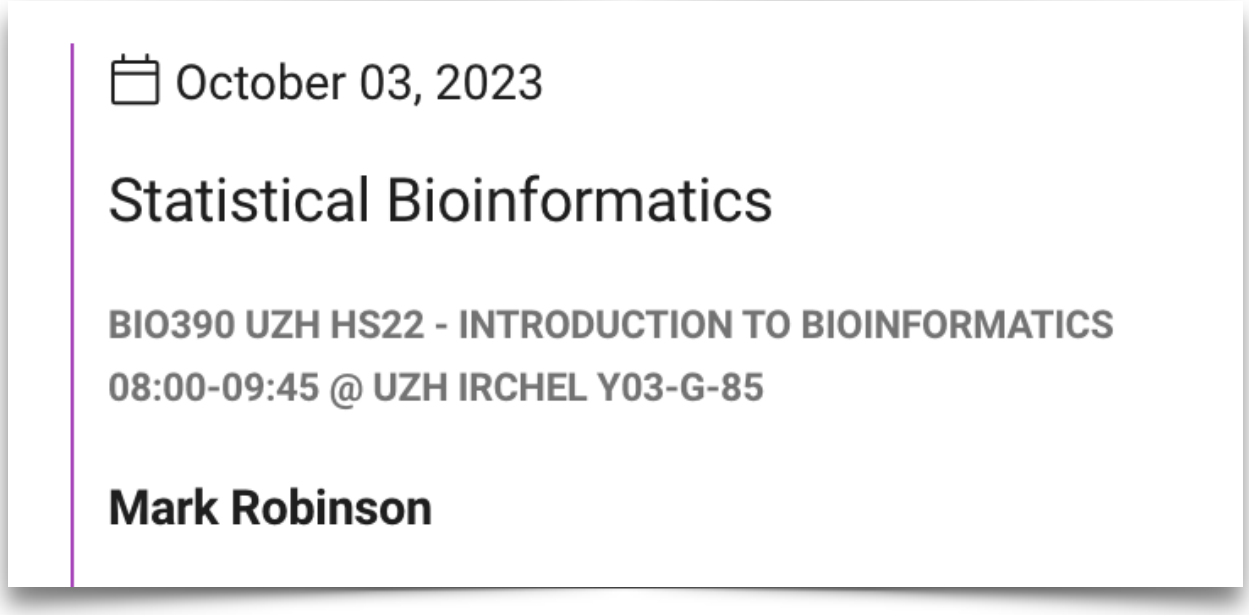
BIO390 Introduction to Bioinformatics

Statistical Bioinformatics: motivation via data examples (1st hour), some fundamental concepts (2nd hour)

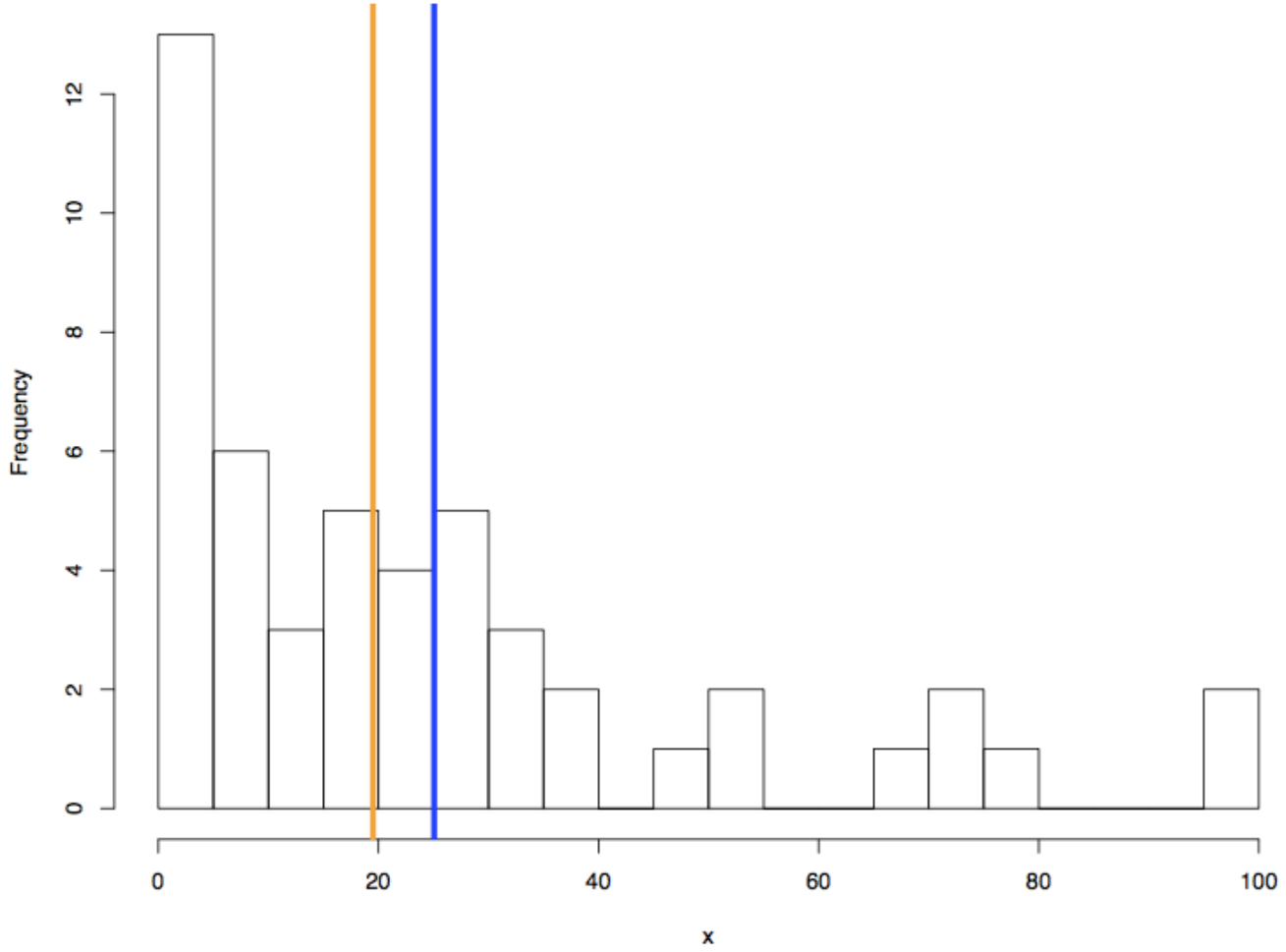


Mark D. Robinson

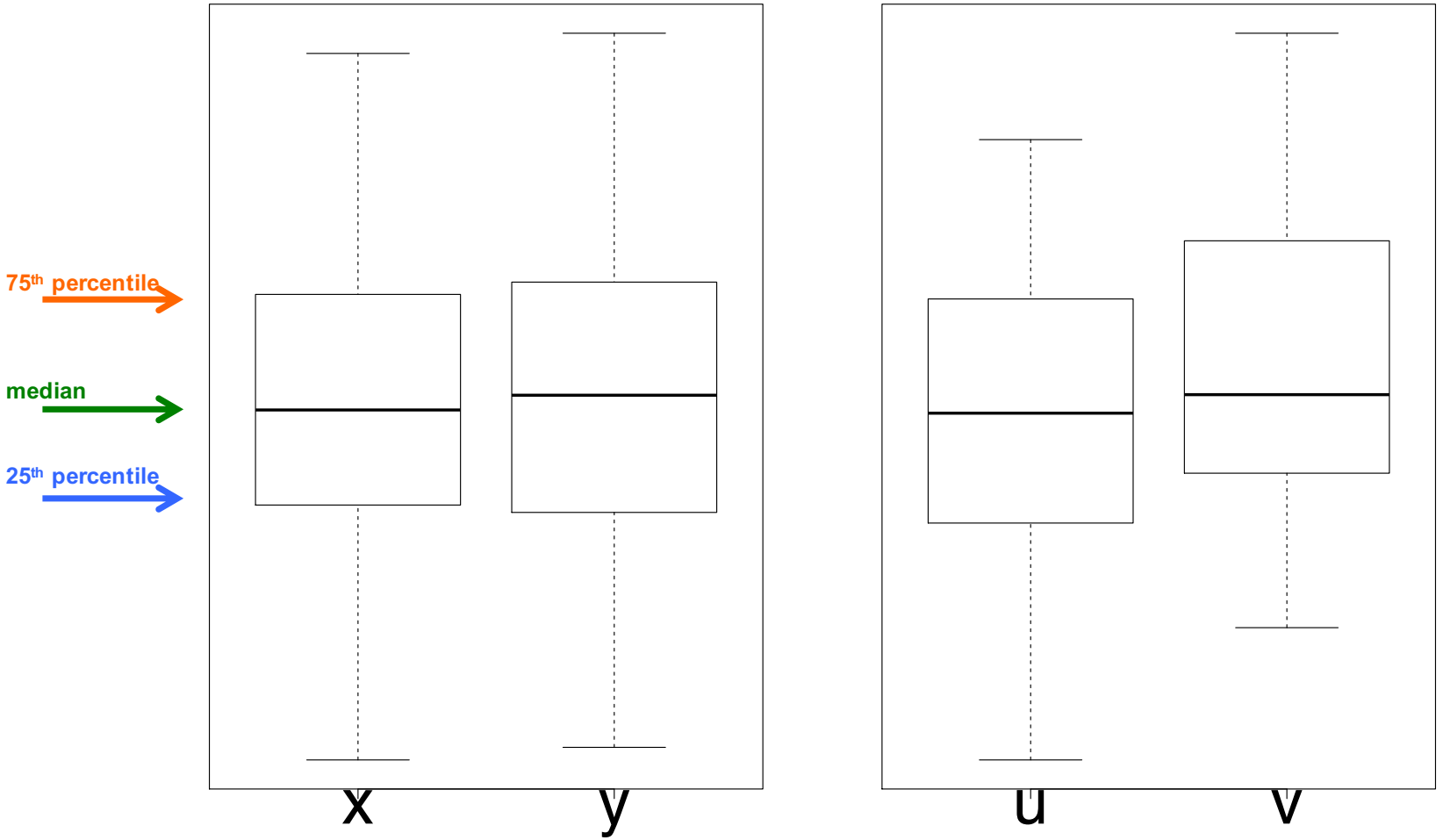
Survey: Statistical Insight

klicker

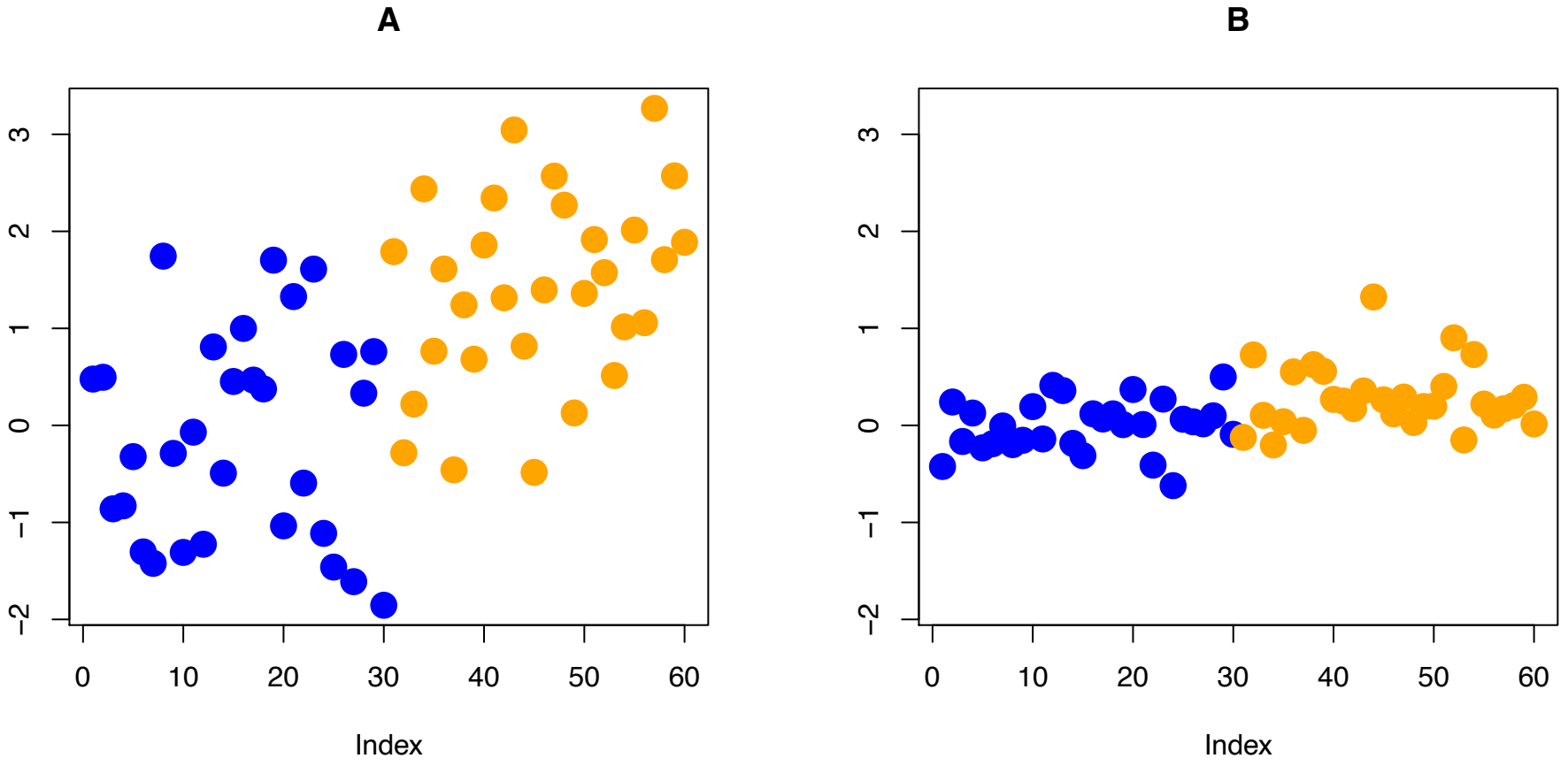
From the histogram, determine whether blue or orange represents the mean/median



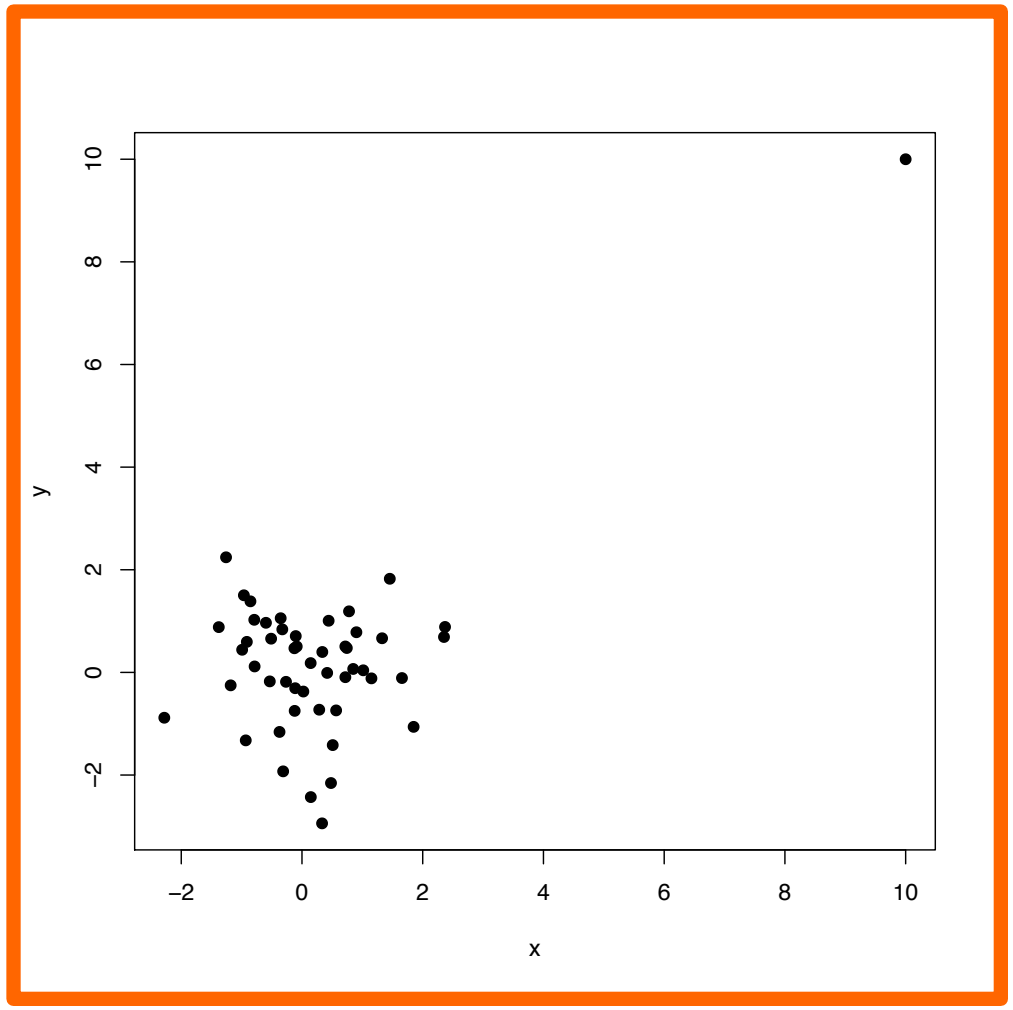
Given these boxplots, which of two underlying distributions are more similar?



Which plot highlights more (statistical) evidence for a change in the population means (between orange and blue)?



In your view, what best describes the associations shown in the plot of 'x' and 'y' ?



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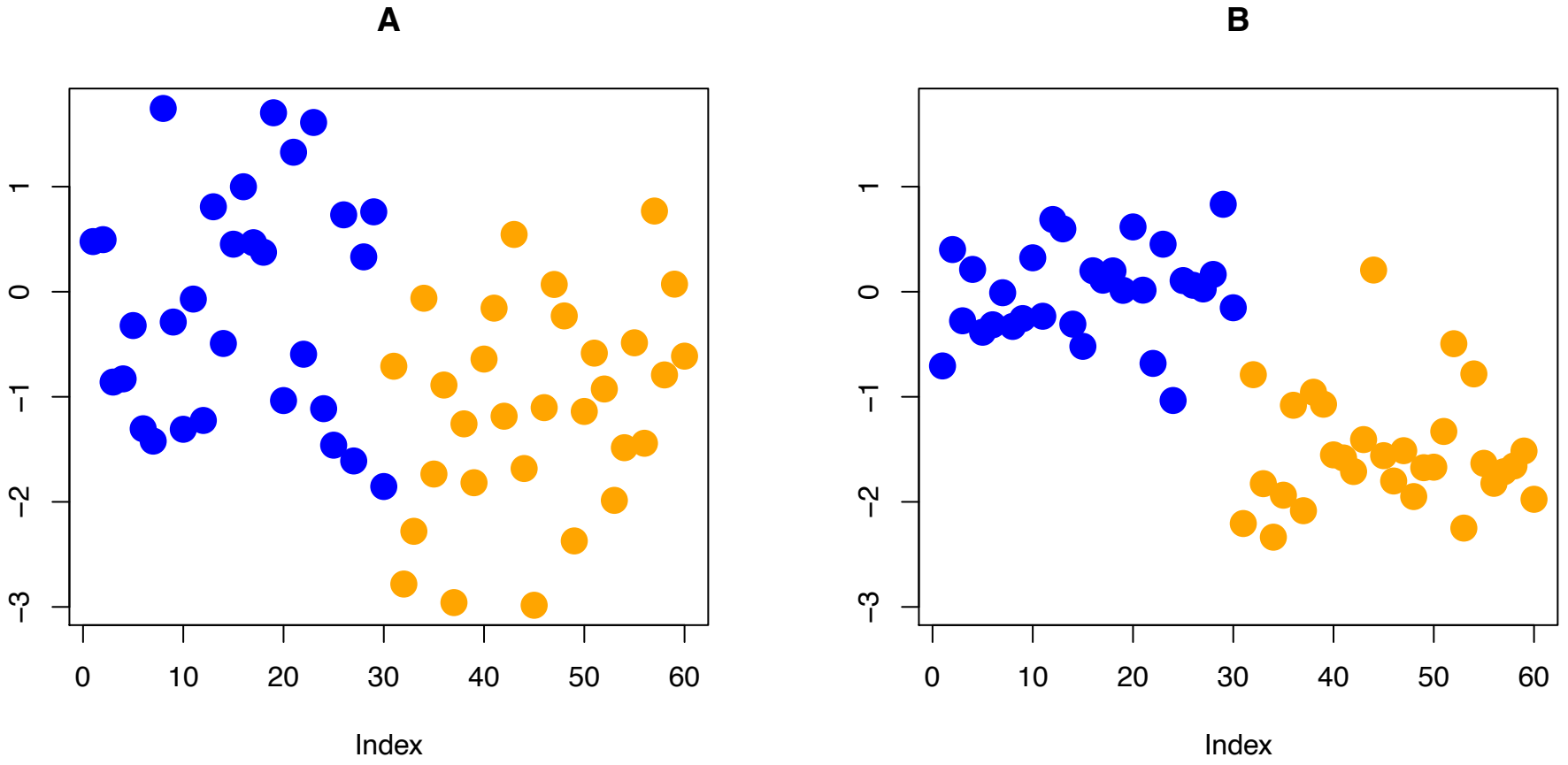


http://en.wikipedia.org/wiki/Statistical_hypothesis_testing

Of these equations, which one resembles the standard two sample t-test ?

1

$$\frac{(\hat{p}_1 - \hat{p}_2)}{\sqrt{\hat{p}(1-\hat{p})(\frac{1}{n_1} + \frac{1}{n_2})}}$$

2

$$\sum^{k} \frac{(\text{observed} - \text{expected})^2}{\text{expected}}$$

3

$$\frac{(\overline{x}_1 - \overline{x}_2) - d_0}{s_p\sqrt{\frac{1}{n_1} + \frac{1}{n_2}}},$$

Outline

- Motivation: nowadays we are inundated with data, such as microarrays, sequencing, cytometry, imaging —> modern biologists need to be data-savvy (data science, statistics, computation)
- Fundamental statistical concepts: central limit theorem, false positives / false negatives, P-values, multiple testing, exploratory data analysis, regression, clustering, dimension reduction, reproducibility, …
- Data science / programming: BIO 134, BIO 144, (BIO 334, STA 426)

http://simplystatistics.org/2013/07/03/repost-the-5-most-critical-statistical-concepts/

Critical skills needed by statisticians (Jeffrey Leek's words):

With all the excitement going on around statistics, there is also increasing diversity. It is increasingly hard to define "statistician" since the definition ranges from very mathematical to very applied. An obvious question is: what are the most critical skills needed by statisticians?

So just for fun, I made up my list of the top 5 most critical skills for a statistician by my own definition. They are by necessity very general (I only gave myself 5).

1. **The ability to manipulate/organize/work with data on computers** - whether it is with excel, R, SAS, or Stata, to be a statistician you have to be able to work with data.
2. **A knowledge of exploratory data analysis** - how to make plots, how to discover patterns with visualizations, how to explore assumptions
3. **Scientific/contextual knowledge** - at least enough to be able to abstract and formulate problems. This is what separates statisticians from mathematicians.
4. **Skills to distinguish true from false patterns** - whether with p-values, posterior probabilities, meaningful summary statistics, cross-validation or any other means.
5. **The ability to communicate results to people without math skills** - a key component of being a statistician is knowing how to explain math/plots/analyses.

Modern biologists

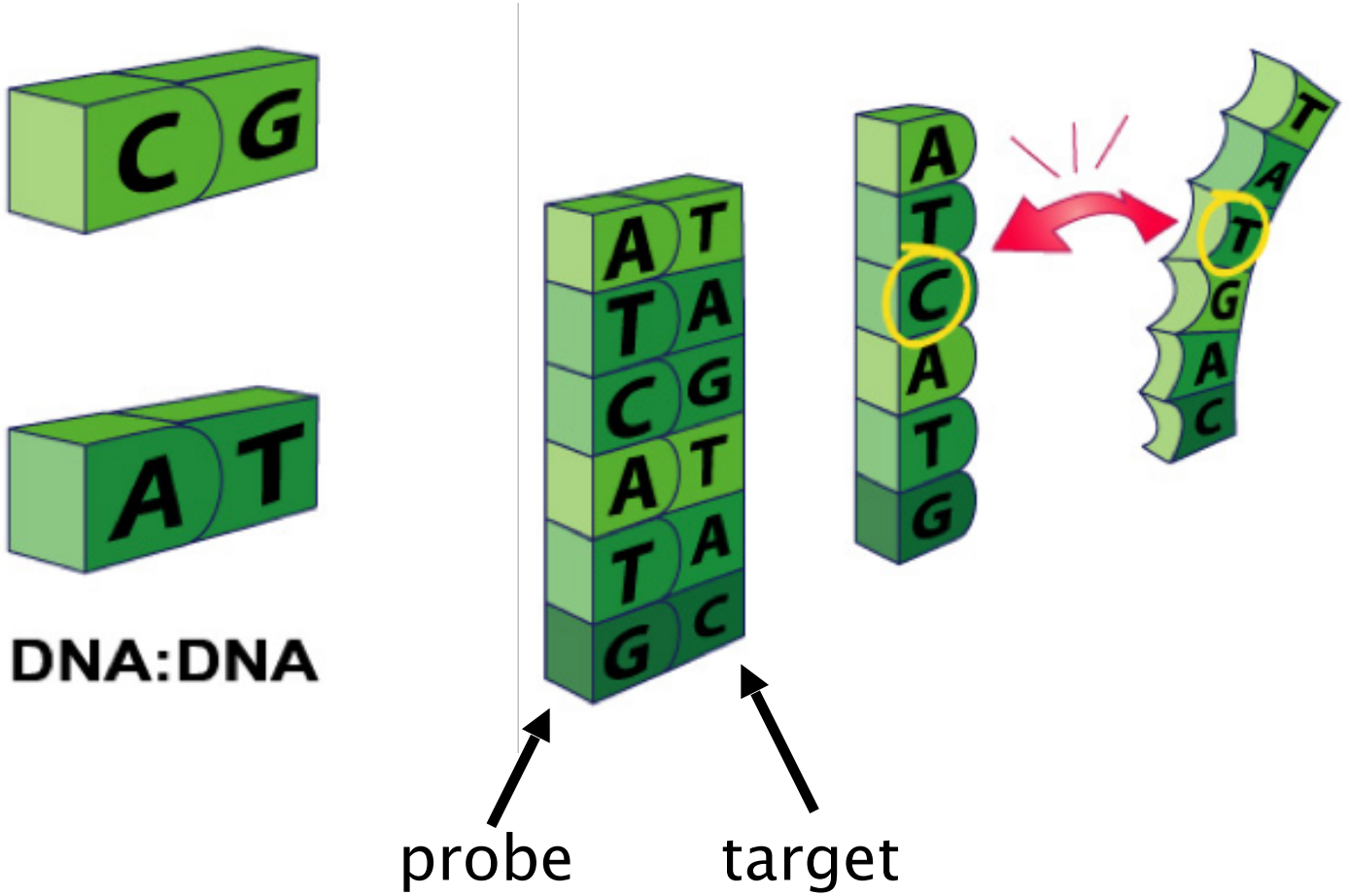
enough knowledge to understand caveats of analysis

Technologies
in my research area

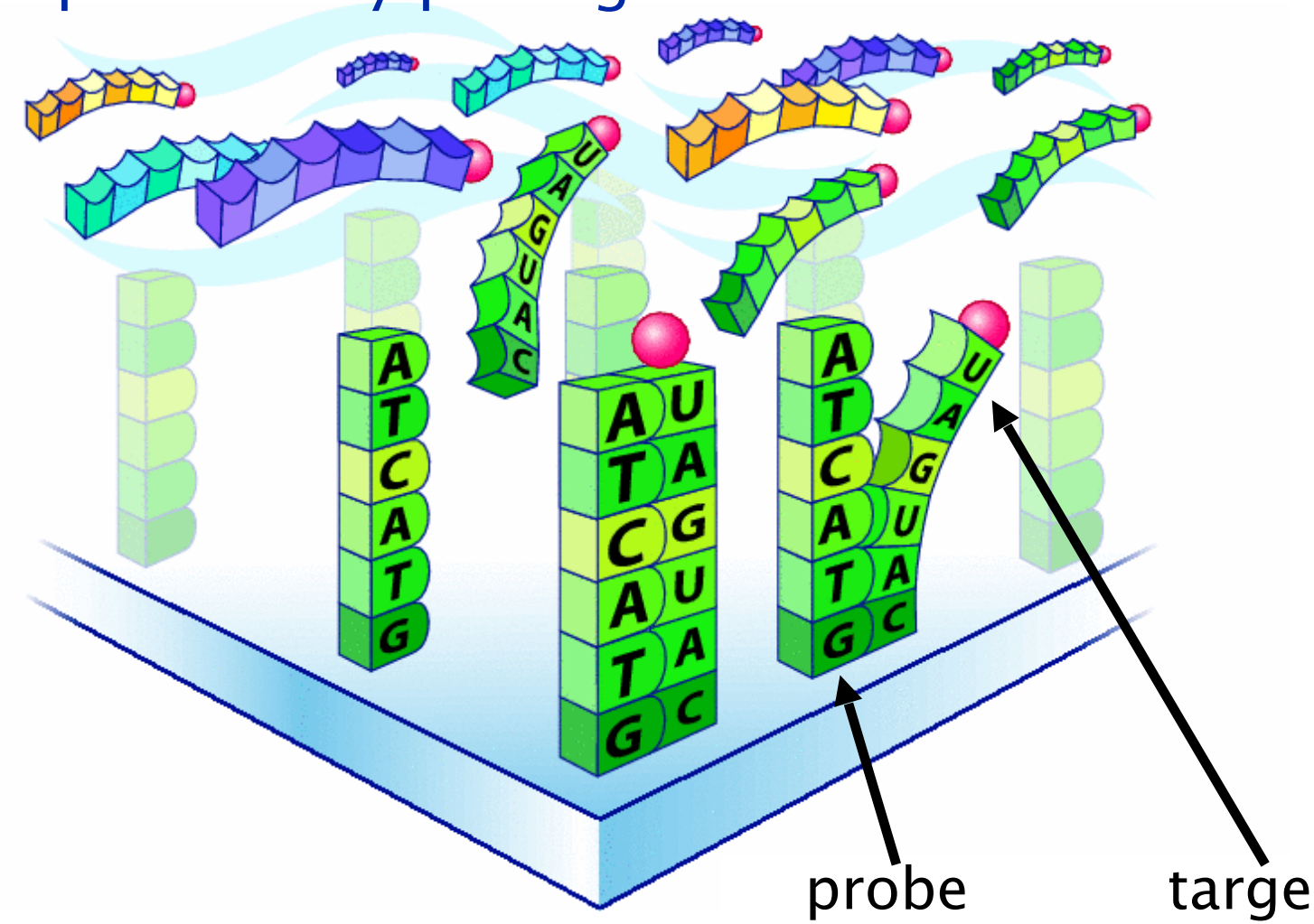
microarray, high-throughput sequencing, single cell, cytometry, etc.

“it’s just data”

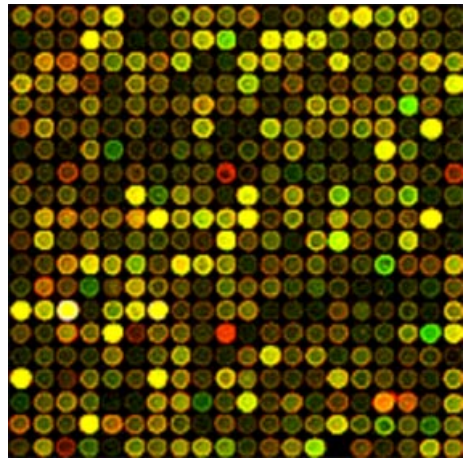
Microarray fundamentals: Nature gives a complementary pairing



DNA microarray: **parallel** northern blots; Nature gives a complementary pairing



Abundance (of complementary DNA species) measured by flouresence intensity



Gene Expression Profiling: questions of interest

- What genes have changed in expression? (e.g. between disease/normal, affected by treatment)
  Gene discovery, differential expression
- Is a specified group of genes all up-regulated in a particular condition?
  Gene **set** differential expression
- Can the expression profile predict outcome?
  Class prediction, classification
- Are there tumour sub-types not previously identified? Do my genes group into previously undiscovered pathways?
  Class discovery, clustering

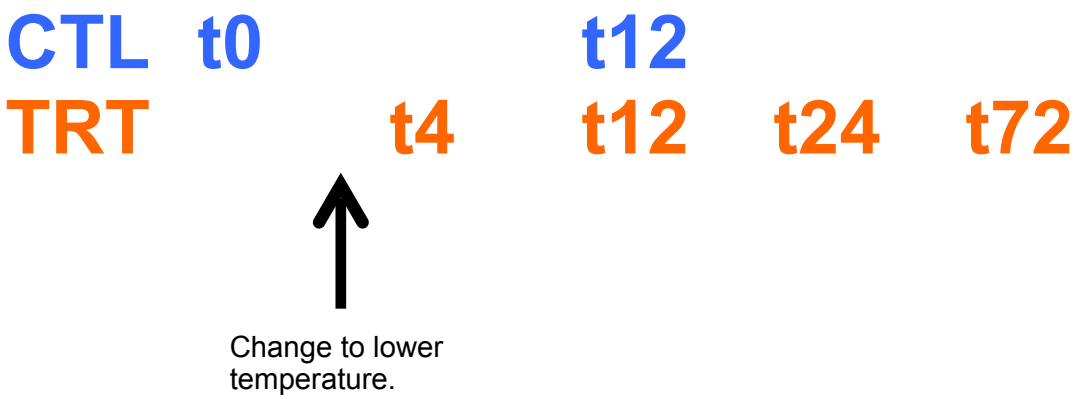
"To consult the statistician after an experiment is finished is often merely to ask [them] to conduct a post mortem examination. [They] can perhaps say what the experiment died of." R. A. Fisher

Motivation for exploratory data analysis: Case Study

(from Stefano, a former M.Sc. student in my Institute)

He is studying gene expression in fruitfly and is interested in transcriptional responses following "heat shock".

Basic schematic of experiment:

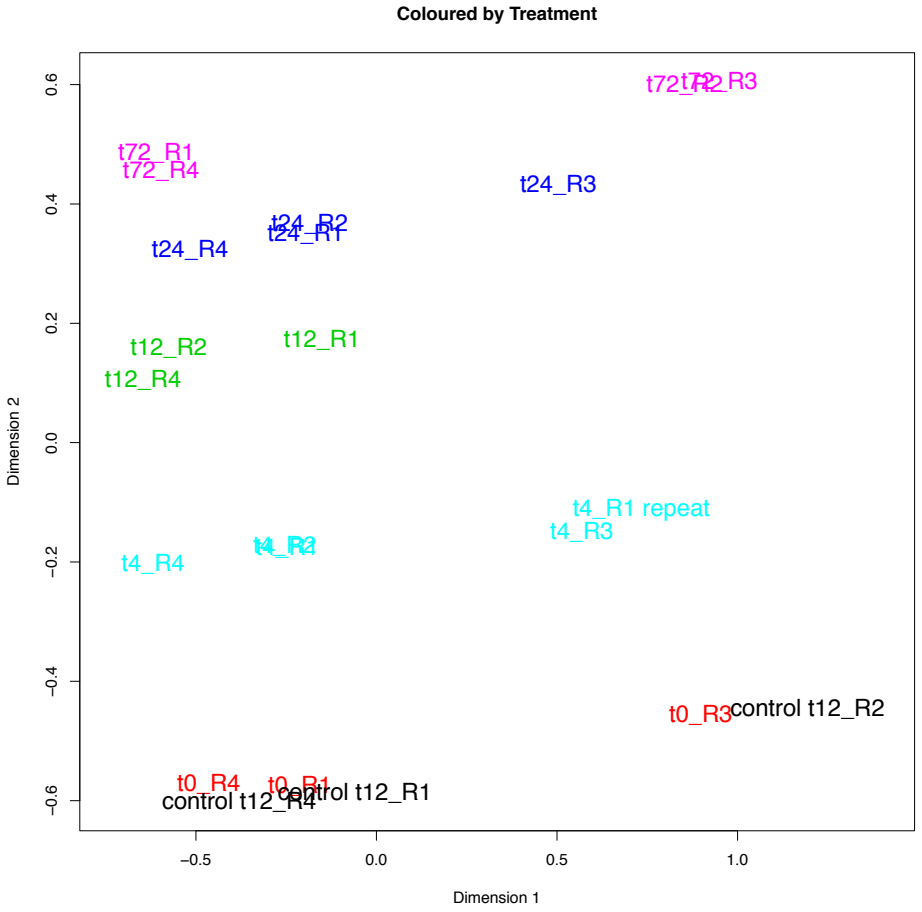


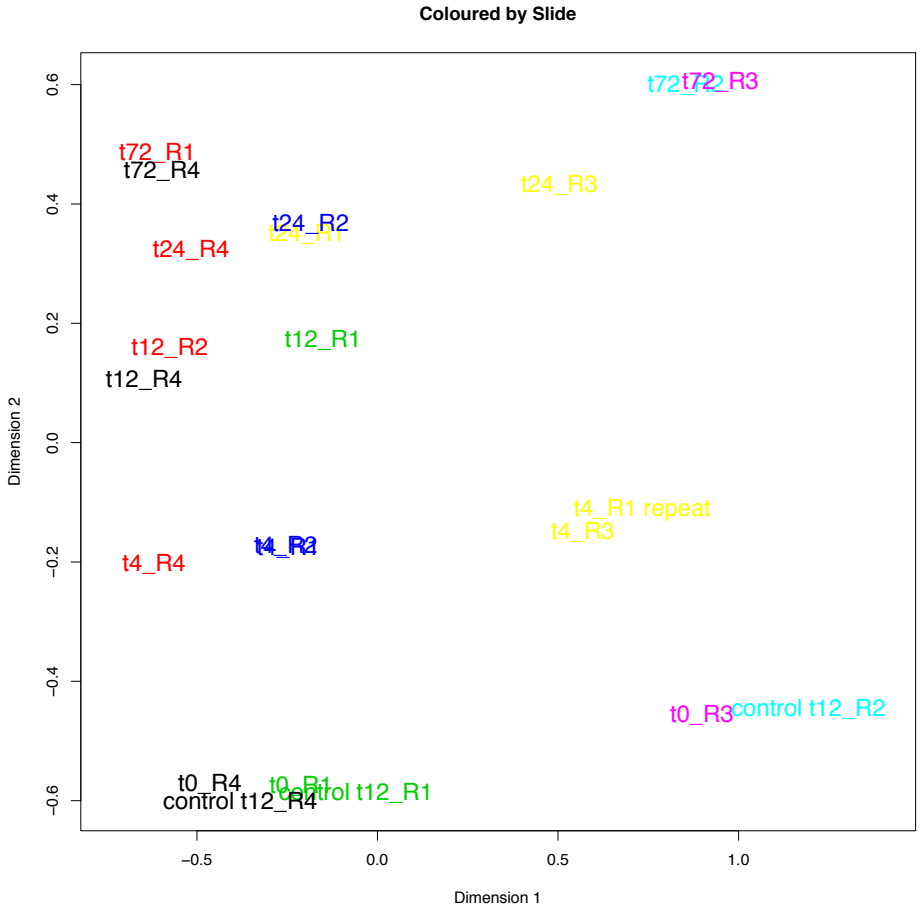
~4 replicates for each condition

http://en.wikipedia.org/wiki/Multidimensional_scaling

```
library(limma)
plotMDS(d)  # 'd' is a matrix
"Plot samples on a two-dimensional scatterplot so that
distances on the plot approximate the typical log2 fold
changes between the samples."
```

Take a close look at where the 24 samples are to each other relative to the X- and Y-axes





22 samples x ~20,000 genes

reduced to 22 samples x 2 dimensions

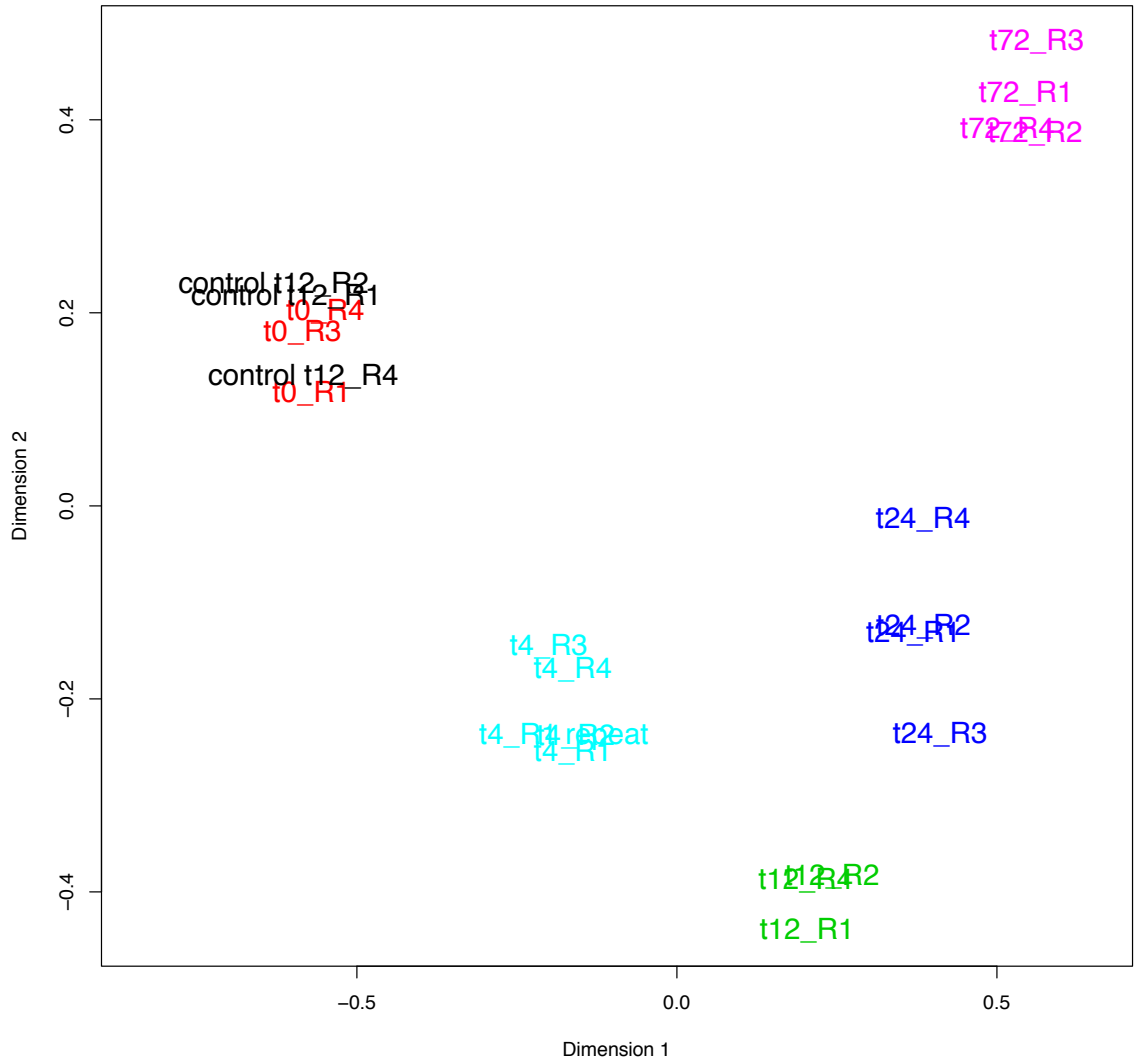
Magic: Surrogate variable analysis to detect and "remove" batch effects

OPEN ACCESS Freely available online PLOS GENETICS

Capturing Heterogeneity in Gene Expression Studies by Surrogate Variable Analysis

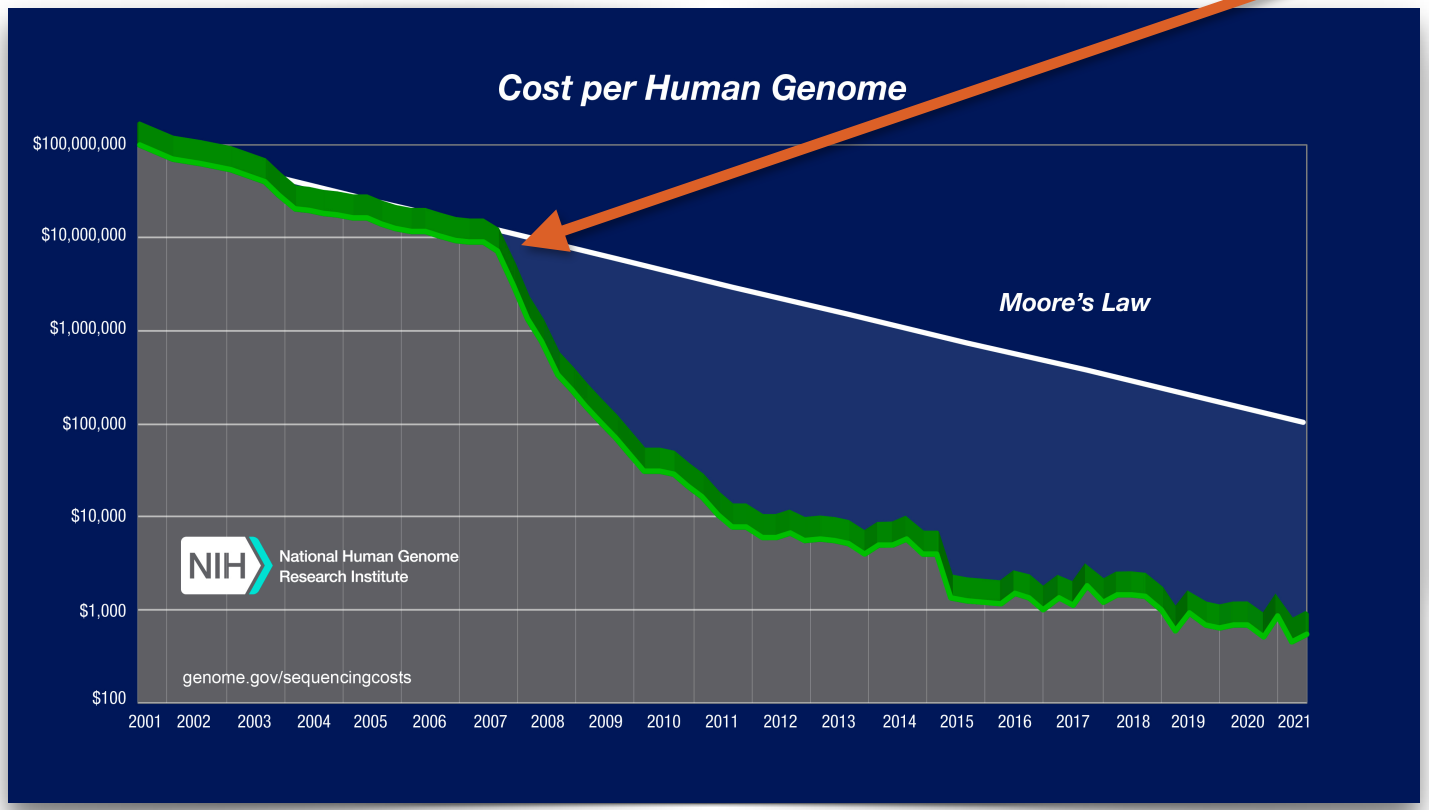
Jeffrey T. Leek[1], **John D. Storey**[1,2*]

1 Department of Biostatistics, University of Washington, Seattle, Washington, United States of America, **2** Department of Genome Sciences, University of Washington, Seattle, Washington, United States of America



High-throughput sequencing

(Solexa) Illumina



https://www.statnews.com/2017/01/09/illumina-ushering-in-the-100-genome/


BUSINESS

STAT+

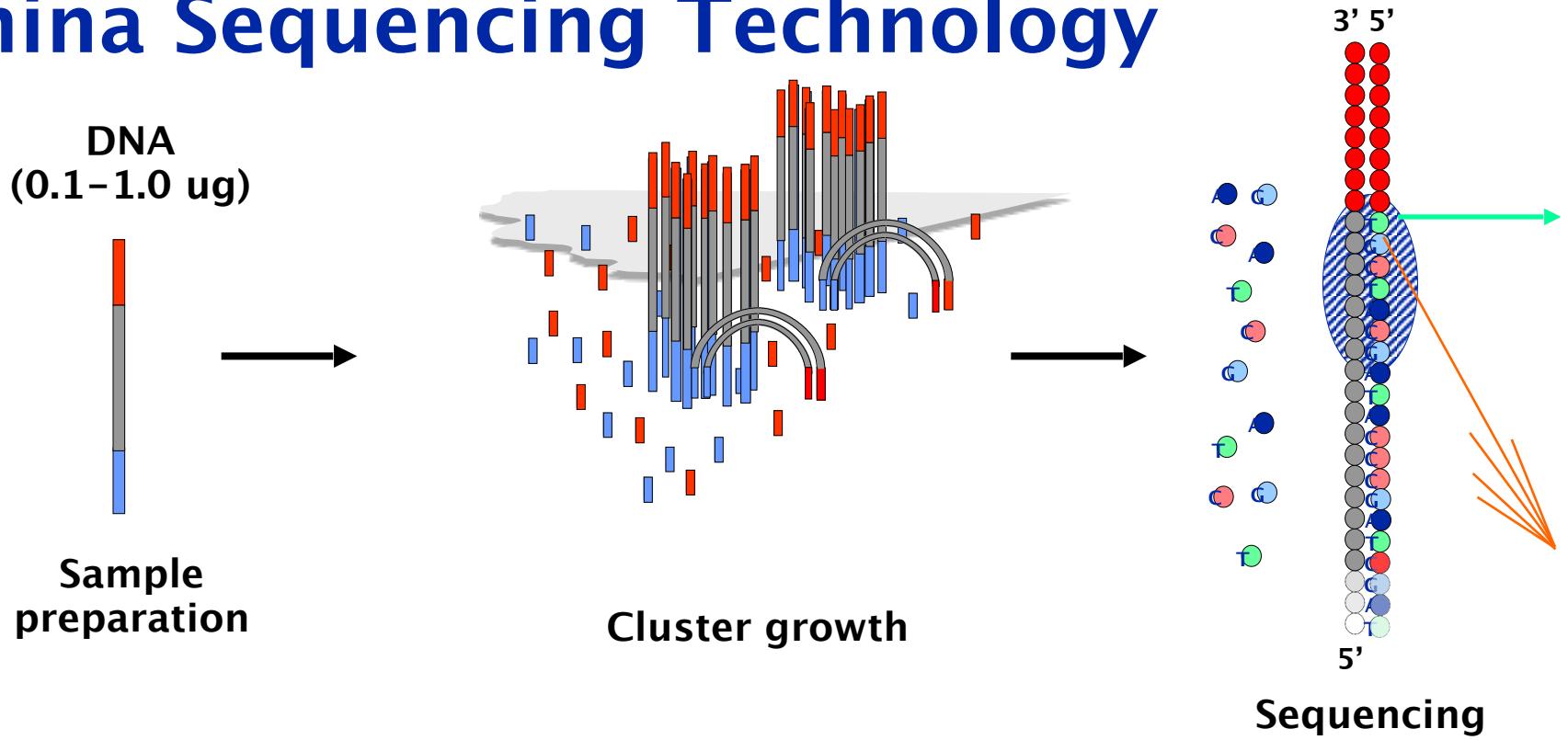
Illumina says it can deliver a $100 genome — soon

By MEGHANA KESHAVAN @megkesh / JANUARY 9, 2017

Sept 29th 2022 - **NovaSeq X Series**, unveiled earlier today, ushers in the era of the genome with revolutionary new production-scale sequencers .. can generate more than 20,000 whole genomes per year – 2.5 times the throughput of prior sequencers – greatly accelerating genomic discovery and clinical insights, to understand disease and ultimately transform patient lives.

Many assays based on sequencing DNA:
https://liorpachter.wordpress.com/seq/

Illumina Sequencing Technology



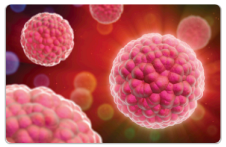
This slide courtesy of Gary Schroth, Illumina

Applications of high-throughput sequencing

Common Sequencing Applications

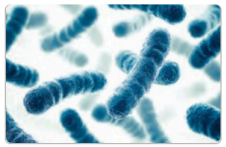
Cancer Research

NGS-based sequencing enables cancer researchers to detect rare somatic variants, tumor subclones, and circulating DNA fragments. Learn more about sequencing for cancer research.



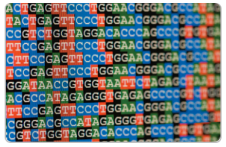
Microbiology Research

From environmental metagenomics studies to infectious disease surveillance and more, NGS-based sequencing can help researchers gain genetic insight into bacteria and viruses. Learn more about microbial genomics.



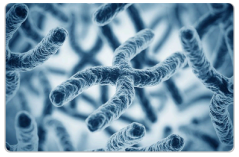
Complex Disease Research

Illumina sequencing is introducing new avenues for understanding immunological, neurological, and other complex disorders on a molecular level. Learn more about complex disease genomics.



Reproductive and Genetic Health

Illumina sequencing and array technologies deliver fast, accurate information that can guide choices along the reproductive and genetic health journey. Find reproductive and genetic health solutions.



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Keyword or person | Departments

Functional Genomics Center Zurich

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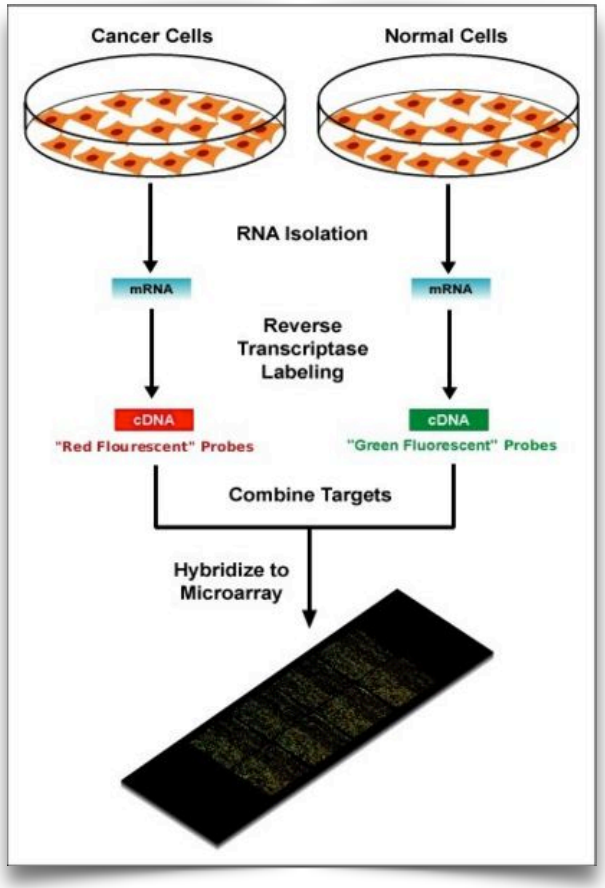
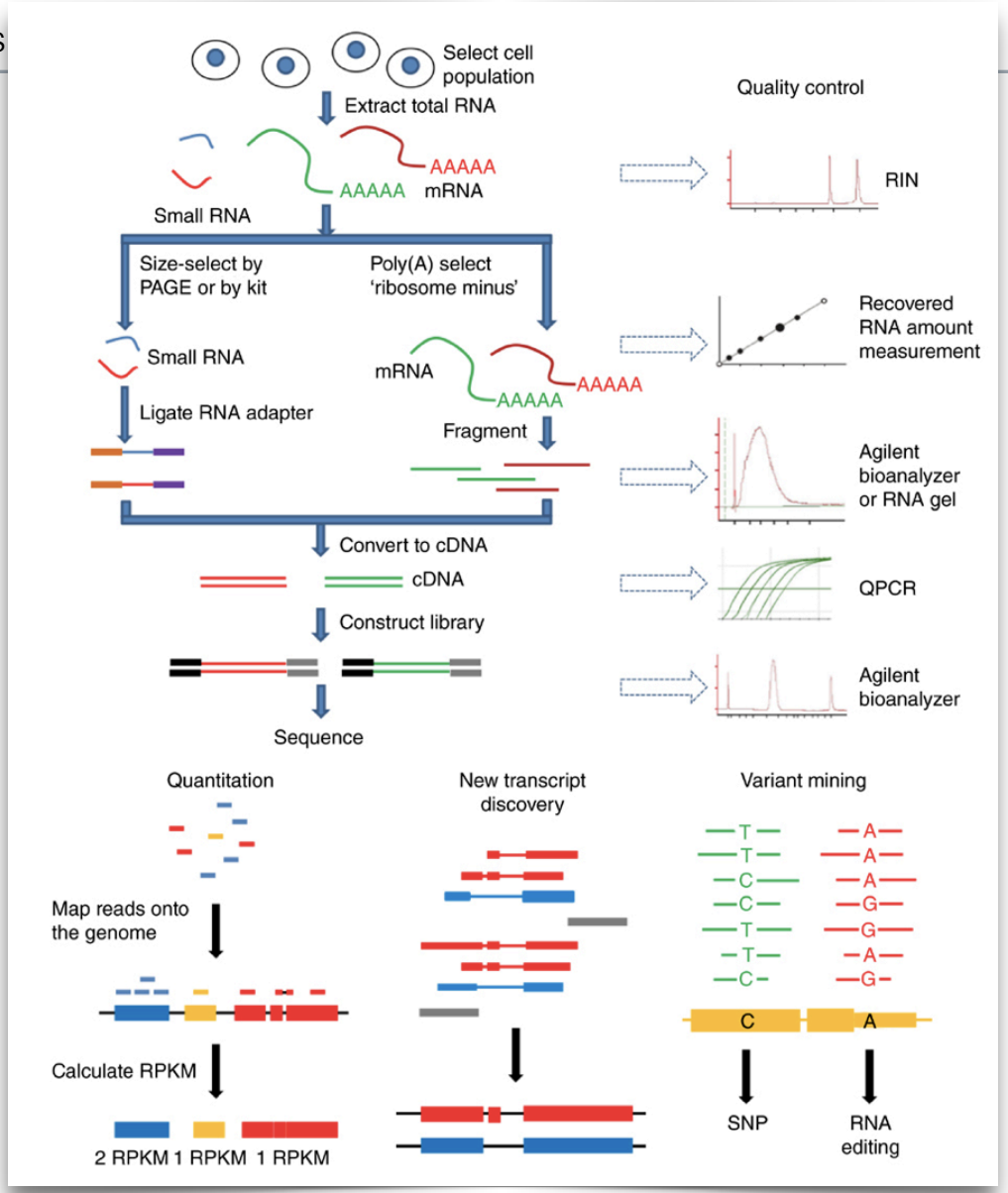
Application Group	Application	Order via B-Fabric
DNA sequencing	Whole Exome Sequencing	Project
DNA sequencing	Methylation Profiling	Project
DNA sequencing	ChIP-Seq	Project
DNA sequencing	Targeted Sequencing and Metagenomics	Project
DNA sequencing	*De novo* Genome Assembly	Project
DNA sequencing	Whole Genome Resequencing	Project
RNA sequencing	Transcriptome Profiling	Project
RNA sequencing	Small RNA Profiling	Project
RNA sequencing	De novo Transcriptome Assembly	Project

Abundance by Fluorescence Intensity (DNA microarray)



http://en.wikipedia.org/wiki/DNA_microarray

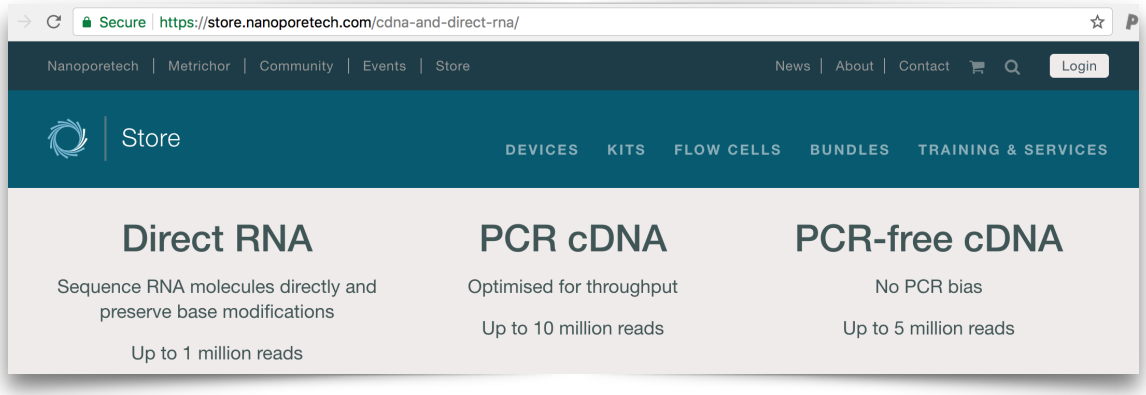
Abundance by Counting (RNA-seq)



Zeng & Mortazavi, Nature Immunology, 2012

https://nanoporetech.com/applications/dna-nanopore-sequencing

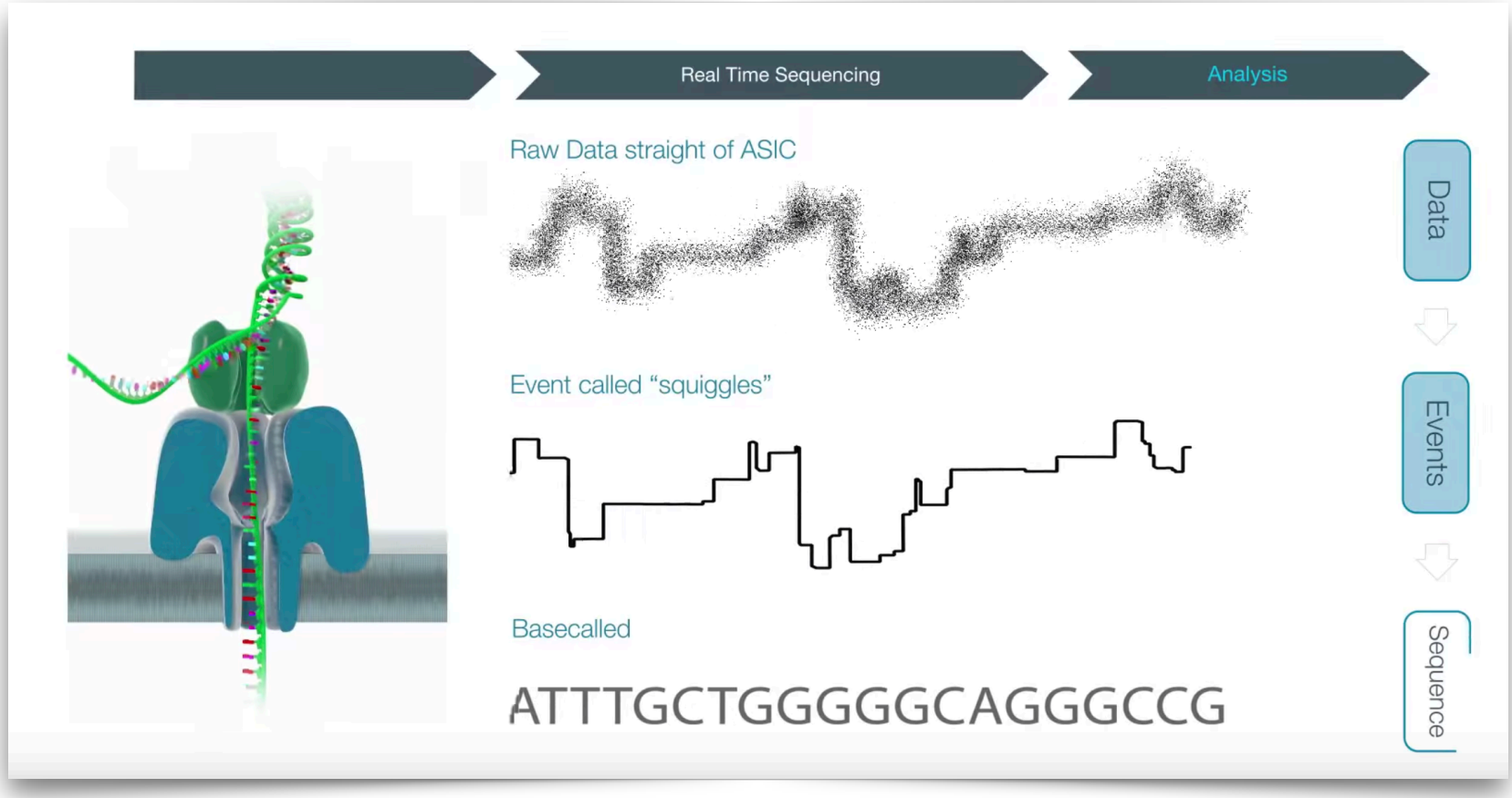
ONT (Oxford Nanopore)



—> attachment of processive enzyme, leads RNA/DNA fragment to pore, combination of nucleotides going through pore creates a “characteristic disruption of the electrical current” —> order of signals can be used to determine the sequence of bases on that single strand.

https://nanoporetech.com/applications/dna-nanopore-sequencing

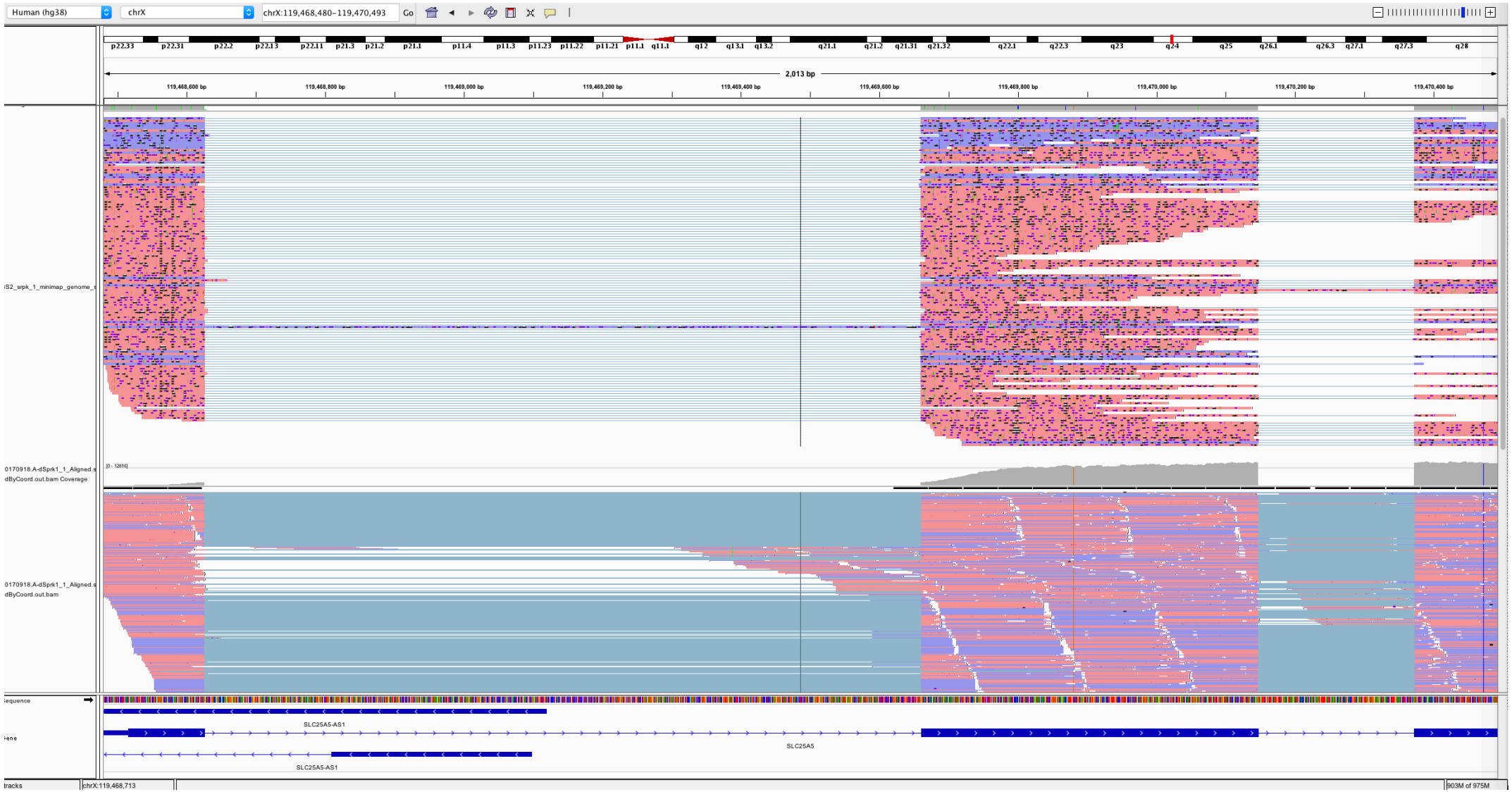
ONT (Oxford Nanopore)



Quick look at reads in a browser

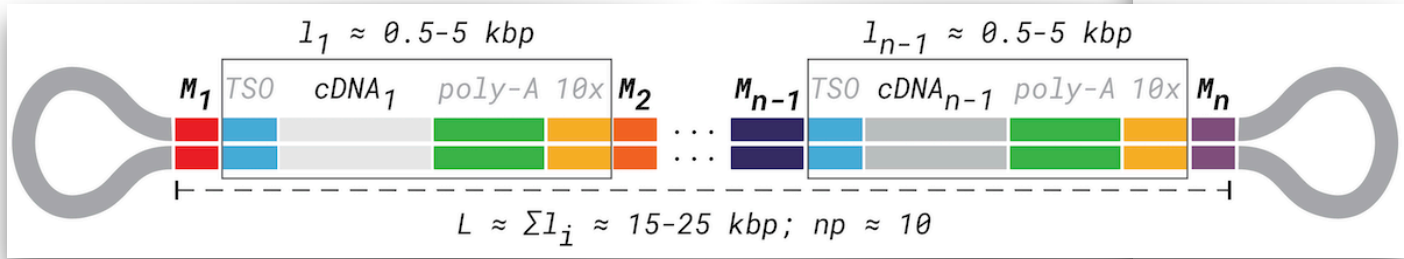
ONT

Illumina



But mRNAs (or corresponding cDNAs) are short —> concatenate them.

MAS = Multiplexed Arrays Sequencing



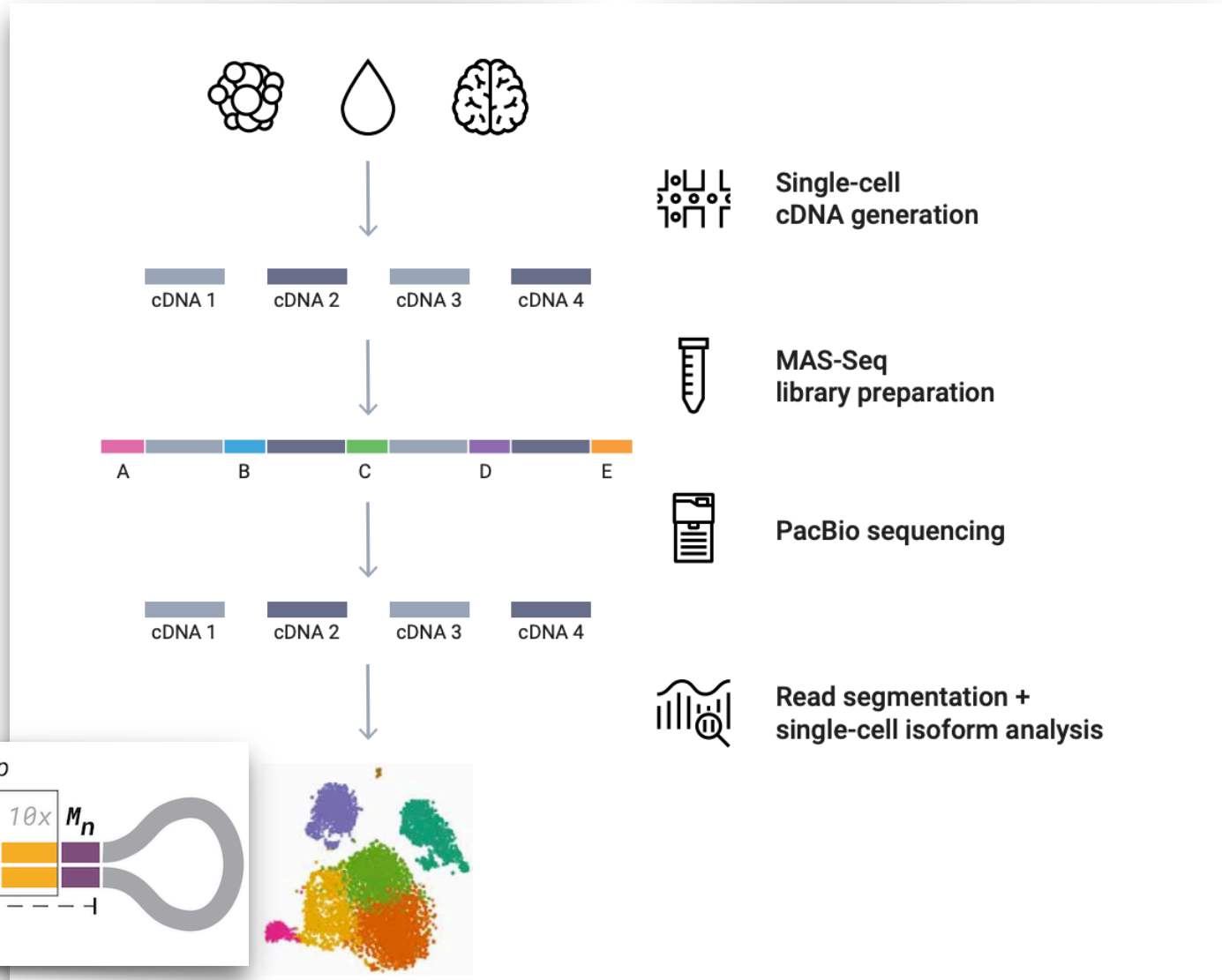


Figure 2. MAS-Seq for single-cell isoform sequencing. Single-cell cDNA molecules are concatenated into a larger insert library and sequenced, then processed using the PacBio software.

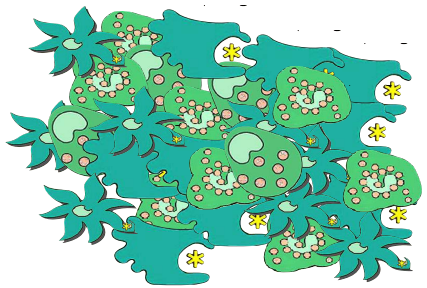
Al'Khafaji et al., 2023

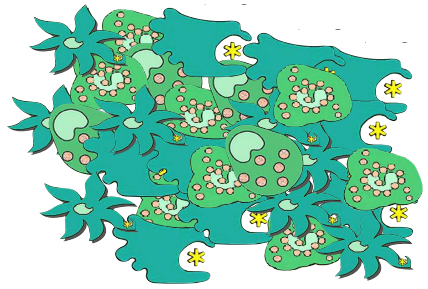
Bulk vs single-cell RNA-sequencing

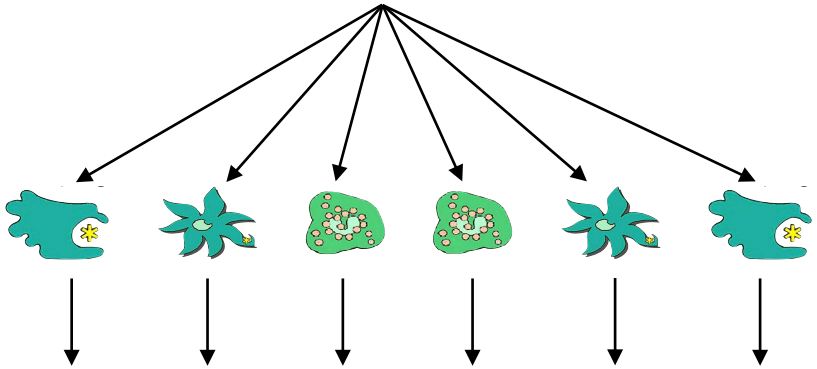
Cell sorting, tissue dissociation

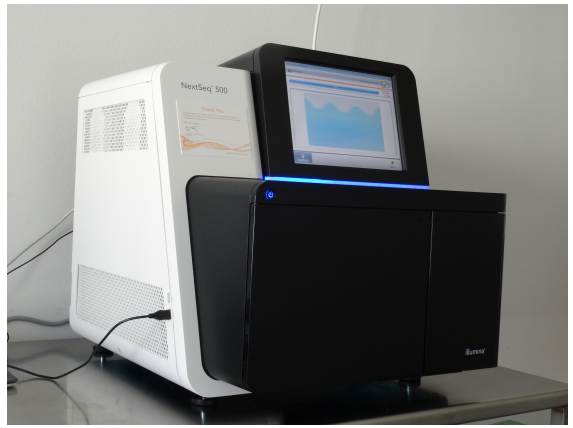
RNA extraction, preparation of cDNA, cell barcoding, UMIs (scRNA-seq only)

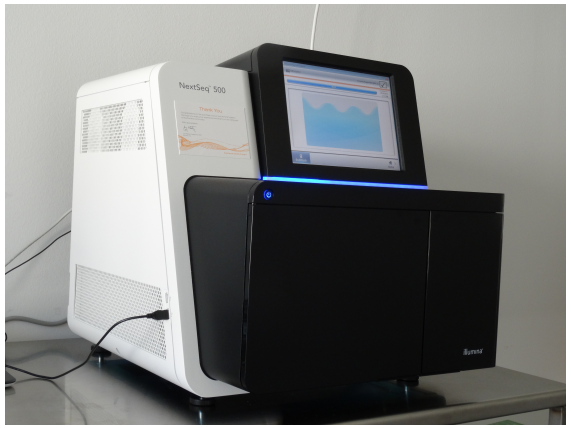
sequencing











Images modified from https://www.flickr.com/photos/konradfoerstner/21264667663 and https://commons.wikimedia.org/wiki/File:Innate_Immune_cells.svg

Diversity of (single cell) data types: sequencing

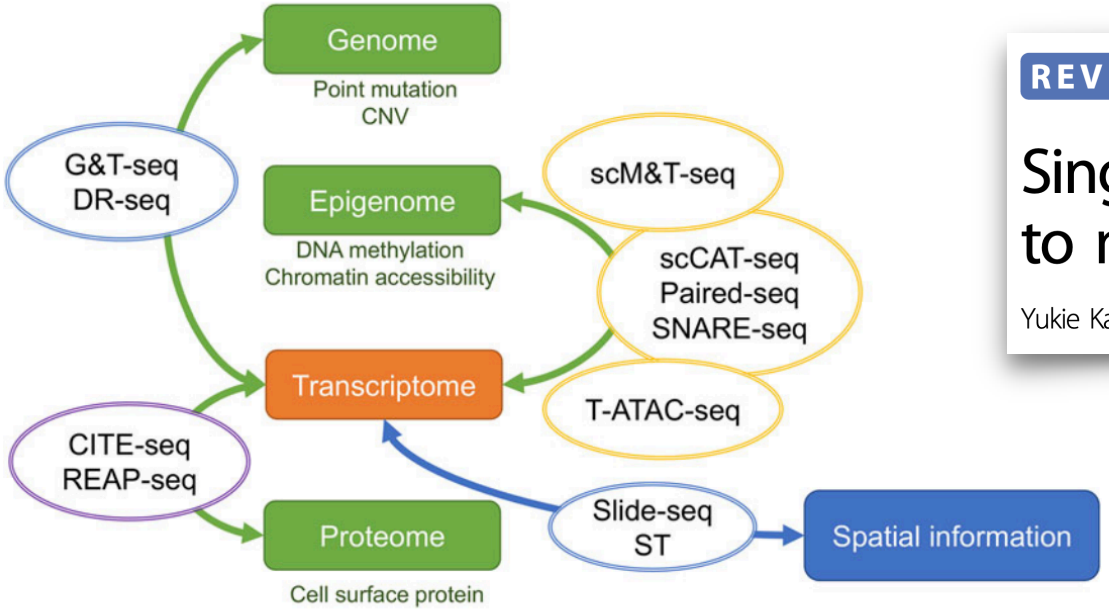


Fig. 3 Multilayered single-cell sequencing. Representative single-cell multimodal sequencing methods. Genomic, epigenomic, and proteomic information can be simultaneously profiled with the transcriptome. Spatial information for a tissue section can also be obtained with gene expression data at the level of one to tens of cells. ST spatial transcriptomics (Visium).

REVIEW ARTICLE Open Access

Single-cell sequencing techniques from individual to multiomics analyses

Yukie Kashima[1,2], Yoshitaka Sakamoto[1], Keiya Kaneko[1], Masahide Seki[1], Yutaka Suzuki[1] and Ayako Suzuki[1]

Motivation: Single-cell RNA-seq: finding cell subpopulation-specific changes in state

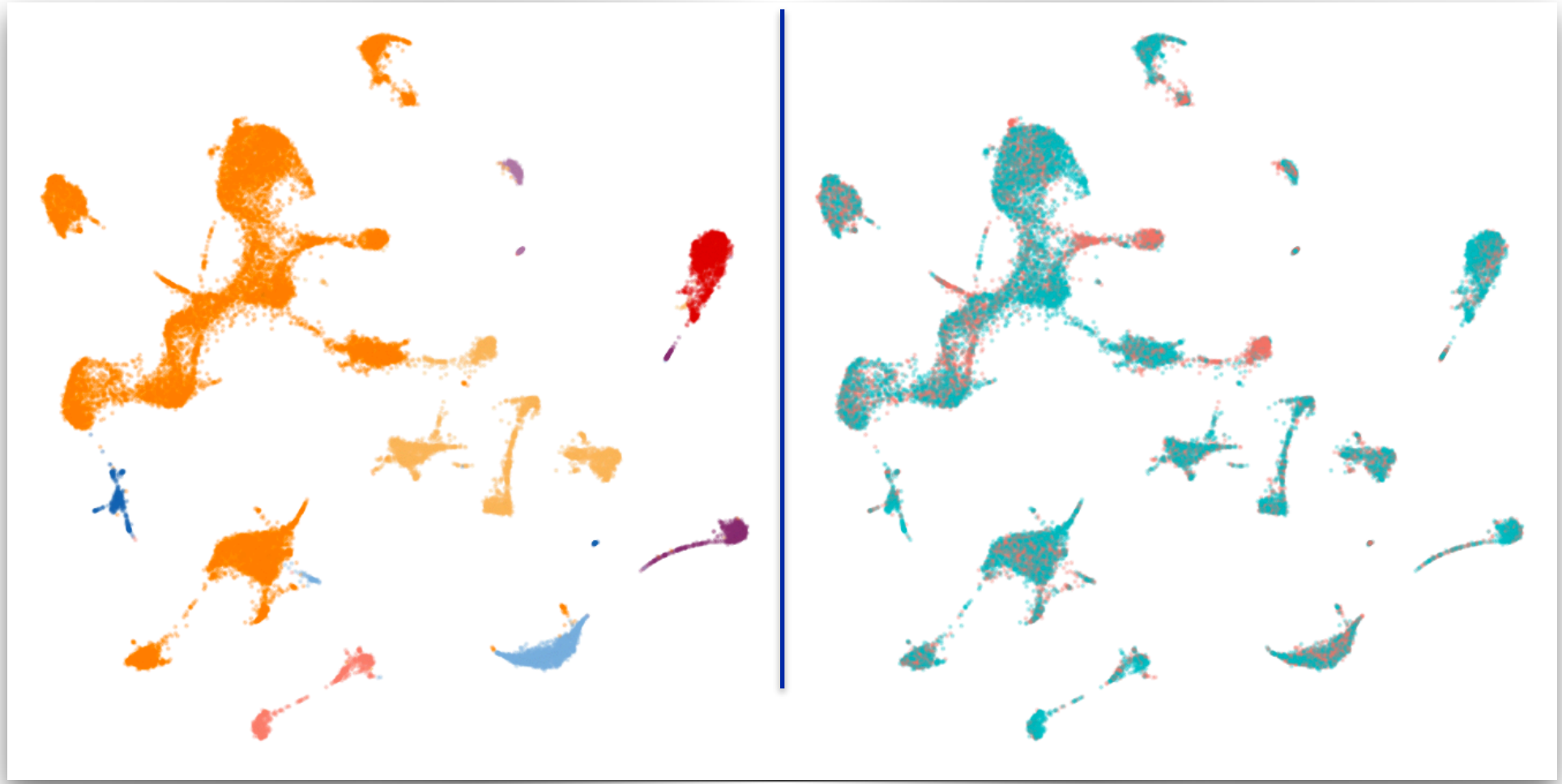
frontal cortex

single nuclei RNA-seq (10x)

Data from:
4 mice vehicle treated
4 mice LPS treated

Each dot is one cell

5000 genes -> 2D "embedding" / "projection".



Flow cytometry

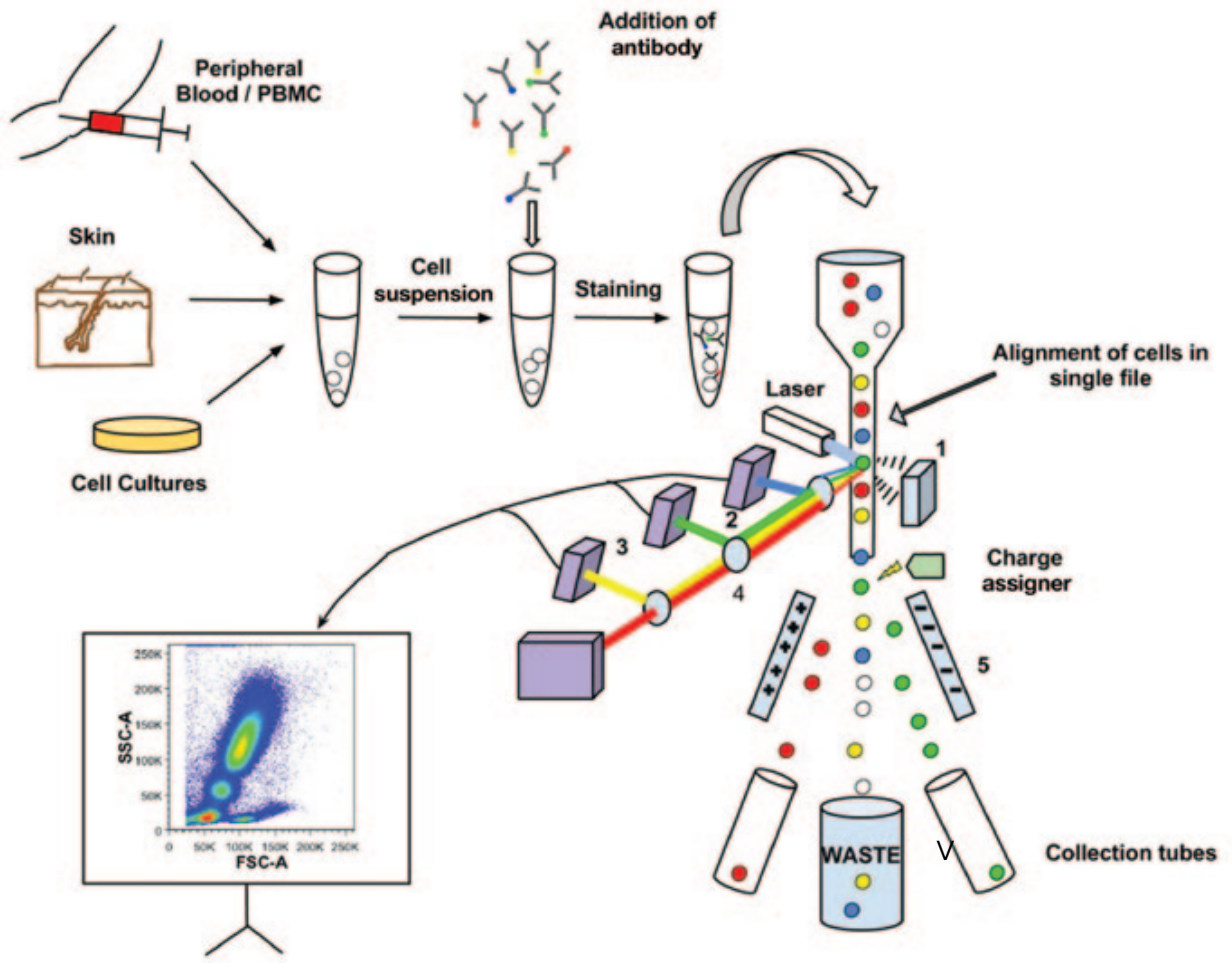
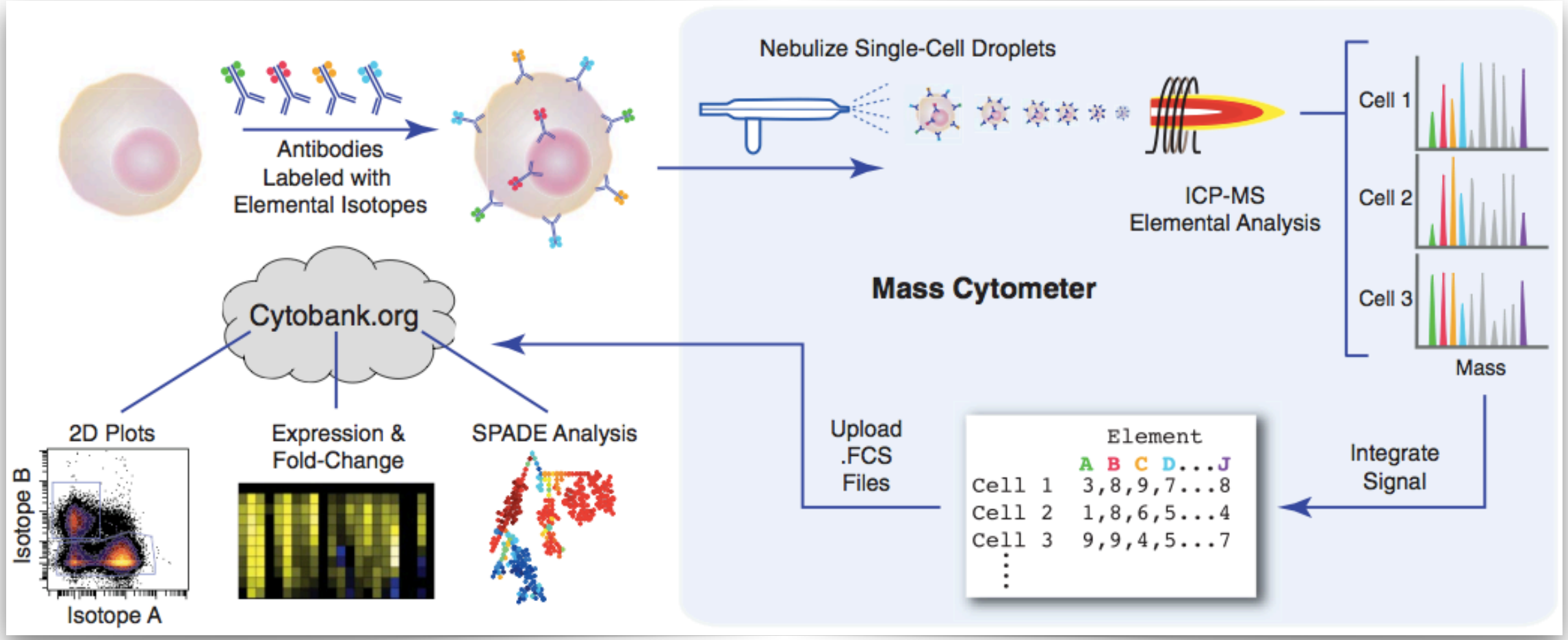


Figure 1. Schematic representation of a flow cytometer. For details please see text. (1) Forward-scatter detector, (2) side-scatter detector, (3) fluorescence detector, (4) filters and mirrors, and (5) charged deflection plates.

Jahan-Tigh et al., Journal of Investigative Dermatology, 2012

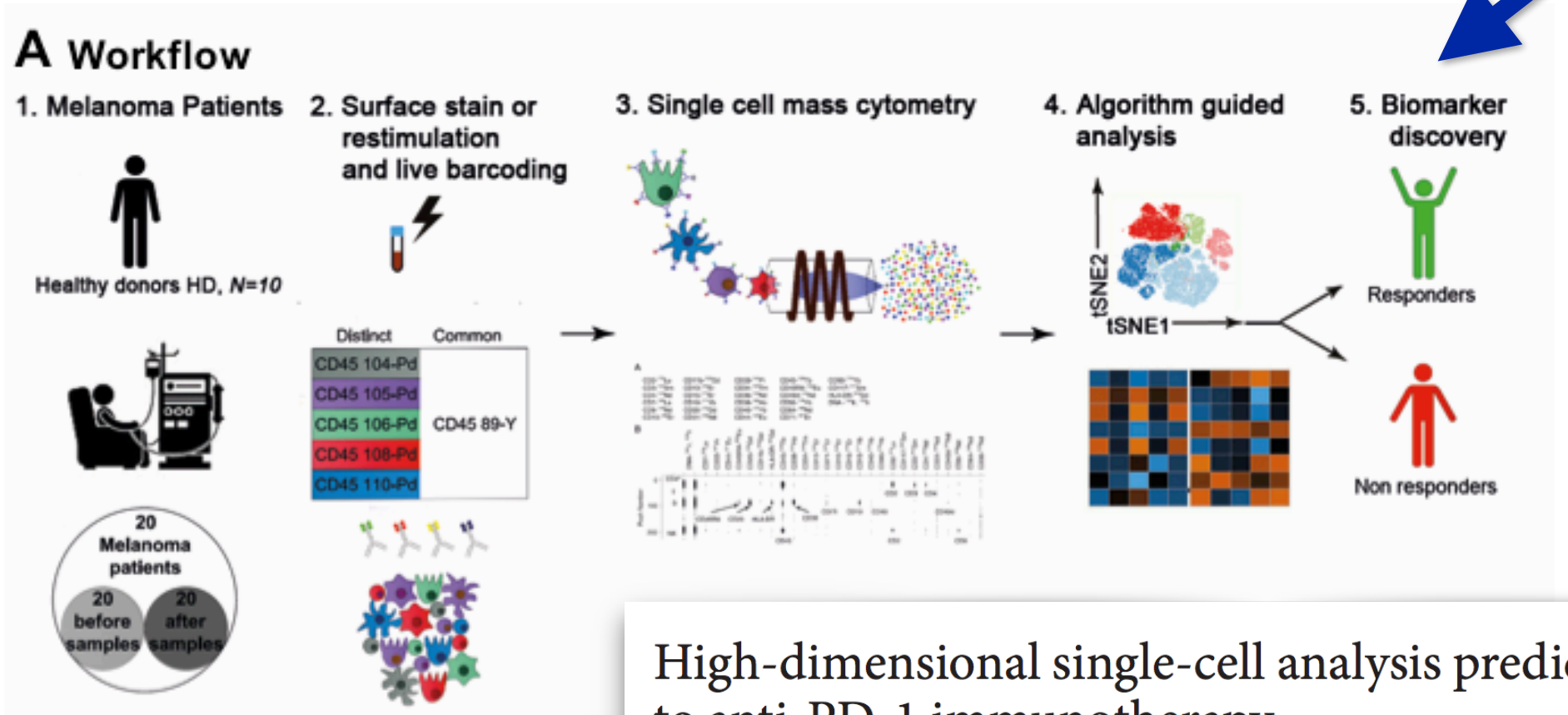
Mass cytometry



Bendall et al. (2011), Fig. 1A

Algorithm guided analysis = statistics

Finding molecular biomarkers associated with drug response

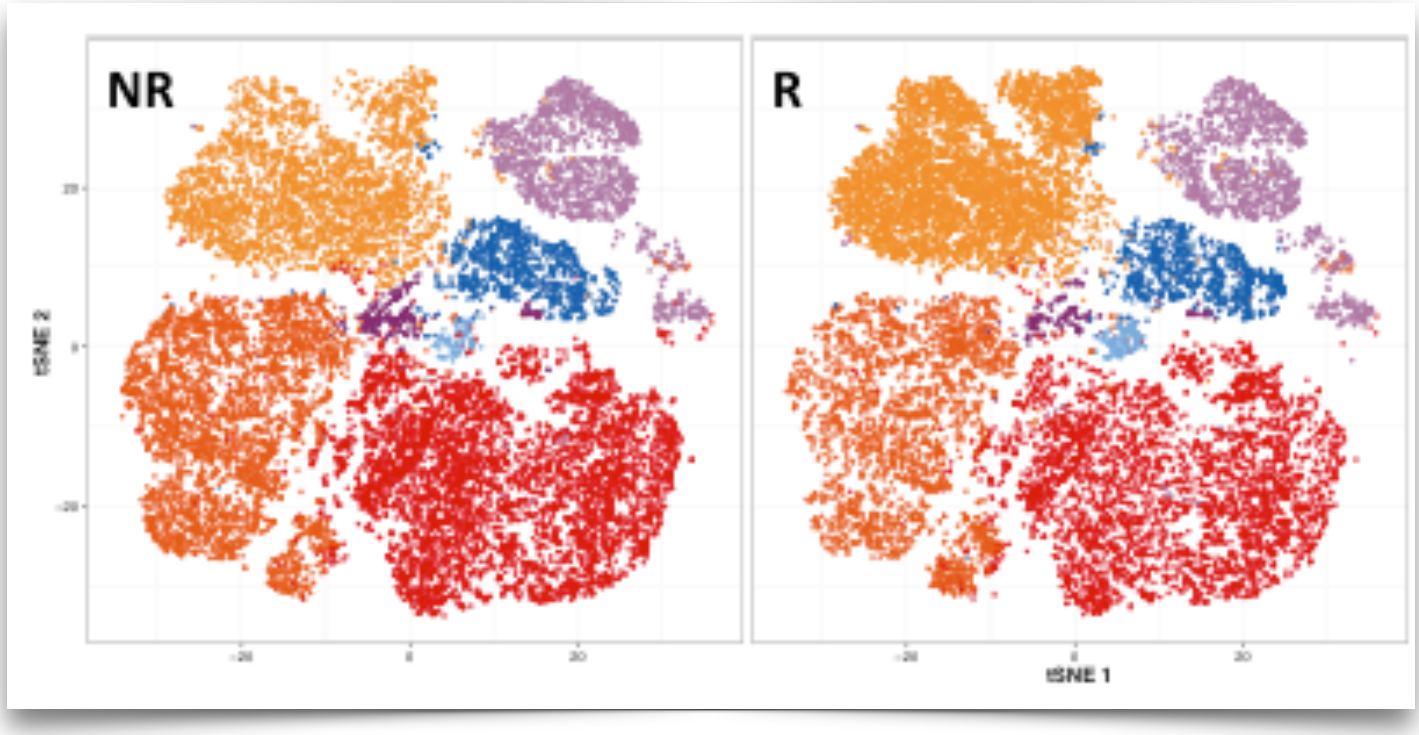


High-dimensional single-cell analysis predicts response to anti-PD-1 immunotherapy

Carsten Krieg[1,6], Malgorzata Nowicka[2,3], Silvia Guglietta[4], Sabrina Schindler[5], Felix J Hartmann[1], Lukas M Weber[2,3], Reinhard Dummer[5], Mark D Robinson[2,3], Mitchell P Levesque[5,7] & Burkhard Becher[1,7]

Differential abundance of cell populations

tSNE projection
(each dot = cell,
cells from multiple
patients)



NR: non-responders
R: responders

Under the hood: Generalized linear mixed model to assess the change in relative abundance of subpopulations.

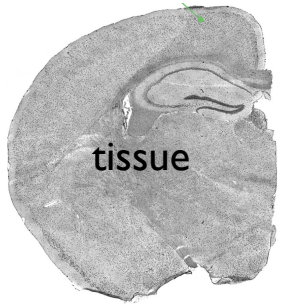
From bulk to single-cell RNA-seq to imaging- & sequencing-based spatially resolved transcriptomics

Slide from Helena Crowell

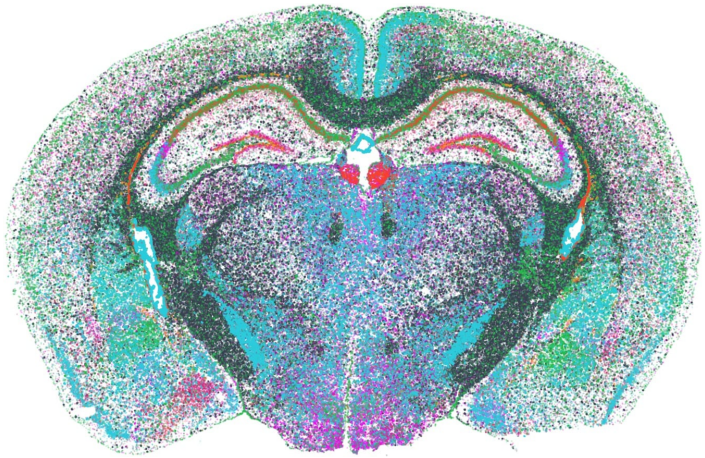
bulk

single-cell

spatial

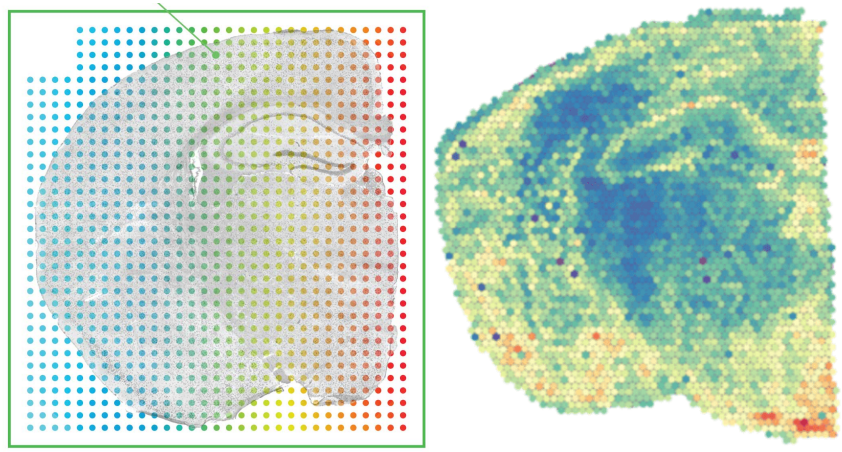


imaging-based



- molecule-level data
- targeted panel (100s of features)
- single-cell resolution requires segmentation

sequencing-based



- spot-level data
- whole transcriptome (10,000s of features)
- single-cell resolutions requires aggregation or deconvolution

Some of the statistical fundamentals that underpin much of our research .. and our discoveries (.. but also underpin analyses that you may do in the future)

- central limit theorem
- false positives / false negatives (error control)
- statistical tests, multiple testing, P-values
- sharing information (limma)
- clustering
- exploratory data analysis, e.g., dimensionality reduction

Central limit theorem

Central limit theorem

The short non-technical version: once you start taking sums (averages), **sampling distributions** of the mean converge to the Gaussian (normal) bell shaped curve as the sample size increases.

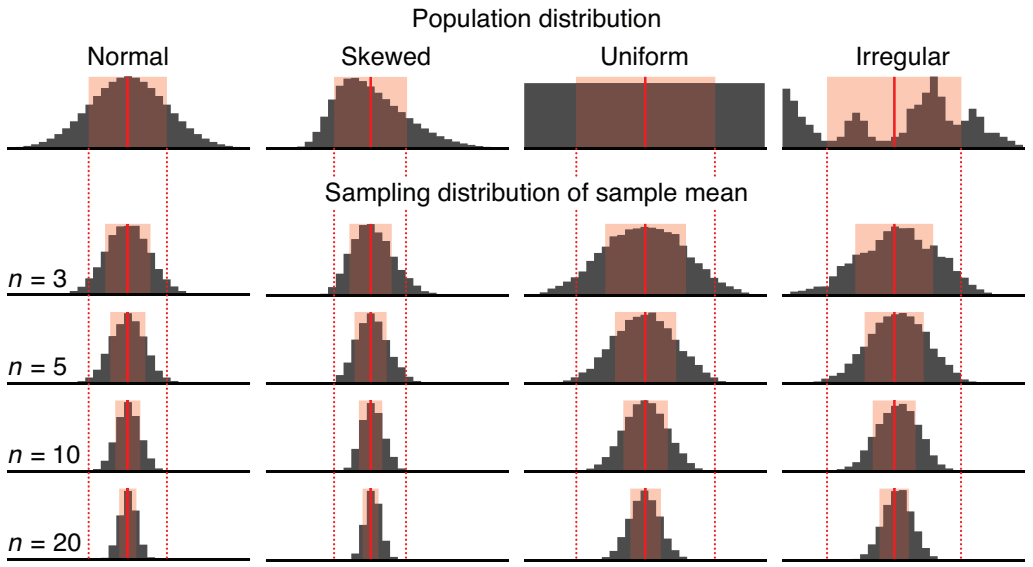


Figure 3 | The distribution of sample means from most distributions will be approximately normally distributed. Shown are sampling distributions of sample means for 10,000 samples for indicated sample sizes drawn from four different distributions. Mean and s.d. are indicated as in **Figure 1**.

If time, demonstrate this in R.

false positives, false negatives, multiple testing, P-values

Hypothesis testing

EDITORIAL · 20 MARCH 2019

It's time to talk about ditching statistical significance

Looking beyond a much used and abused measure would make science harder, but better.

- Method of making a decision
- Is this result "statistically significant"? ("Is my finding likely to occur by chance?")
- (Controversial)
- Statistical significance != Biological significance

"Researchers should seek to analyse data in multiple ways to see whether different analyses converge on the same answer."

Operationally, it works (something) like:

– Define "null hypothesis" (usually some kind of baseline setting)

– Define alternative: non-null

– Calculate test statistics (e.g. where the sampling distribution under the null is known) and/or P-value

– If P-value < some (magic) cutoff, decide to reject the null hypothesis in favour of the alternative; otherwise, accept the null hypothesis

NHST (Null hypothesis statistical testing): Hypothetical example

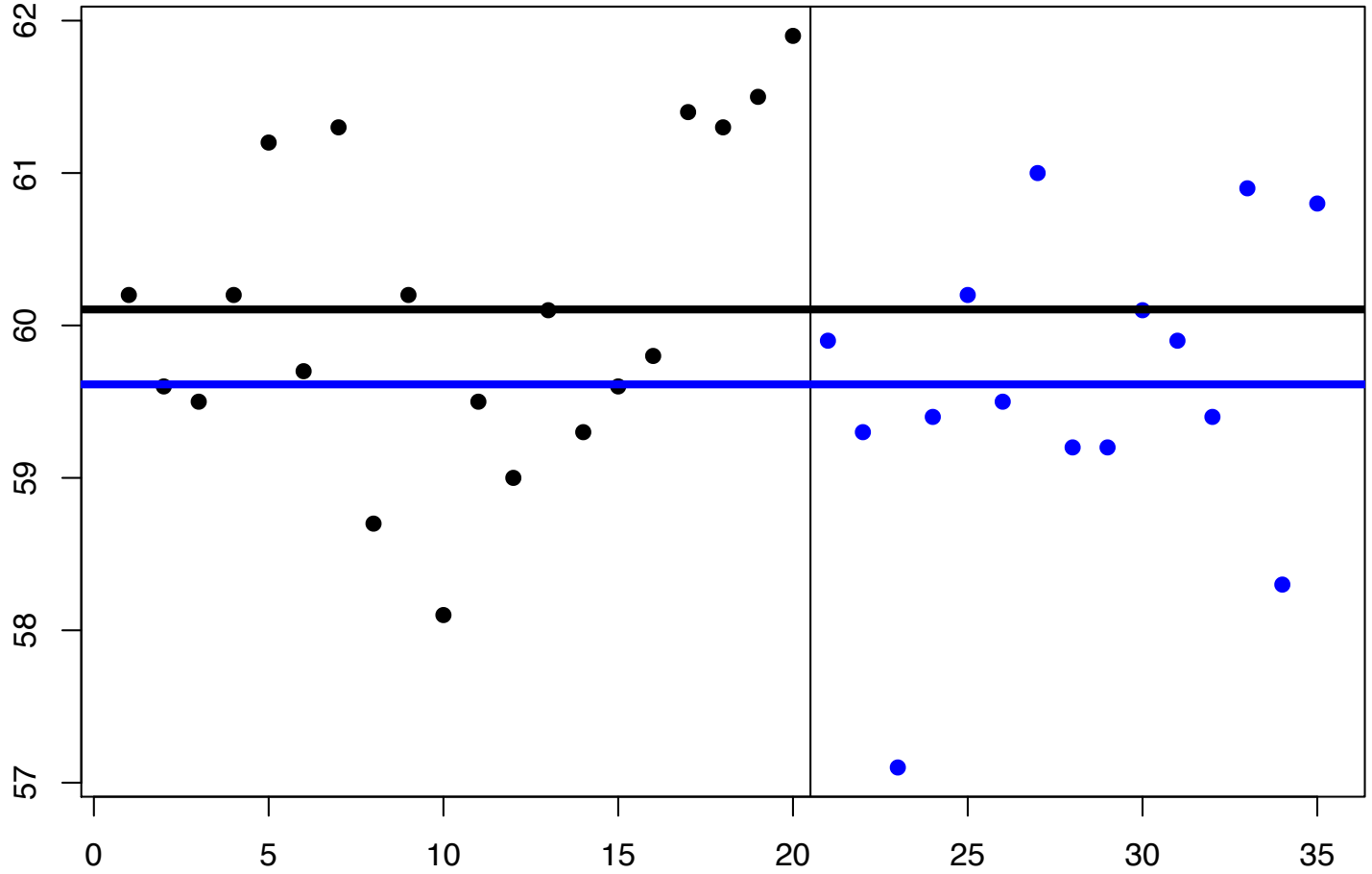
Say we wanted to know whether ETHZ students are scoring better or worse in a particular course than UZH students. First, we take a random sample from each population.

Null hypothesis: population mean of ETHZ scores = population mean of UZH scores
Alternative: means are different

Critical point: Assume that null hypothesis is true (i.e., means are equal), calculate a test statistic that we know the distribution of (under the null). Calculate the probability of observing something as or more extreme than our test statistic.

We'll use a t-statistic.

There are some variations of the t-test, but let us assume that the variances are equal



$$t = \frac{\bar{X}_1 - \bar{X}_2}{s_{X_1X_2} \cdot \sqrt{\frac{1}{n_1} + \frac{1}{n_2}}}$$

$$s_{X_1X_2} = \sqrt{\frac{(n_1 - 1)s_{X_1}^2 + (n_2 - 1)s_{X_2}^2}{n_1 + n_2 - 2}}.$$

Where does the t-test come from?

VOLUME VI MARCH, 1908 No. 1

BIOMETRIKA.

THE PROBABLE ERROR OF A MEAN.

BY STUDENT.

If the number of experiments be very large, we may have precise information as to the value of the mean, but if our sample be small, we have two sources of uncertainty:—(1) owing to the "error of random sampling" the mean of our series of experiments deviates more or less widely from the mean of the population, and (2) the sample is not sufficiently large to determine what is the law of distribution of individuals. It is usual, however, to assume a normal distribution, because, in a very large number of cases, this gives an approximation so close that a small sample will give no real information as to the manner in which the population deviates from normality: since some law of distribution must be assumed it is better to work with a curve whose area and ordinates are tabled, and whose properties are well known. This assumption is accordingly made in the present paper, so that its conclusions are not strictly applicable to populations known not to be normally distributed; yet it appears probable that the deviation from normality must be very extreme to lead to serious error. We are concerned here solely with the first of these two sources of uncertainty.

https://en.wikipedia.org/wiki/Student%27s_t-test

OK, but mathematically, where does the t-distribution come from?

$$Z = \left(\overline{X}_n - \mu\right) \frac{\sqrt{n}}{\sigma}$$

$$V = (n-1)\frac{S_n^2}{\sigma^2}$$

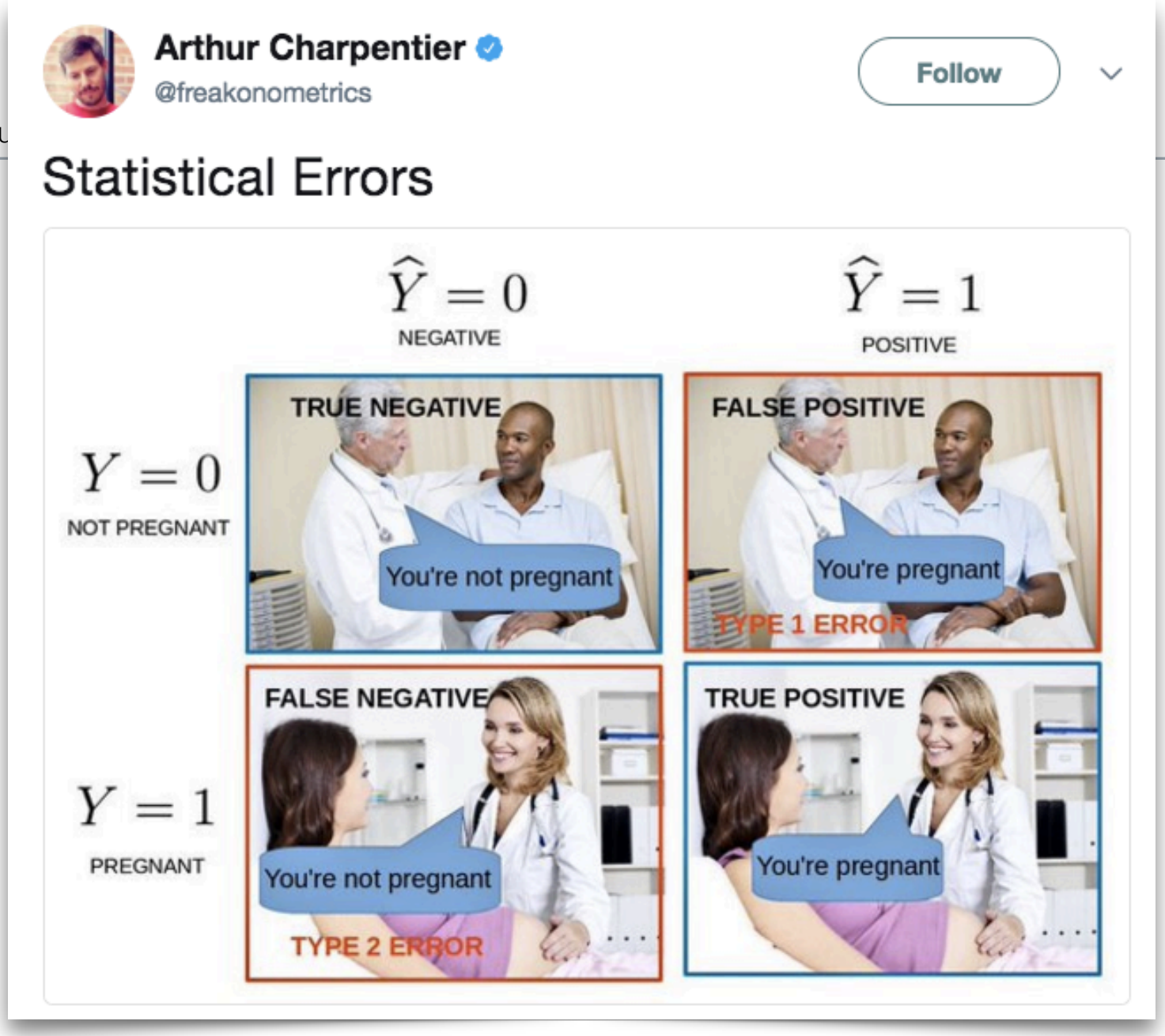
$$T \equiv \frac{Z}{\sqrt{V/\nu}} = \left(\overline{X}_n - \mu\right) \frac{\sqrt{n}}{S_n},$$

Clever discovery by William Gosset (i.e. “Student”)
The variance parameter cancels out —> straightforward extension to the 2-sample problem.

False positives / false negatives

Most statistical testing regimes set an error rate (5%)

Type I error = false positive
Type II error = false negative



https://twitter.com/freakonometrics/status/779060142239260672

limma (sharing information)

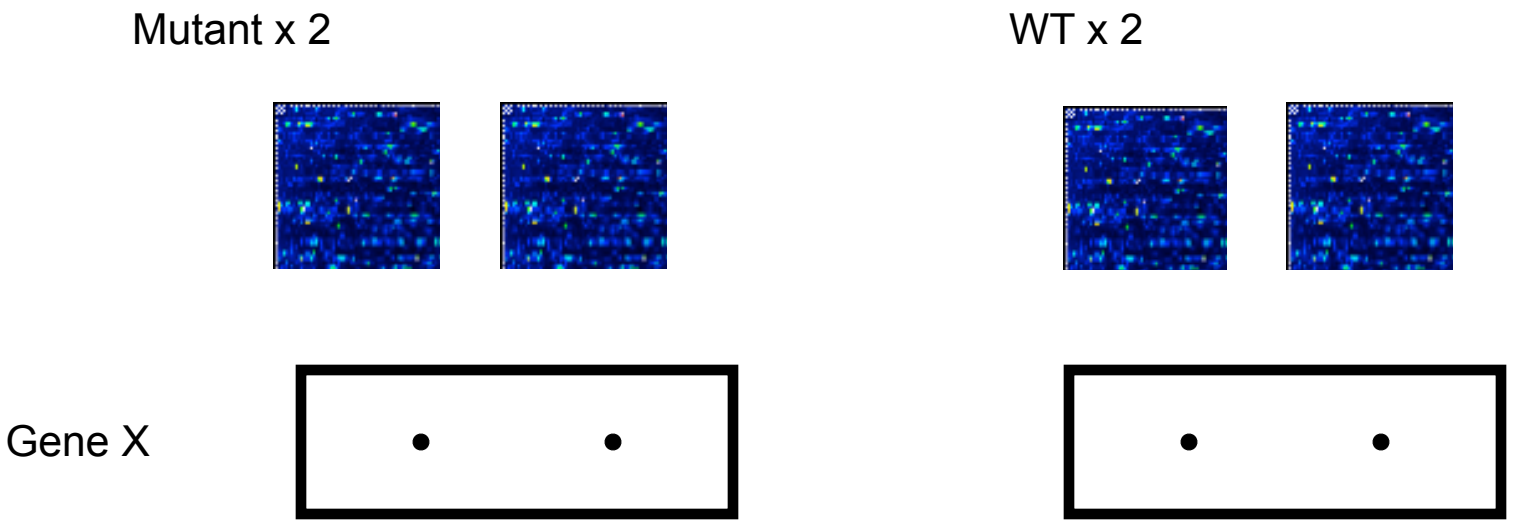
Differential expression, small sample inference

- Table of data (e.g., microarray gene expression data with replicates of each of condition A, condition B)
  - *rows* = features (e.g., genes), *columns* = experimental units (samples)
- Most common problem in statistical bioinformatics: want to infer whether there is a **change in the response** → a statistical test for each row of the table.

```
> head(y)
          group0     group0      group0     group1     group1      group1
gene1 -0.1874854  0.2584037 -0.05550717 -0.4617966 -0.3563024 -0.03271432
gene2 -3.5418798 -2.4540999  0.11750996 -4.3270442 -5.3462622 -5.54049106
gene3 -0.1226303  0.9354707 -1.10537767 -0.1037990  0.5221678 -1.72360854
gene4 -2.3394536 -0.3495697 -3.47742610 -3.2287093  6.1376670 -2.23871974
gene5 -3.7978820  1.4545702 -7.14796503 -4.0500796  4.7235714 10.00033769
gene6  1.4627078 -0.3096070 -0.26230124 -0.7903434  0.8398769 -0.96822312
```

[1] http://www.stat.ubc.ca/~rollin/stats/ssize/n2.html

A very common experiment: microarray or RNA sequencing



Which genes are differentially expressed?

$n_1 = n_2 = 2$ Affymetrix arrays

~30,000 probe-sets

In genomics, there is often a **multiple testing** problem

- You often make multiple tests (e.g., for every gene). Say, you set your cutoff such that you had a 5% false positive rate.
- In doing 20,000 tests (for 20,000 genes), ~1000 would be rejected just by chance.
- There are various ways to "correct" for multiple testing. Two popular ones include:
    1. False discovery rate (weak)
    2. Bonferroni correction (strong)

Classical 2-sample t-tests

$$t_g = \frac{\overline{y}_{\text{mu}} - \overline{y}_{\text{wt}}}{s_g \, c}$$

give very high false discovery rates

$$c = \sqrt{\frac{1}{n_1} + \frac{1}{n_2}}$$

Residual df = 2

t-tests with **common** variance (pooled over all genes measured)

$$t_{g,\text{pooled}} = \frac{\overline{y}_{\text{mu}} - \overline{y}_{\text{wt}}}{s_0\, c}$$

with residual standard deviation s_0 pooled across genes

More stable, but ignores gene-specific variability

$$c = \sqrt{\frac{1}{n_1} + \frac{1}{n_2}}$$

A better compromise: moderate between

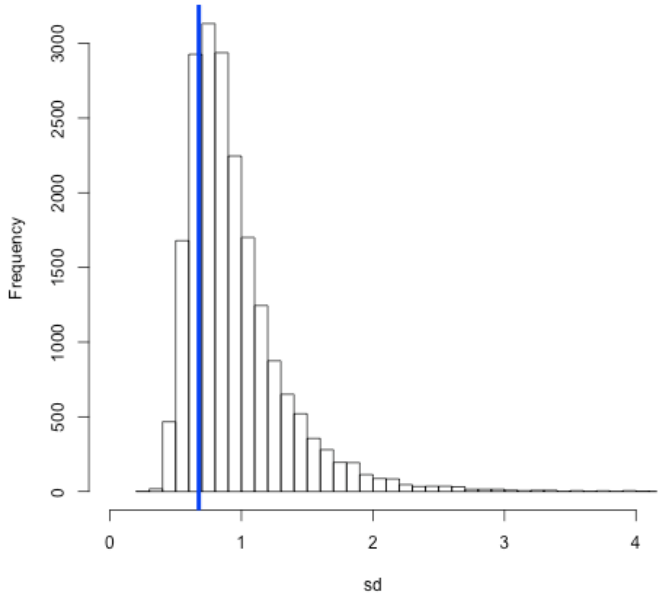
Shrink standard deviations towards common value

$$\tilde{s}_g^2 = \frac{d_0 s_0^2 + d_g s_g^2}{d_0 + d_g}$$

d = degrees of freedom

Moderated t-statistics

$$\tilde{t}_g = \frac{\overline{y}_{\text{mu}} - \overline{y}_{\text{wt}}}{\tilde{s}_g \, u}$$



Exact distribution for moderated t

An unexpected piece of mathematics shows that, under the null hypothesis,

$$\tilde{t}_g \sim t_{d_0+d_g}$$

The degrees of freedom add.

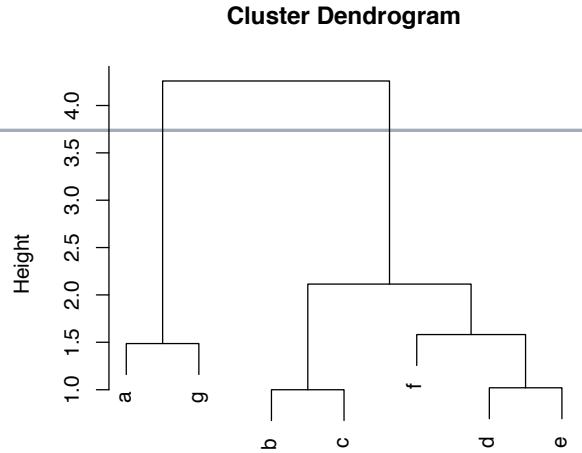
In effect, the moderated variance adds d_0 extra samples to the analysis, thus increasing the statistical power.

Smyth 2004

clustering (hierarchical)

http://en.wikipedia.org/wiki/Hierarchical_clustering

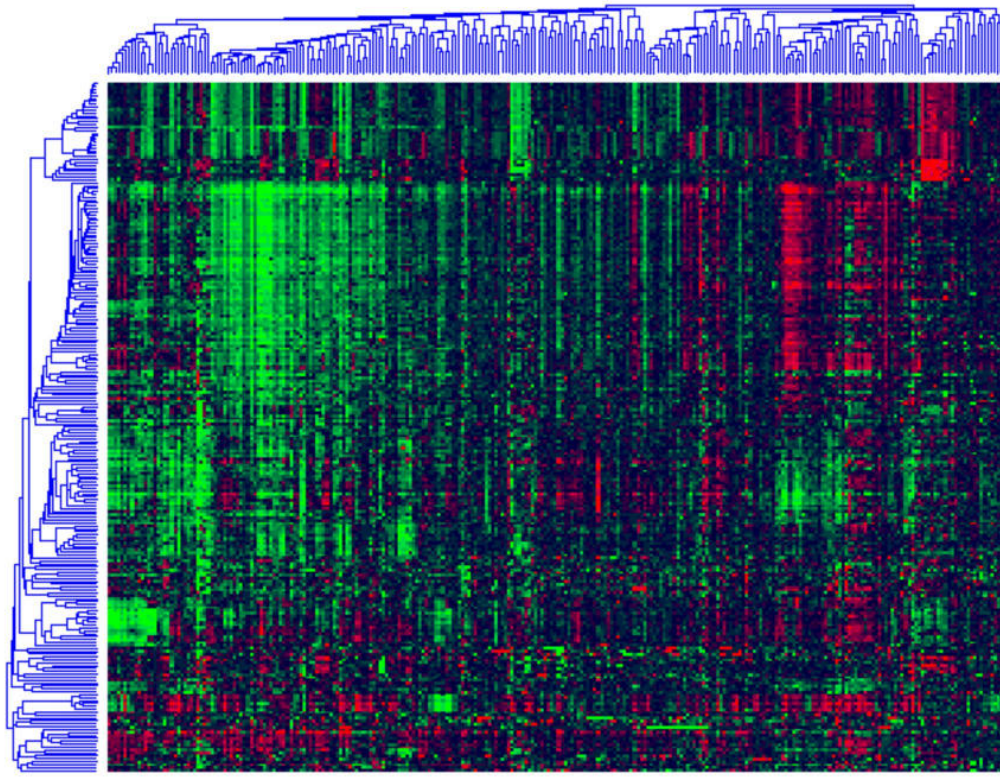
Hierarchical (Agglomerative) Clustering



Divisive (all features start as 1 cluster, then subsequently split) versus Agglomerative (every feature is it's own cluster, then subsequently merged)

Metric: to define how similar any two vectors are.

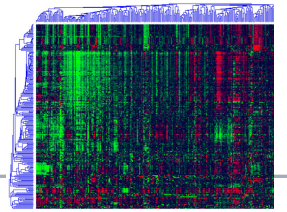
Linkage: determines how clusters are merged into a tree



http://en.wikipedia.org/wiki/Euclidean_distance

Euclidean distance:

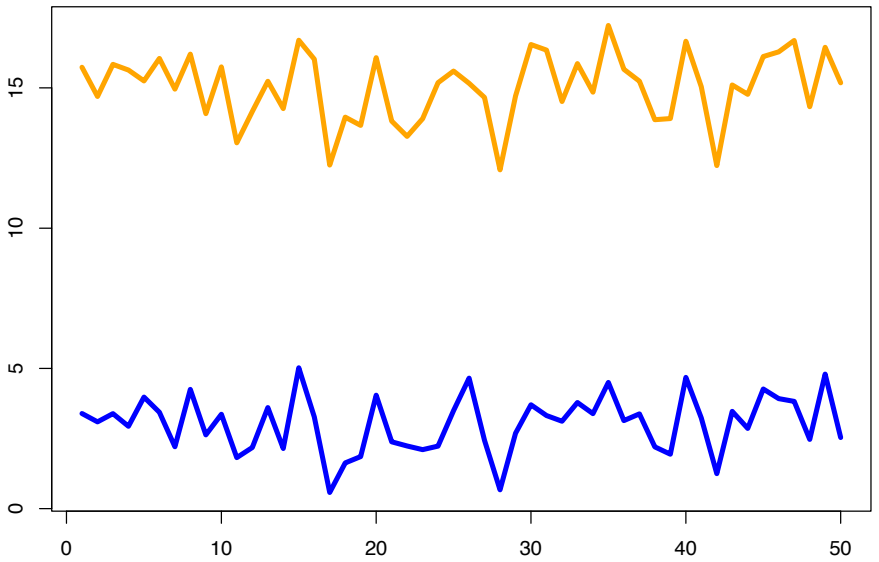
$$\mathrm{d}(\mathbf{p},\mathbf{q}) = \mathrm{d}(\mathbf{q},\mathbf{p}) = \sqrt{(q_1-p_1)^2+(q_2-p_2)^2+\cdots+(q_n-p_n)^2} = \sqrt{\sum_{i=1}^{n}(q_i-p_i)^2}.$$

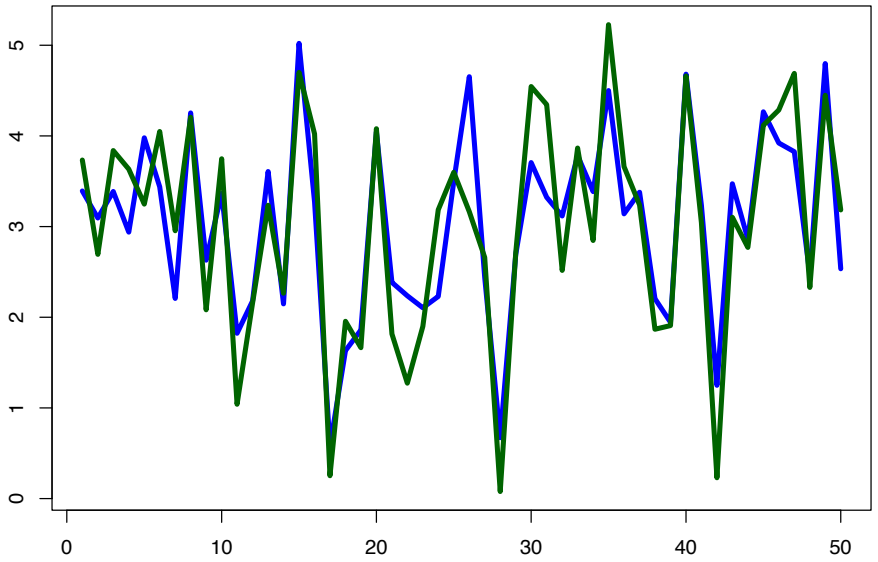


Are these “vectors” similar ?

```
> sqrt(sum((x-(y-12))^2))
[1] 3.926007
> sqrt(sum((x-y)^2))
[1] 84.84028
```

It depends how you define similar.

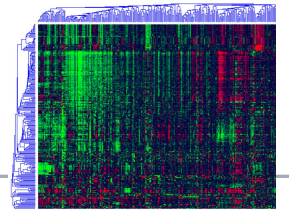




Euclidean distance: 84.84 3.92

http://en.wikipedia.org/wiki/Correlation_and_dependence

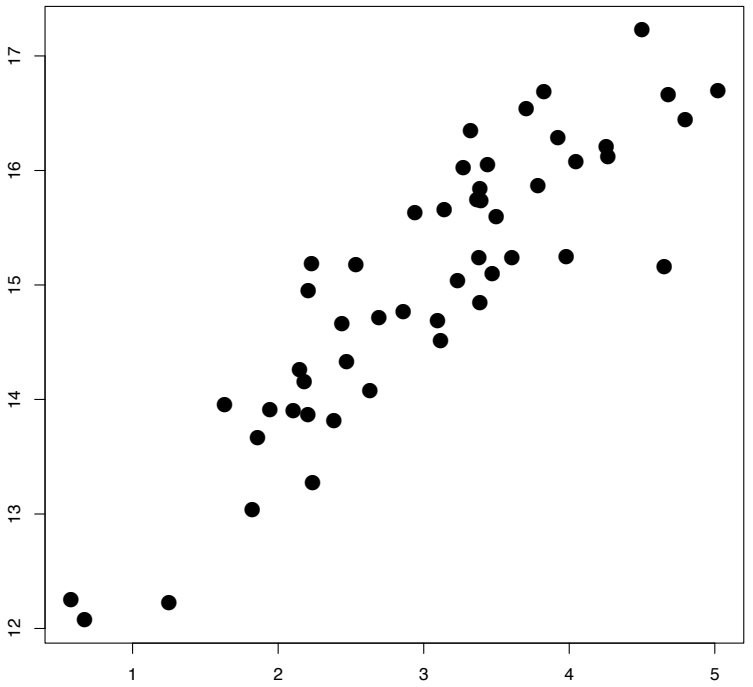
$$r_{xy} = \frac{\sum_{i=1}^{n}(x_i - \bar{x})(y_i - \bar{y})}{(n-1)s_x s_y} = \frac{\sum_{i=1}^{n}(x_i - \bar{x})(y_i - \bar{y})}{\sqrt{\sum_{i=1}^{n}(x_i - \bar{x})^2 \sum_{i=1}^{n}(y_i - \bar{y})^2}},$$

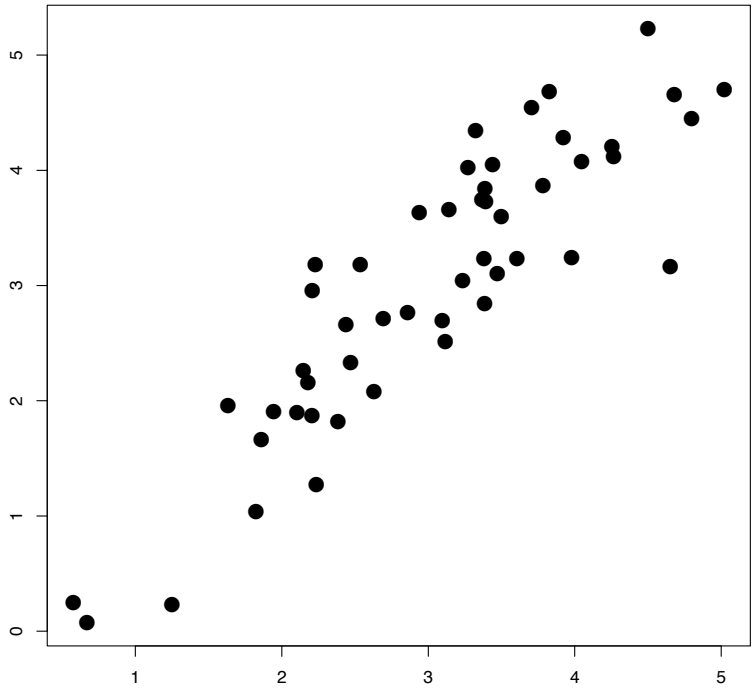


Are these “vectors” similar ?

```
> cor(x,y)
[1] 0.8901139
> cor(x,y-12)
[1] 0.8901139
```

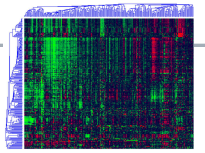
It depends how you define similar.





Correlation: 0.89 0.89

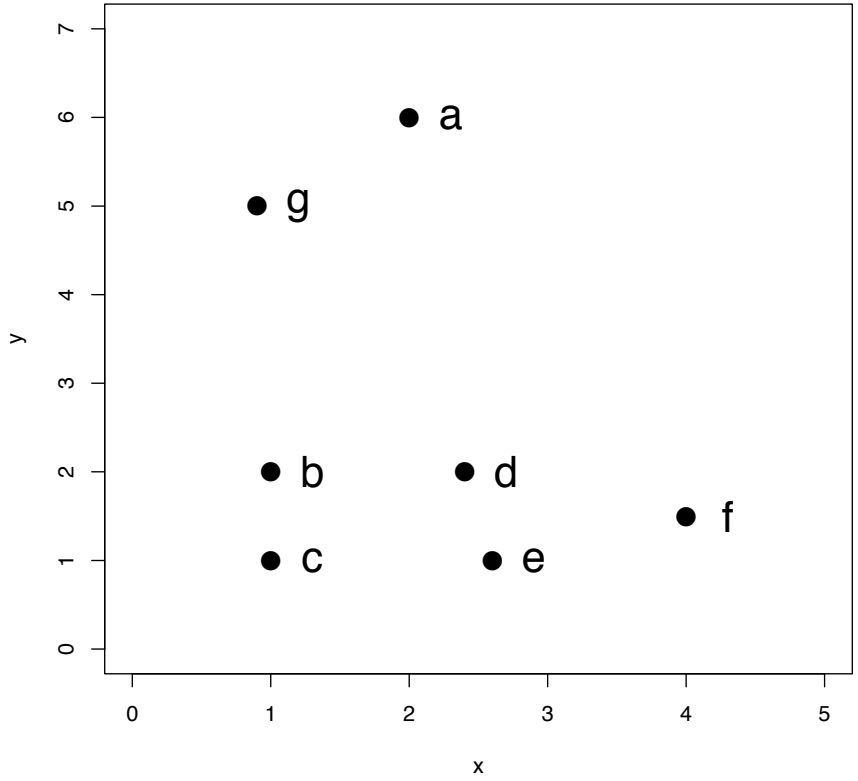
http://en.wikipedia.org/wiki/Hierarchical_clustering



Hierarchical (Agglomerative) Clustering

Start with distances.

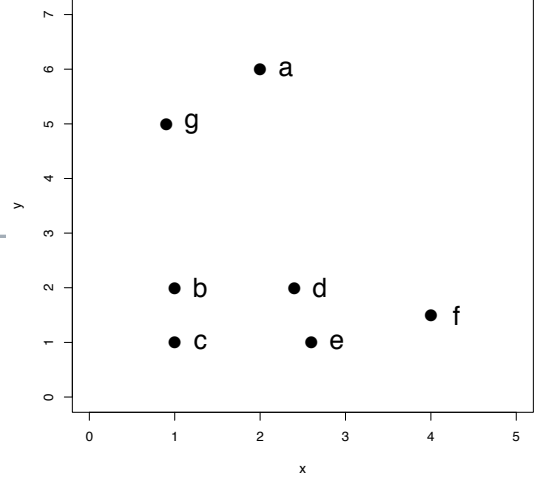
Linkage: determines how clusters are merged into a tree.

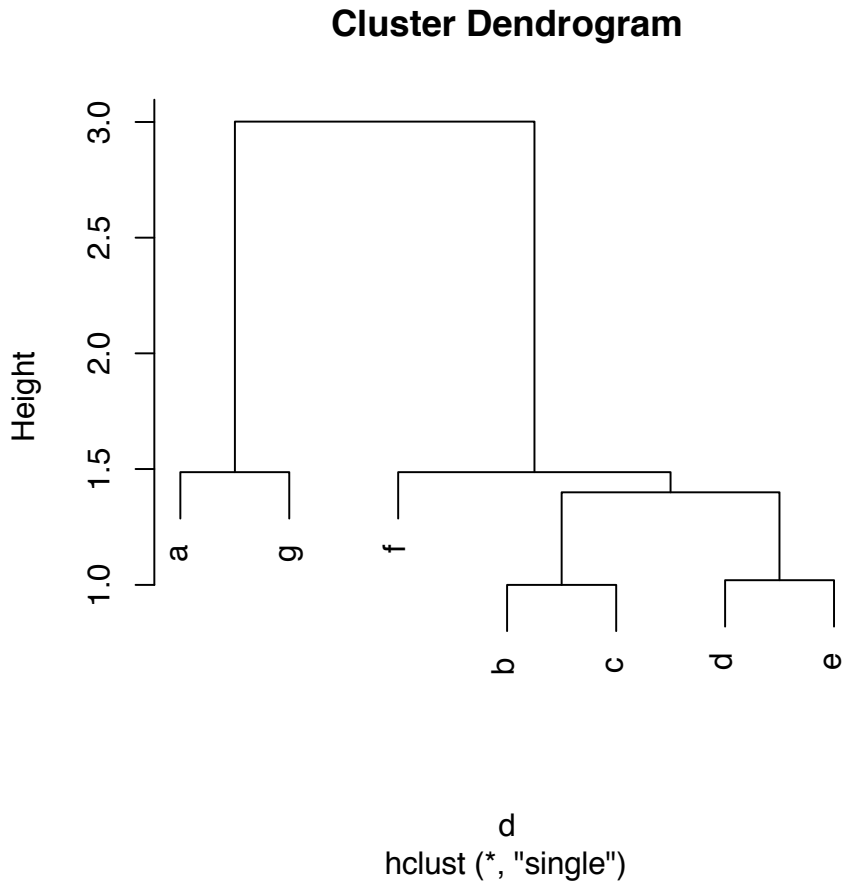


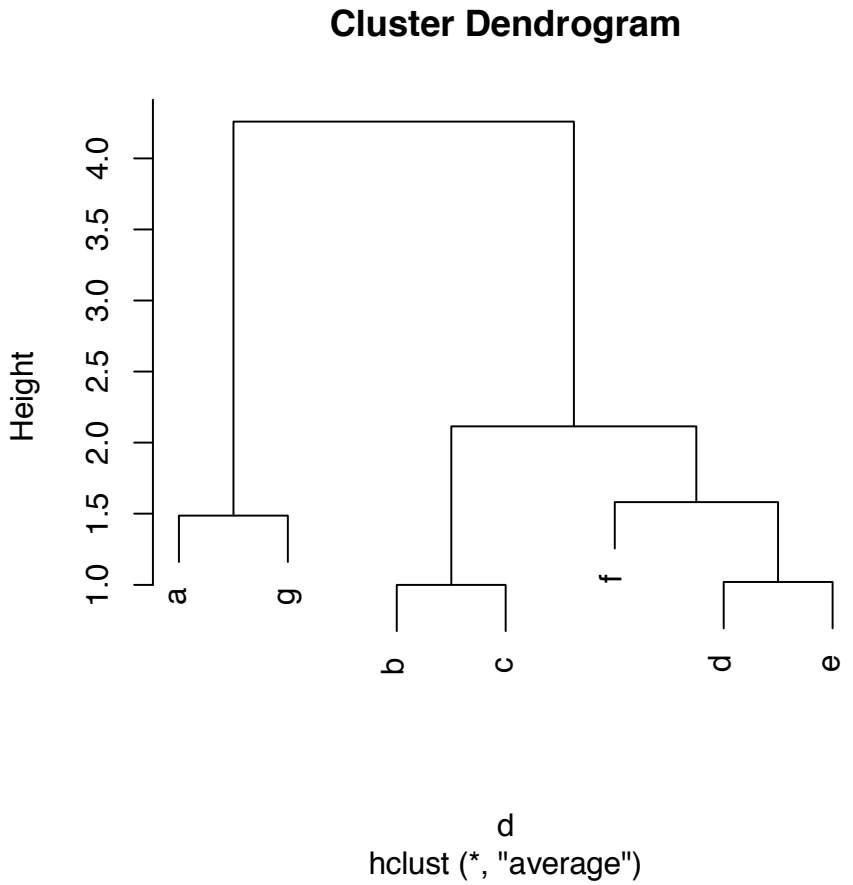
From eyeballing, here is a likely set of merges:

b,c
d,e
a,g,
(d,e),f
(b,c),((d,e),f)
ALL

Different linkages







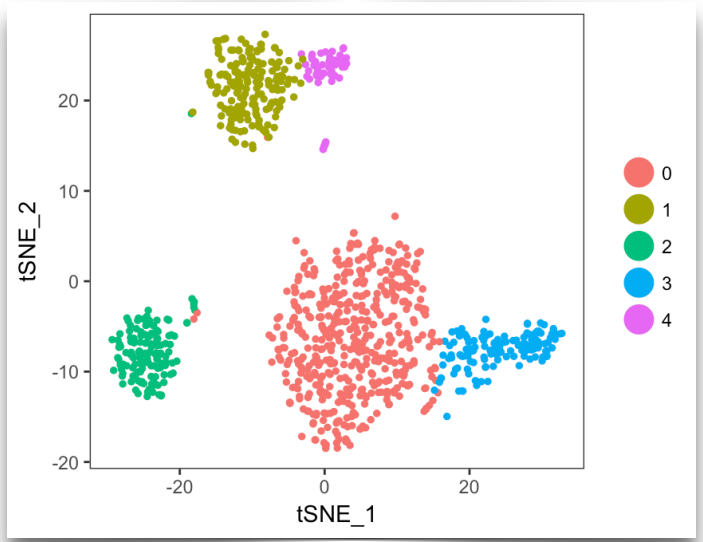
dimension reduction
(exploratory data analysis)

Dimension reduction: general introduction

- Many types of data come as a matrix of N samples (e.g., cells, patients) x G features (e.g., genes, proteins)
- Each sample is a point in G-dimensional space
- Goal: represent the data in 2-3 dimensions, but preserve **structure** as best as possible (i.e., points that are **close** in G dimensions should be close in 2 or 3 dimensions)

Dimension reduction is versatile

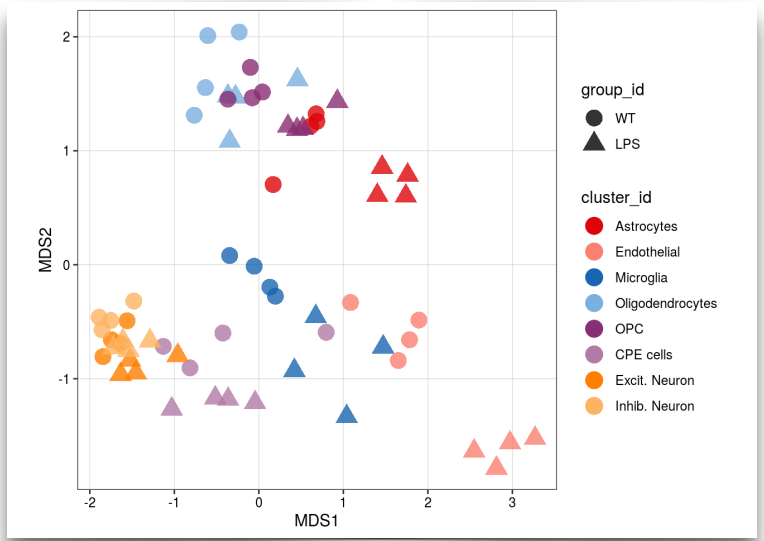
K features x N cells —> 2 dimensions x N cells



Each point = **single cell**

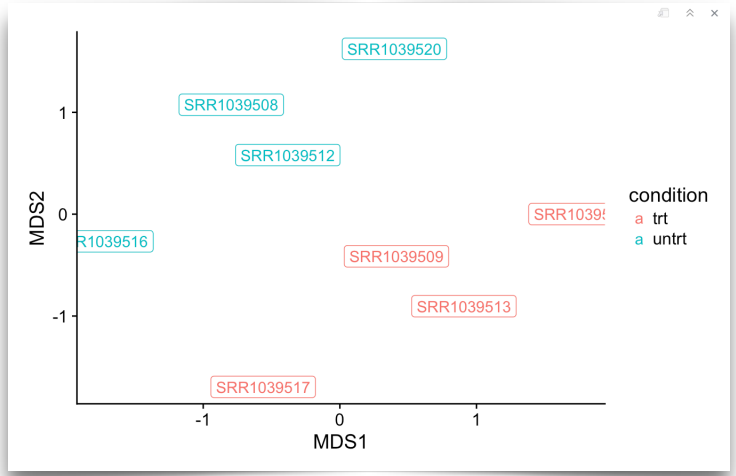
(10x PBMC)

N cells x K features —> N cell subpopulations x 2 dimensions



Each point = **subpopulation from a single sample** (LPS mouse cortex)

P samples x K features —> P samples x 2 dimensions



Each point = **sample**

(airway)

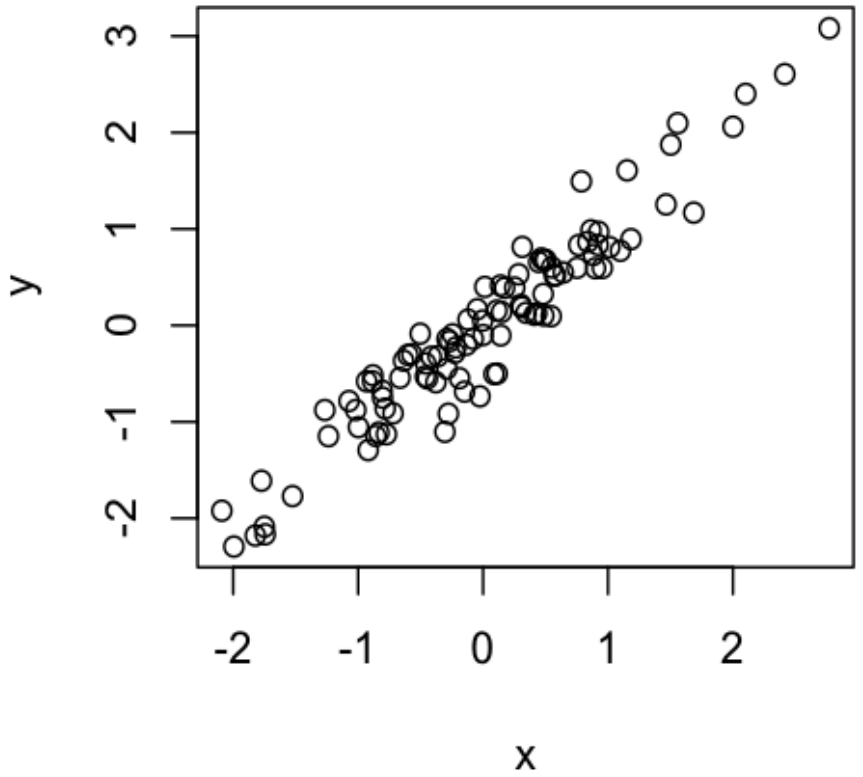
Introduction to dimension reduction: PCA (principal components analysis)

- Form successive *linear* combinations of the features that are: orthogonal, ordered by variance

$$Y = XA$$

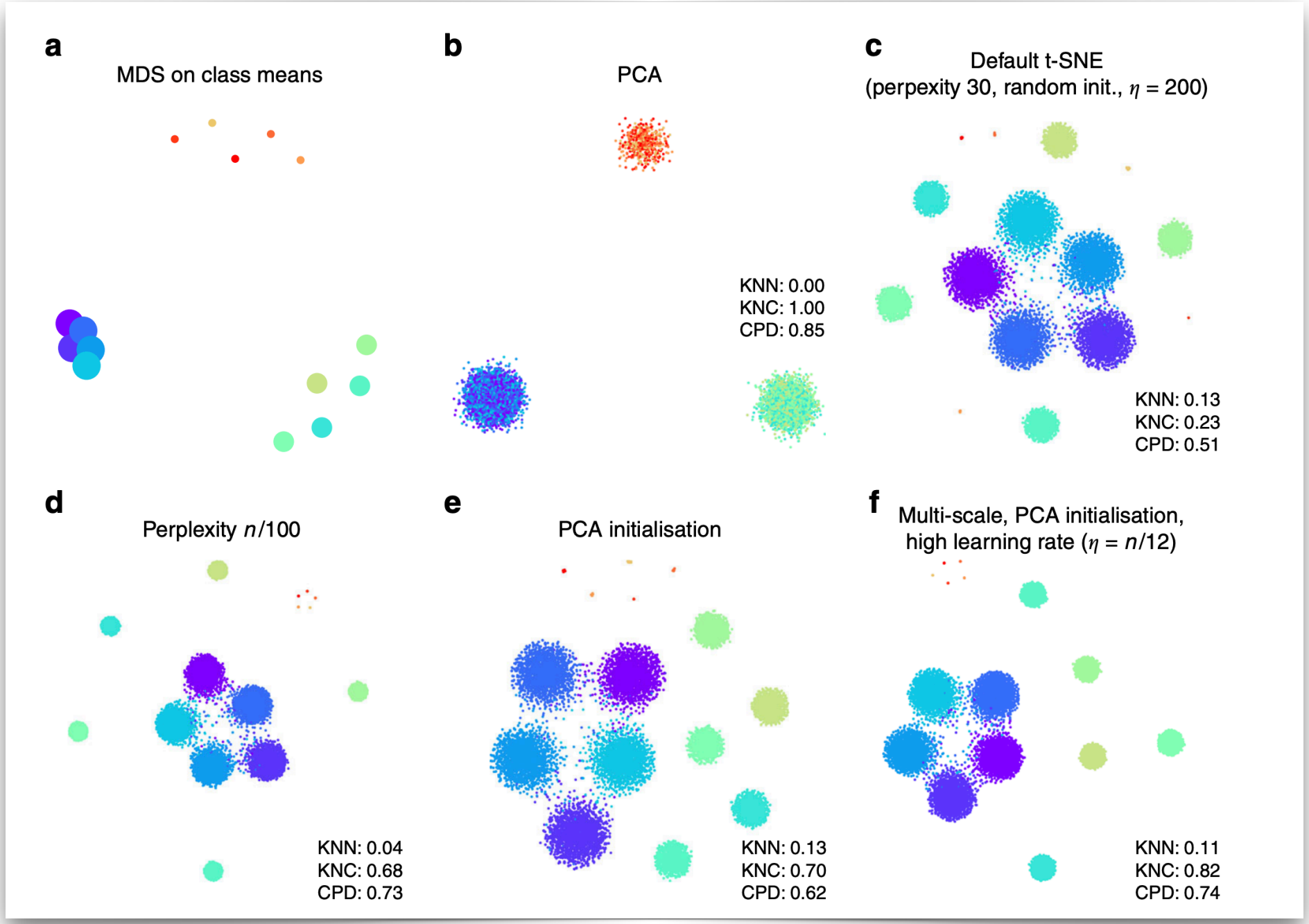
$$Y_{rk} = a_{1k}x_{r1} + a_{2k}x_{r2} + \cdots + a_{pk}x_{rp}$$

- A is the loadings matrix
- Typically, first 2-3 columns ('principal components') of Y are retained for visualisation; often top P PCs are retained for other analyses (e.g., clustering)



https://strata.uga.edu/software/pdf/pcaTutorial.pdf

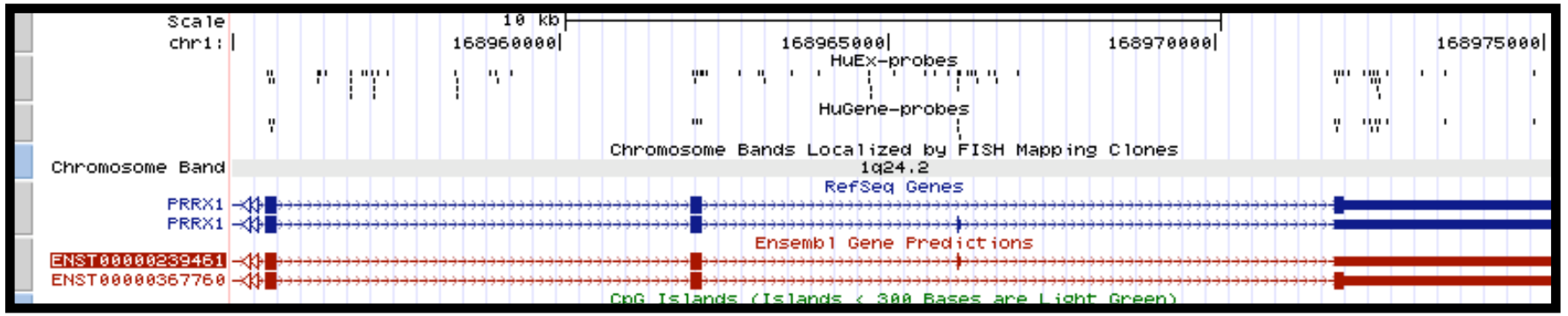
Many variations (linear/non-linear), many notions of distance, many ways to "compress"

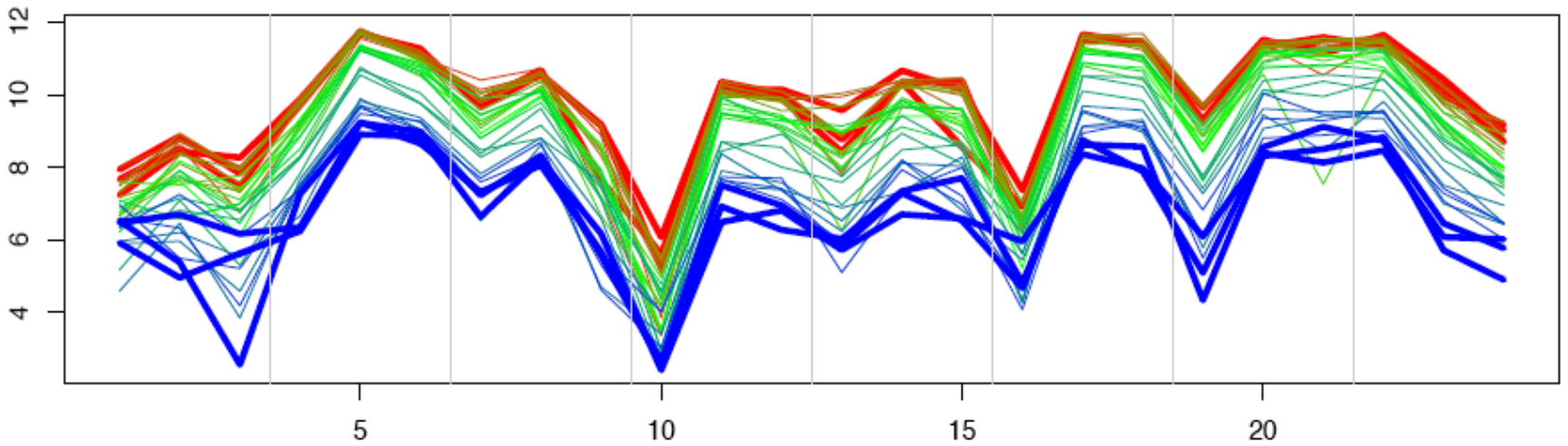


Kobak et al. 2019

Another data example .. a regression model to separate interesting signal (gene expression) from technical effects (probes)

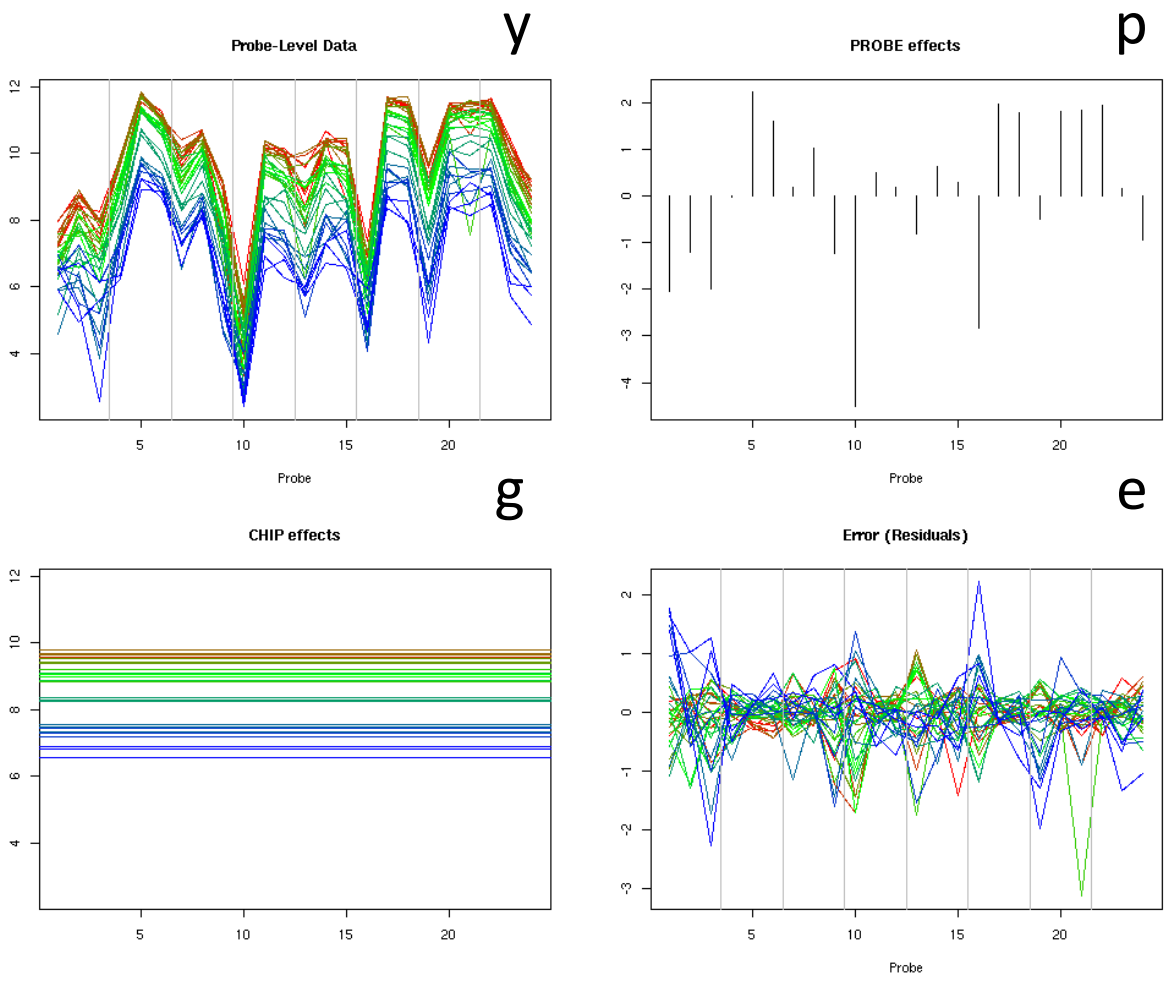
The nature of Affymetrix Probe Level Data





- Data for one gene that is differentially expressed between heart (red is 100% heart) and brain (blue is 100% brain).
- 11 mixtures x 3 replicates = 33 samples (33 lines)
- Note the parallelism: probes have different **affinities**

Linear model decomposes the probe-level data into **PROBE** effects and **CHIP** effects



Linear model:

$y_{ik} = g_i + p_k + e_{ik}$

Robust Multichip Analysis (RMA) uses this model. Irizarry et al. 2003, Biostatistics

Parameters are estimated robustly, meaning a small number of outliers have minimal effect

Tissue mixture dataset