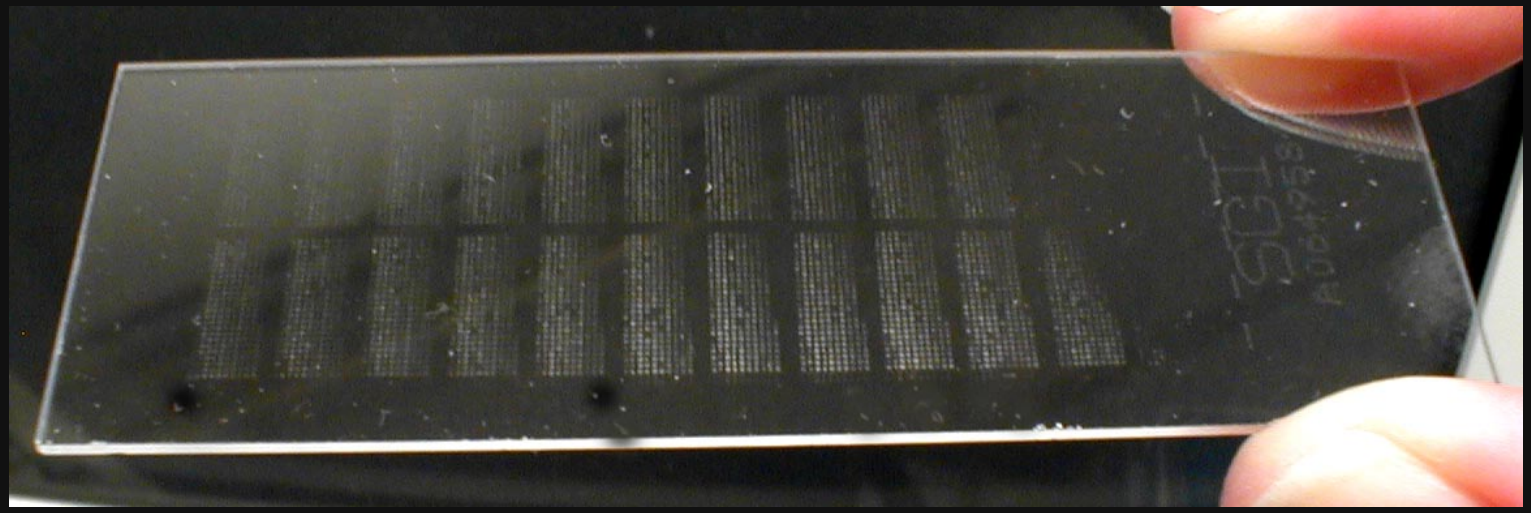
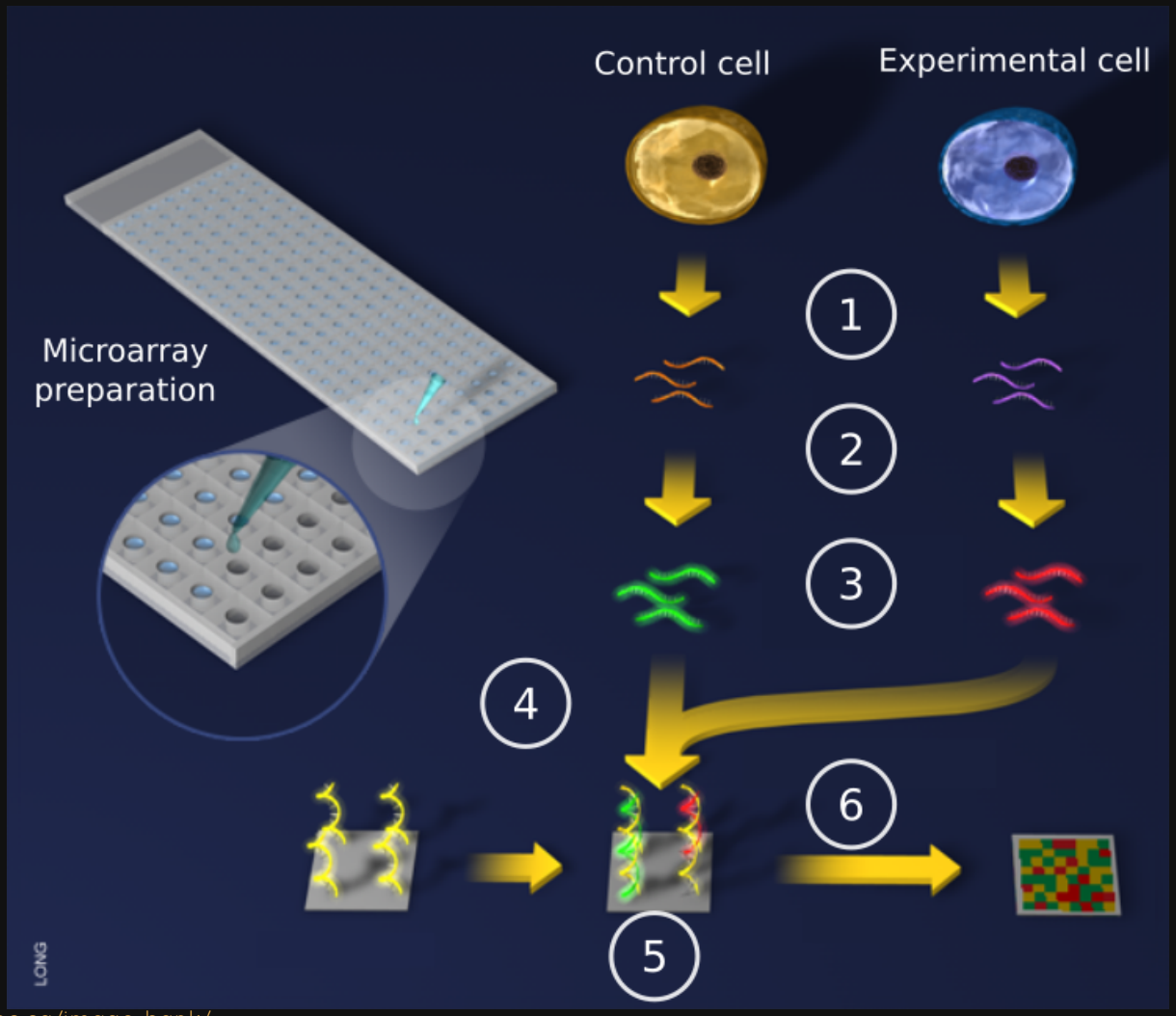
What are microarrays?

*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**.*

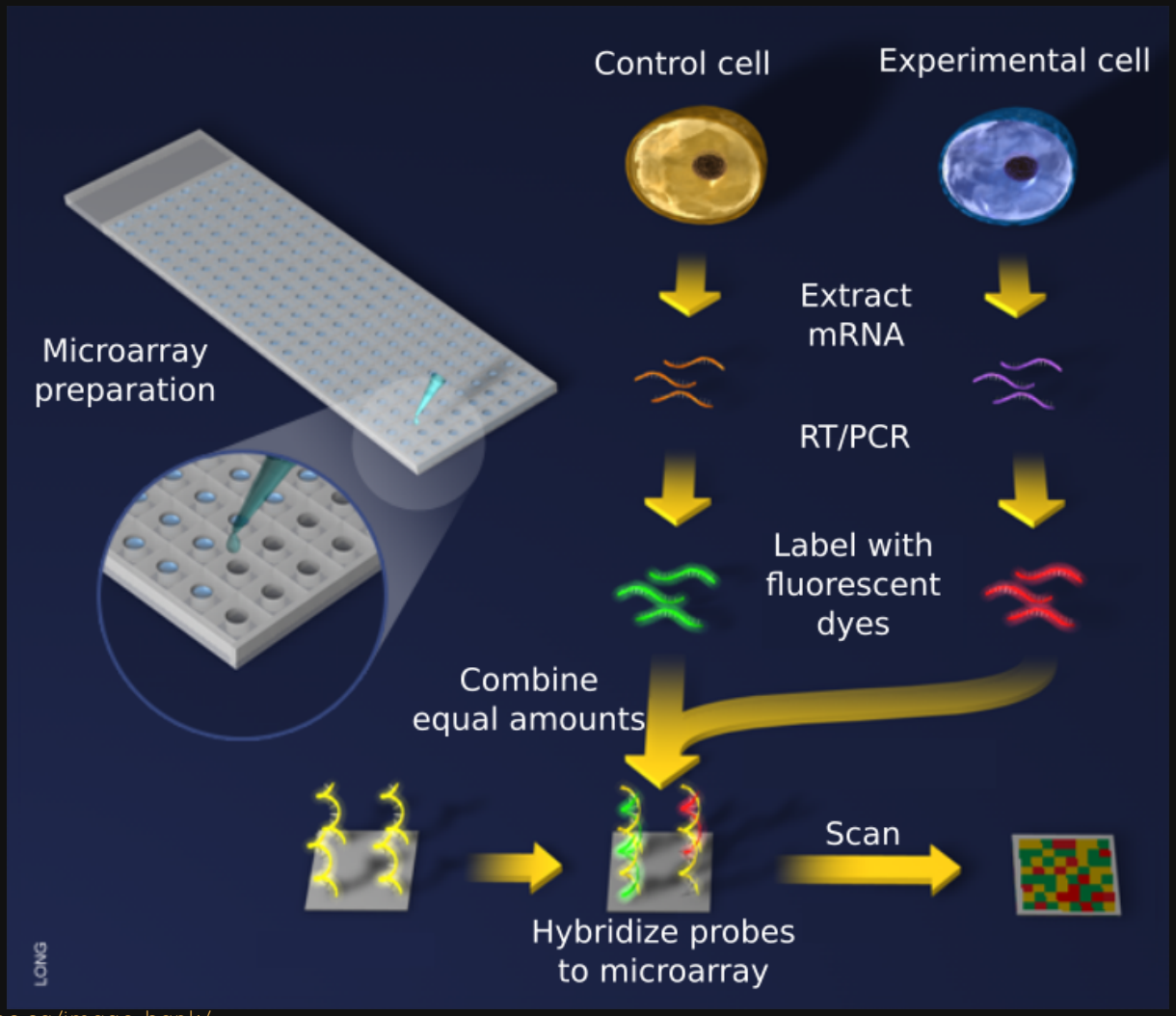


Microarray analysis principle



Imge from: http://www.scq.ubc.ca/image-bank/

Microarray analysis principle



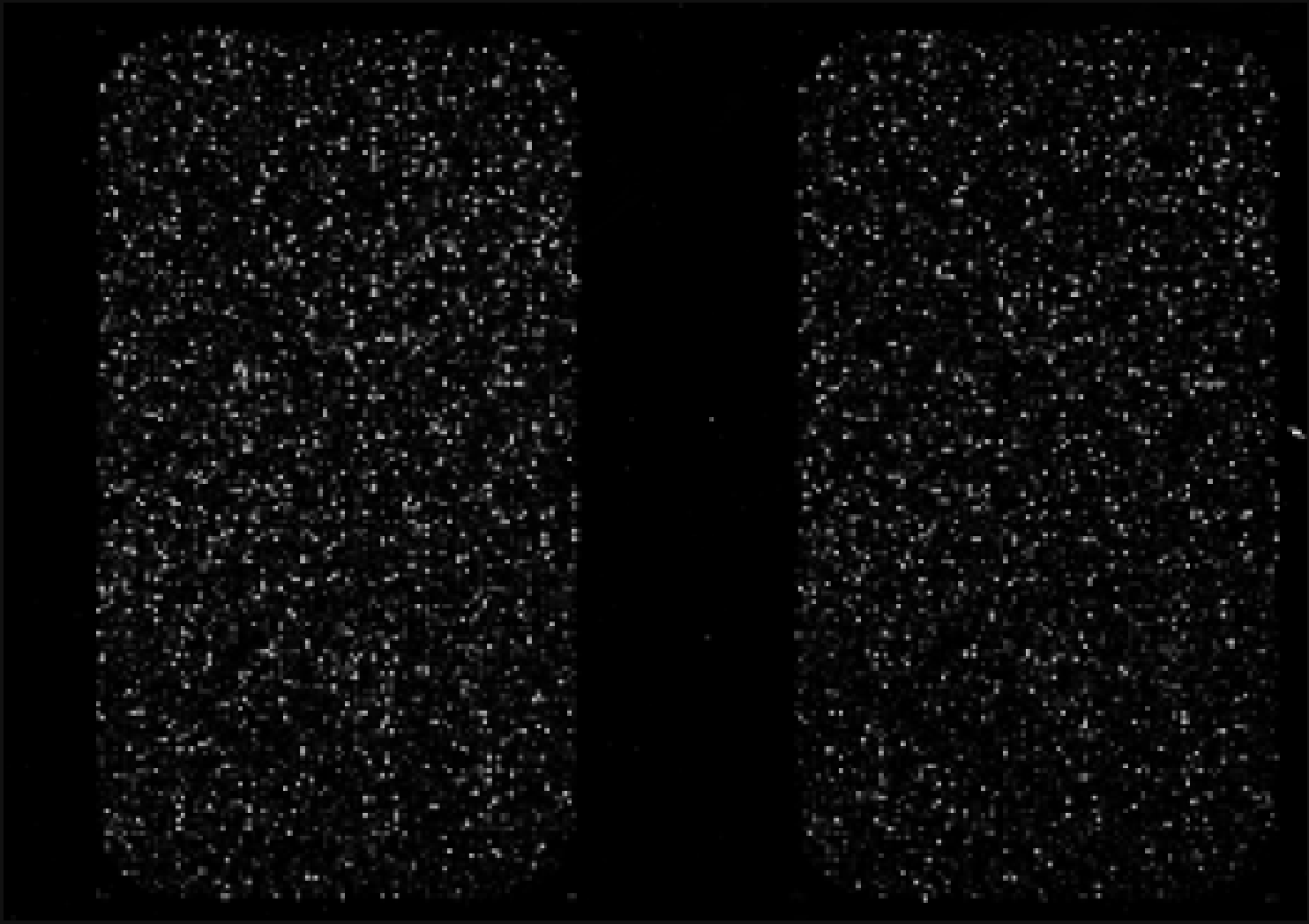
Imge from: http://www.scq.ubc.ca/image-bank/

Competitive hybridization

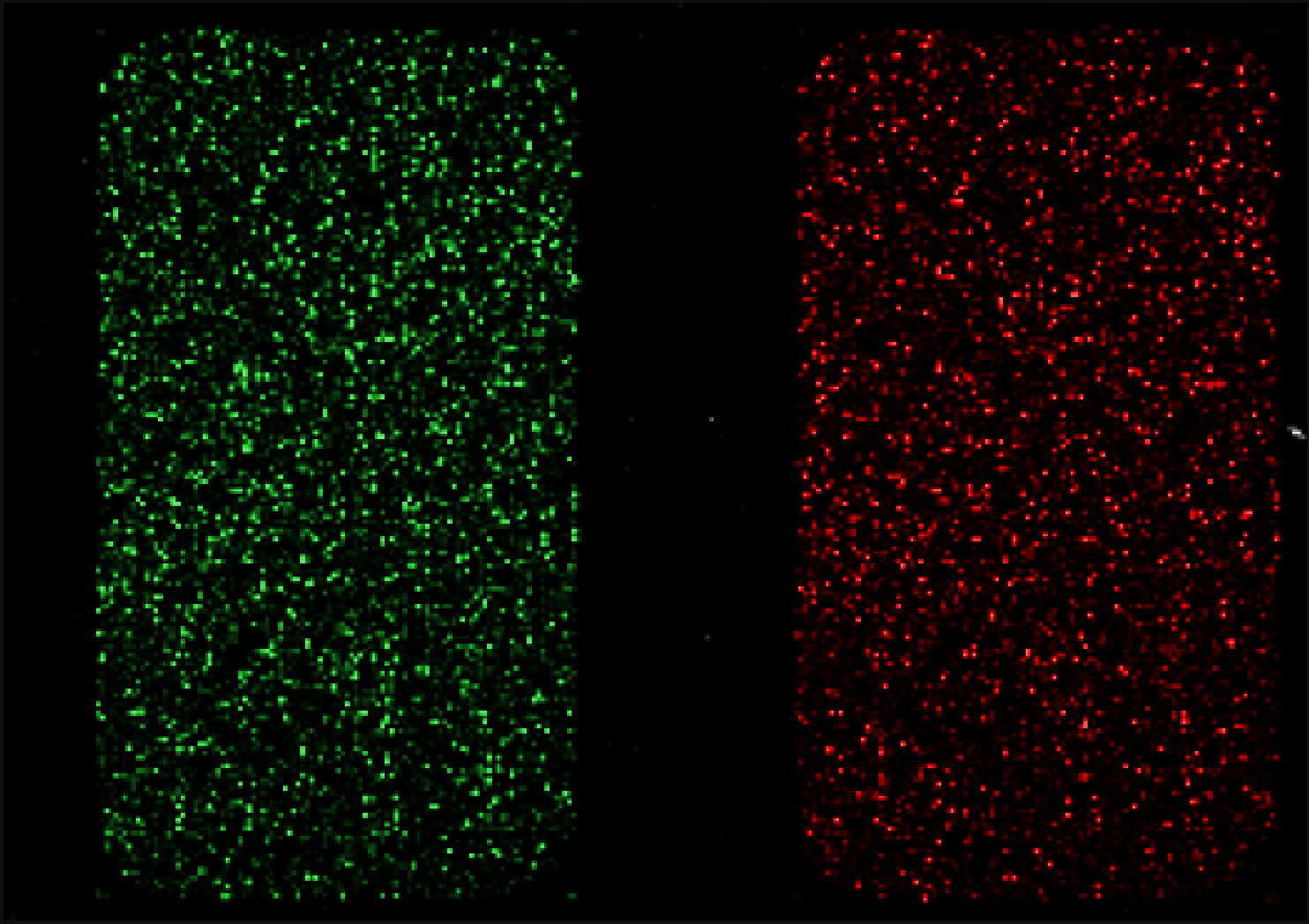
It is possible to represent **different** samples on **one** microarray using **different fluorescent molecules (fluorophores)**

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

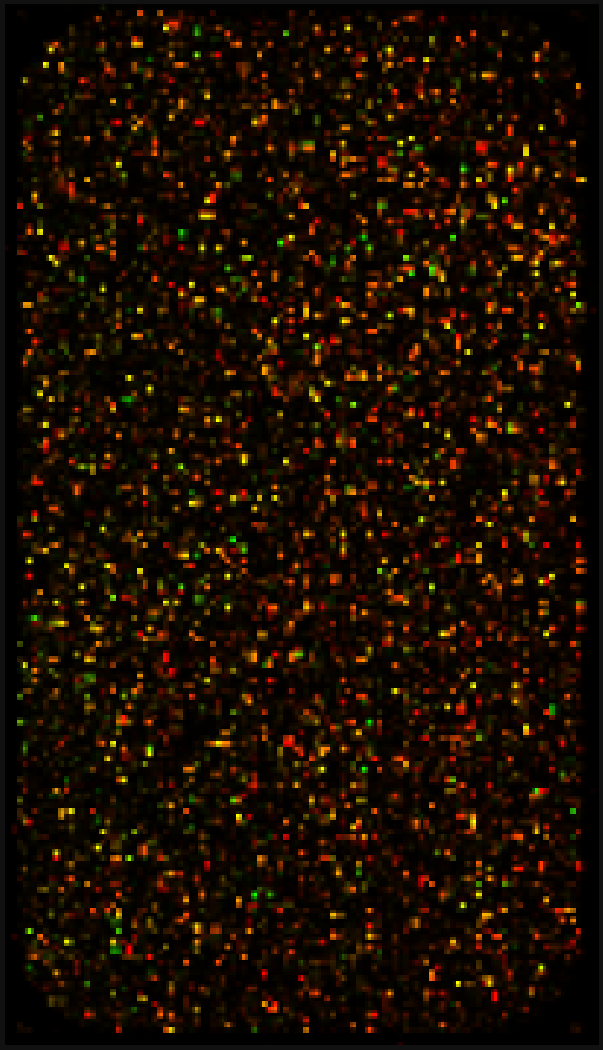
Competitive hybridization



Competitive hybridization



Competitive hybridization



Microarray study pipeline

Question driven

Goals? Hypothesis? Questions?

Microarray study pipeline

- **Platform**
  - What technology?
  - Source of the gene probes?
  - Cross-species hybridization?

Microarray study pipeline

- Platform
- **Experimental design**
  - What statistics?
  - What analysis software?
  - Replication level
  - Hybridization scheme

Microarray study pipeline

- Platform
- Experimental design
- **Laboratory steps**
  - Sample preparation and labelling
  - Hybridization
  - Washing
  - Image acquisition

Microarray study pipeline

- Platform
- Experimental design
- Laboratory steps
- **Bioinformatics steps**

- Data transformation and normalization
- Analysis of differentially expressed genes (**statistical tests**, gene ontology, ...)
- Visualization (graphics)
- Data storage (databases, MIAME standards)

Microarray study pipeline

- Platform
- Experimental design
- Laboratory steps
- Bioinformatics steps
- **Data interpretation**

- Answers?
- New hypotheses?
- Follow-up experiments?
- Validation?

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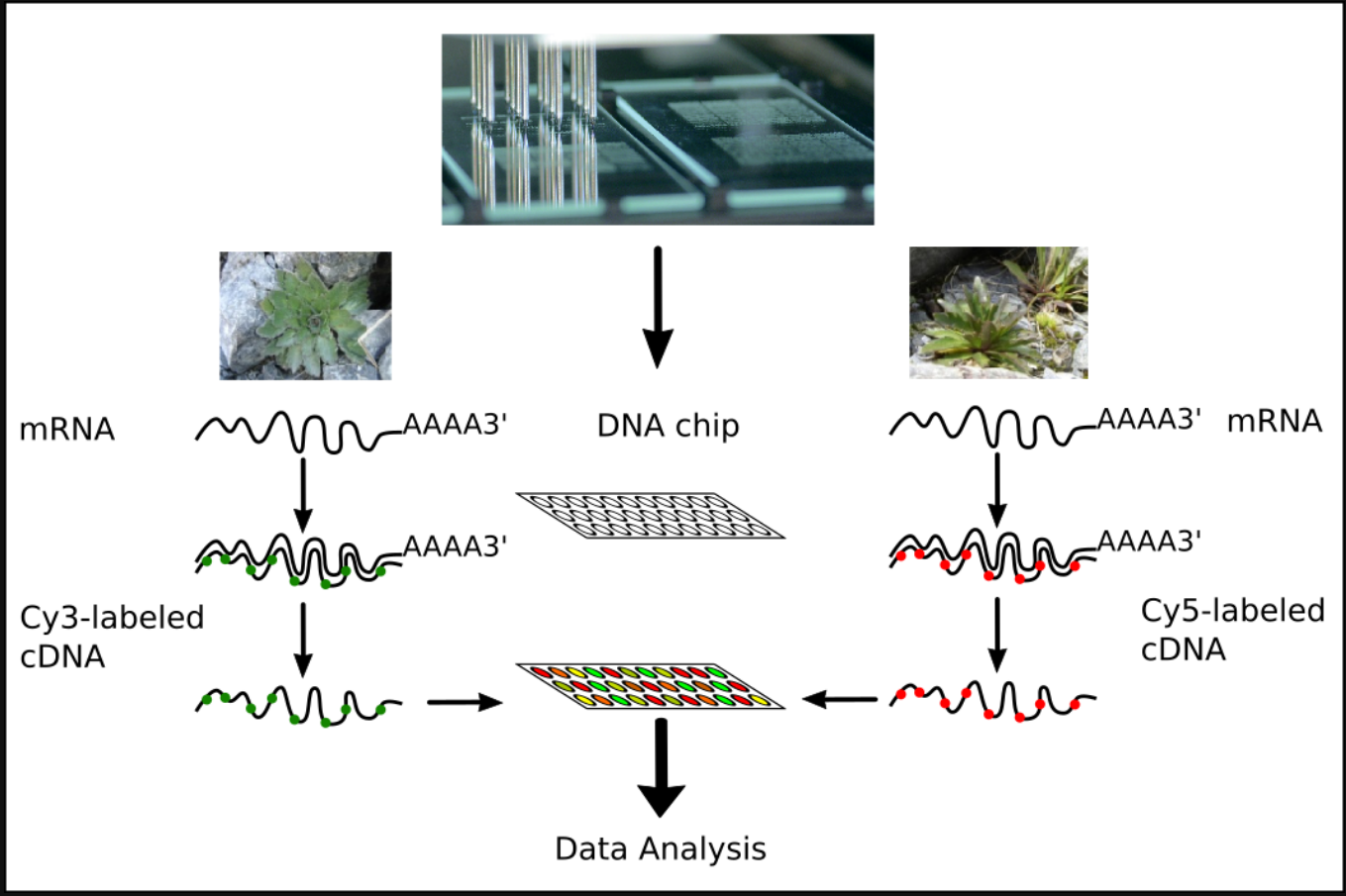
Log Fold Ratio



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

Log Fold Ratio

- $Cy3 = Sample1$ (Green)
- $Cy5 = Sample2$ (Red)
- $Cy5 > Cy3$: higher expression in sample 2
- $Cy3 > Cy5$: higher expression in sample 1
- M(log fold ratio) = $log_2(\frac{Cy5}{Cy3})$
- M(log fold ratio) = $log_2(Cy5) - log_2(Cy3)$

Log Fold Ratio

Reminder: $log_2(x)$ is the unique real number y such that: $b^y = x$.
For example: $log_2(8) = 3$ because $2^3 = 8$

$log_2(Cy5/Cy3)$	$Cy5/Cy3$
2	4
1.58	3
1	2
0.58	1.5
0	1
-0.58	0.66
-1	0.5
-1.58	0.33
-2	0.25

Hypothesis testing

T-test

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

Mean:

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

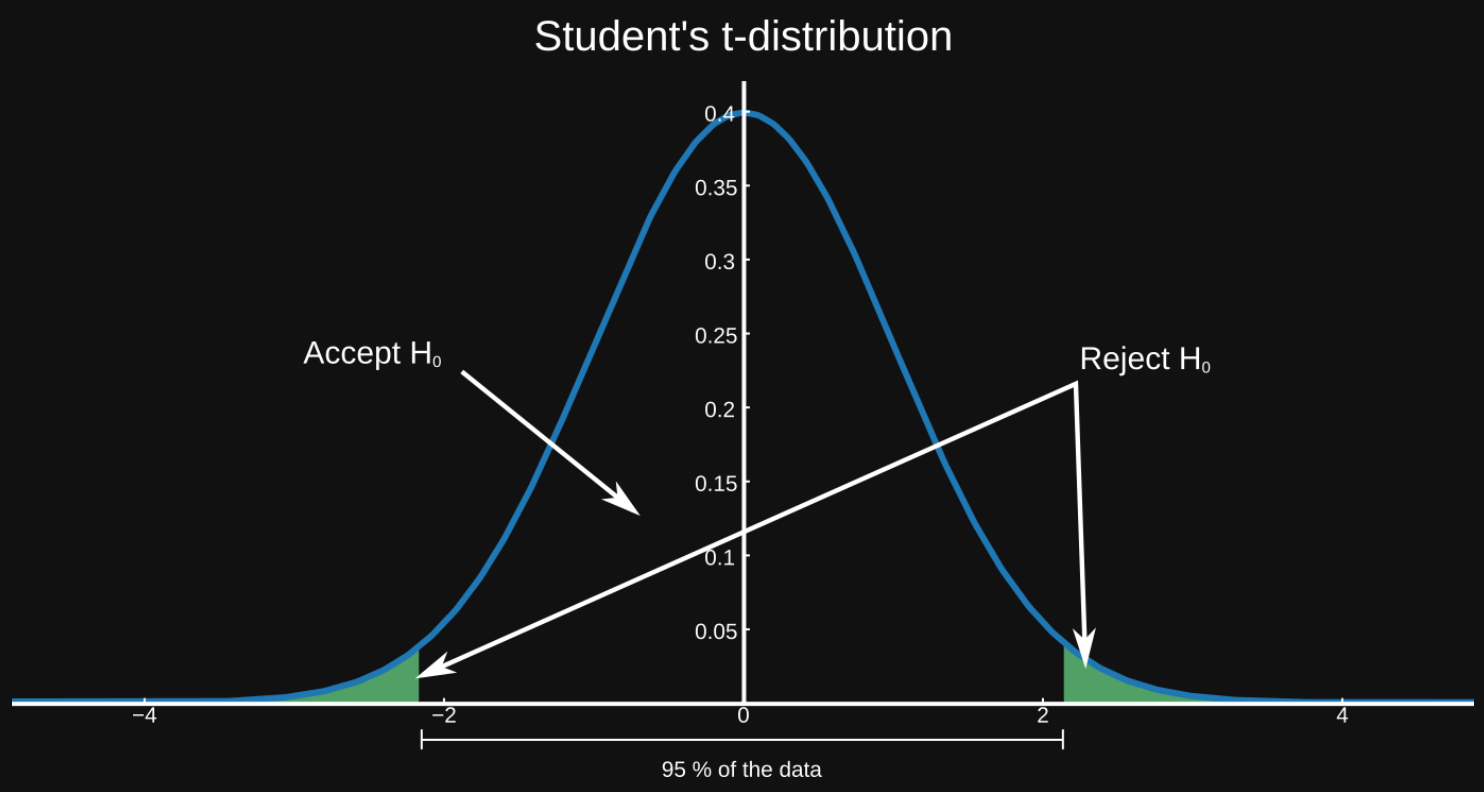
Variance:

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

T-statistic:

$$T_x = \frac{\overline{x_{S_1}} - \overline{x_{S_2}}}{\sqrt[2]{\frac{S_{x_{S_1}}^2}{M} + \frac{S_{x_{S_2}}^2}{N}}}$$

T-test and P-value



T-test is used only to compare two samples. To compare more, ANOVA (ANalysis Of VAriance) is used.

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 $p < \mathbf{0.05}$ 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 analyzing 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.

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

*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.

FDR = 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!

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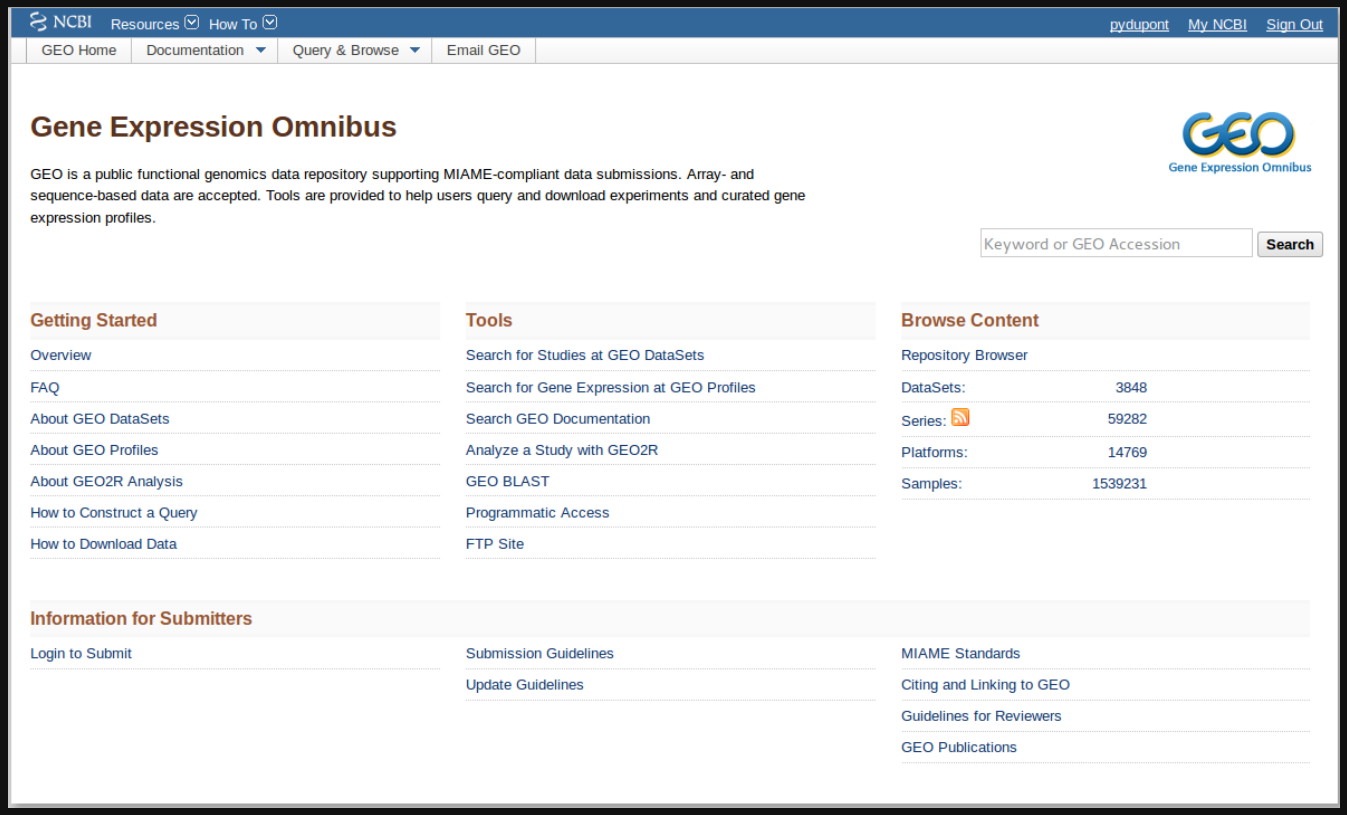
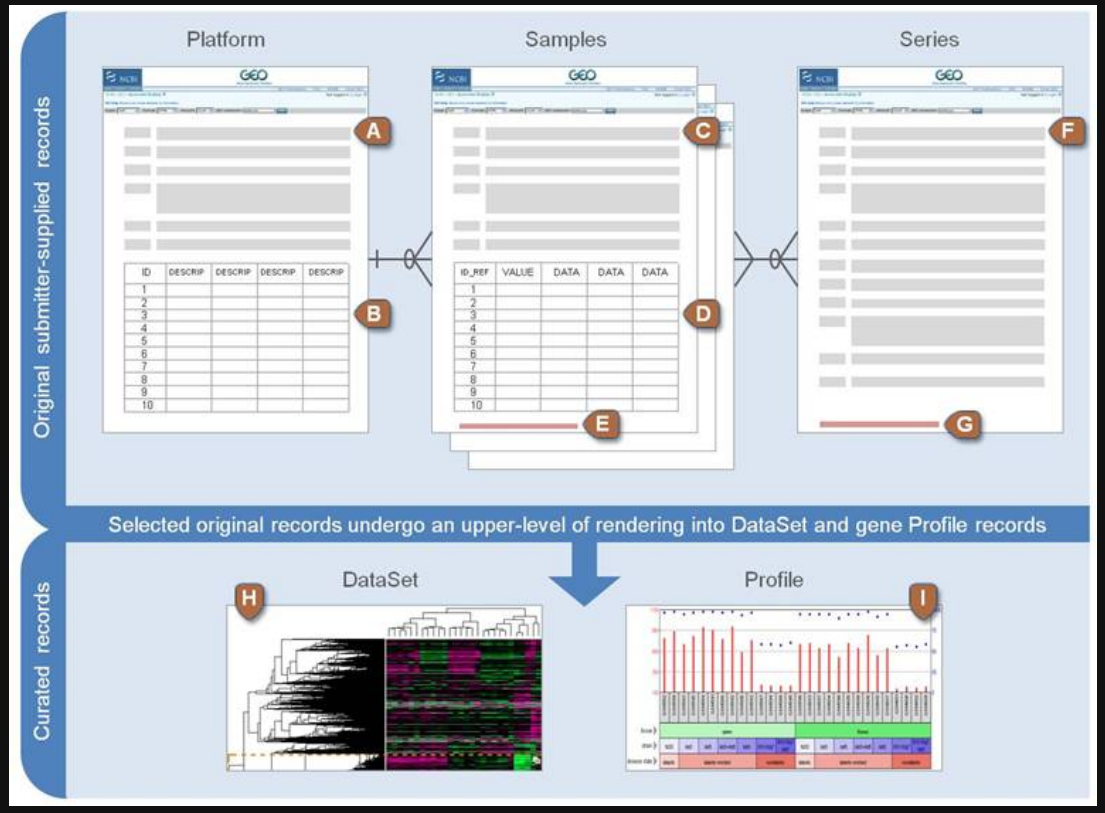
Gene expression databases

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



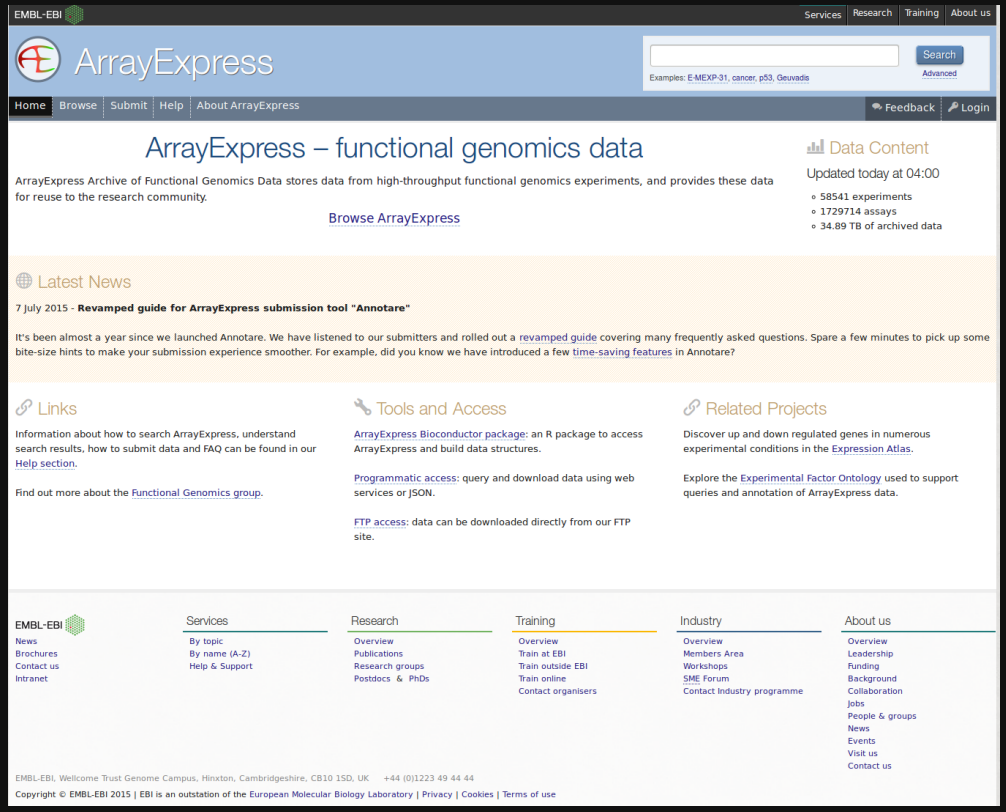
Gene expression databases

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



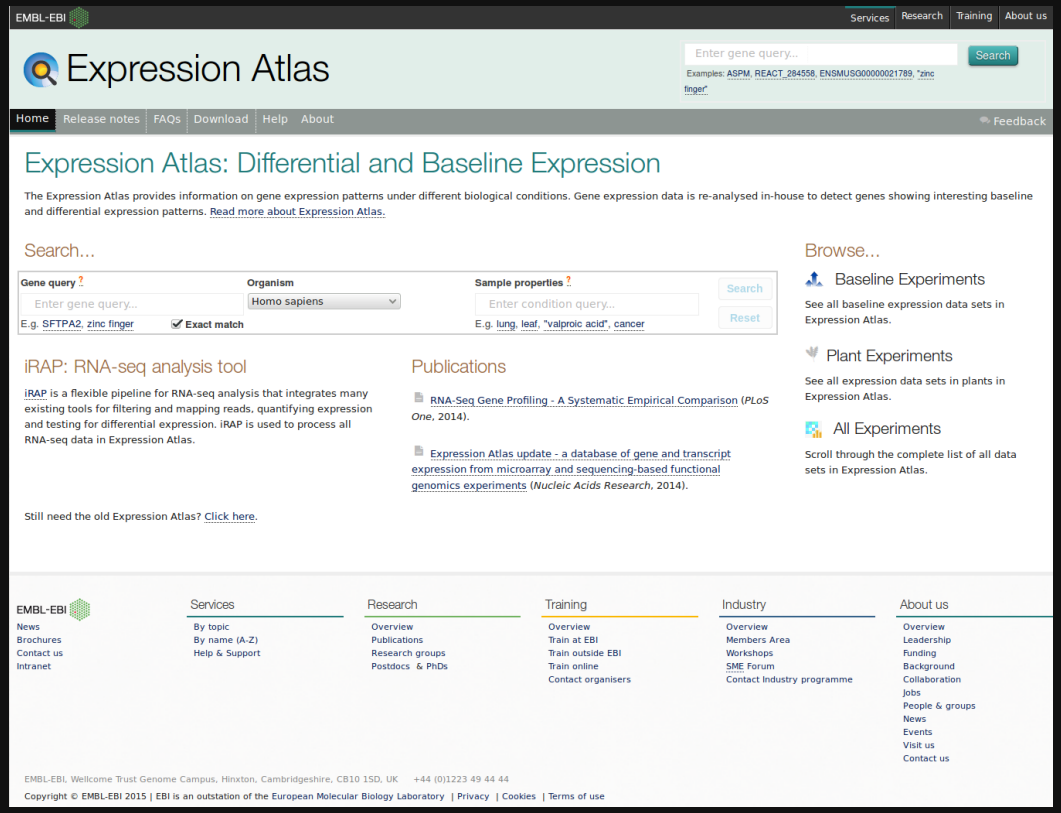
Gene expression databases

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



Gene expression databases

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



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 hybridizations are technical and biological replicates)
5. Sufficient **array annotation** (e.g. gene identifiers, probe sequences)
6. Essential **laboratory and data processing protocols** (e.g. normalization method used to obtain the final data)

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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*

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Lab case study

Herbivory in Nicotiana attenuata (Solanaceae)