Exploratory data analysis & experimental design

Keegan Korthauer

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with slide contributions from Paul Pavlidis

Learning objectives

- Understand general principles on organizing your data
- Be familiar with the main components of exploratory data analysis (EDA)
- Recognize and avoid common pitfalls of data visualization
- Understand the impacts of data quality and experimental design

Where to find code to make these plots

- These slides were made using this R Markdown file; it contains the code used to generate the R plots shown here
- The companion notes (GitHub Markdown document) explore multiple ways of making these plots and more detailed info
    - Source Rmd for these notes here

Recall the CHD8 RNA-seq experiment

- Gompers et al. (Nature Neuroscience 2017) analyzed 26 Chd8+/del5 and 18 WT littermates
- “Using a statistical model that accounted for sex, developmental stage and sequencing batch, we tested for differential expression across 11,936 genes that were robustly expressed in our data sets”
- We'll use this dataset throughout this lecture to illustrate EDA

Data organization

- As is often the case, this data was obtained in two separate files
  - Main data file: expression values - one per gene, per sample (G genes $\times$ N samples)
  - Metadata file: several covariates/experimental design variables for each sample (N samples $\times$ P covariates)
- We read these into R (see Rmd source code and companion notes) as a `data.frame` or `tibble` - matrix-like objects that have column names, and variable types for each column:

```
# data (expression) matrix
dim(d)
```

```
## [1] 12158    44
```

```
# metadata
dim(m)
```

```
## [1] 44  8
```

Small but important detail

Columns of main data matrix should match the rows of the metadata matrix *exactly*

These two objects should represent the same set of samples (and be in the same order)

```
head(colnames(d))
```

```
## [1] "Sample_ANAN001A" "Sample_ANAN001B" "Sample_ANAN001C" "Sample_ANAN001D"
## [5] "Sample_ANAN001E" "Sample_ANAN001F"
```

```
head(m$Sample)
```

```
## [1] "Sample_ANAN001A" "Sample_ANAN001B" "Sample_ANAN001C" "Sample_ANAN001D"
## [5] "Sample_ANAN001E" "Sample_ANAN001F"
```

```
identical(colnames(d), m$Sample)
```

```
## [1] TRUE
```

Data matrix

```
head(d)
```

```
##               Sample_ANAN001A Sample_ANAN001B Sample_ANAN001C Sample_ANAN001D
## 0610007P14Rik        5.238647        5.042397        5.364602        5.207641
## 0610009B22Rik        4.251070        4.568908        4.625527        4.568743
## 0610009O20Rik        4.807282        4.743503        4.921915        4.813903
## 0610010F05Rik        4.830067        5.063055        4.861288        4.957009
## 0610010K14Rik        5.958641        5.810707        5.851939        6.043380
## 0610011F06Rik        4.619302        4.579443        4.900748        4.802624
##               Sample_ANAN001E Sample_ANAN001F Sample_ANAN001G Sample_ANAN001H
## 0610007P14Rik        5.222326        5.129427        5.239697        5.127338
## 0610009B22Rik        4.555767        4.412691        4.253776        4.205859
## 0610009O20Rik        4.812471        4.807076        4.828430        4.708997
## 0610010F05Rik        5.065935        4.948113        5.067492        5.124294
## 0610010K14Rik        5.997013        5.979884        5.902530        5.846258
## 0610011F06Rik        4.756396        4.828523        4.575162        4.868060
##               Chd8.e14.S12 Chd8.e14.S13 Chd8.e14.S14 Chd8.e14.S16 Chd8.e14.S17
## 0610007P14Rik     4.841569     5.197562     4.825154     5.181760     5.070708
## 0610009B22Rik     4.452122     4.746104     4.747871     4.789378     4.862351
## 0610009O20Rik     4.455875     4.649412     4.338314     4.555878     4.490673
## 0610010F05Rik     5.314438     5.387970     5.523043     5.308837     5.514546
## 0610010K14Rik     5.855191     6.148984     6.148754     6.098012     6.174872
```

Metadata

```
m
```

```
## # A tibble: 44 × 8
##    Number Sample            DPC Sex   Group  SeqRun MappedReads FeatureCounts
##     <dbl> <chr>           <dbl> <fct> <fct>  <fct>        <dbl>          <dbl>
##  1      1 Sample_ANAN001A  12.5 F     Mutant A          42452222       37655856
##  2      2 Sample_ANAN001B  12.5 M     WT     A          54503162       47106938
##  3      3 Sample_ANAN001C  12.5 M     WT     A          44978512       40448118
##  4      4 Sample_ANAN001D  12.5 F     Mutant A          50099336       44993589
##  5      5 Sample_ANAN001E  12.5 F     Mutant A          47163546       41840678
##  6      6 Sample_ANAN001F  12.5 F     WT     A          43893480       39483622
##  7      7 Sample_ANAN001G  12.5 F     Mutant A          53208684       47697298
##  8      8 Sample_ANAN001H  12.5 F     WT     A          38925414       35056860
##  9      9 Chd8.e14.S12     14.5 M     WT     B          23622816       19586363
## 10     10 Chd8.e14.S13     14.5 F     WT     B          23974703       21019829
## # … with 34 more rows
```

Organizing your data - two main issues

- How you set up your input files for easy reading into R
  - Easiest to work with are text files (e.g. tab-delimited `.tsv`)
  - Excel files not uncommon but not recommended
  - Almost always some data cleaning/wrangling involved (e.g. checking consistency, recoding, renaming variables)
- Within R, being able to refer to subsets of both data sets (main data & metadata) and other manipulations that require “matching” the expression data with the sample information (“slicing and dicing”)
  - For example: “Get me the expression level data for CHD8 in the female adult wild type mice” - this uses information from both sets
  - In practice, you may have to do it multiple ways to play nice with different R packages (e.g. one way for visualization, and another for downstream analysis)

Organizing your data: Option 1 - Separated

Keep main data and metadata tables separate

Pros:

- Minimal startup effort / extra code
- Can be compatible with downstream analysis methods (e.g. Bioconductor)

Cons:

- Risky: easy to make a mistake when subsetting and/or reordering samples - extra sanity checks required
- Not a convenient format for visualization since main data is separated from its metadata

Overall: *not recommended*

Organizing your data: Option 2 - Tidy way

The tidy way - combine main data & metadata into one 'long' table

Pros:

- Unambiguous - keeps all data in one object with one row per observation (e.g. each sample/gene combination is one row, along with all its metadata)
- Plays nice with tidyverse tools (e.g. dplyr manipulations, ggplot2 visualization)

Cons:

- 'long' format is inefficient data storage - sample information is repeated
- Not compatible with many tools for downstream analysis (e.g. Bioconductor)

Overall: *recommended for EDA/visualization*

The Tidy way

```
d_long
```

```
## # A tibble: 534,952 × 10
##    gene          Sample   Expression Number   DPC Sex   Group SeqRun MappedReads
##    <chr>         <chr>         <dbl>  <dbl> <dbl> <fct> <fct> <fct>        <dbl>
##  1 0610007P14Rik Sample_…       5.24      1  12.5 F     Muta… A          42452222
##  2 0610009B22Rik Sample_…       4.25      1  12.5 F     Muta… A          42452222
##  3 0610009O20Rik Sample_…       4.81      1  12.5 F     Muta… A          42452222
##  4 0610010F05Rik Sample_…       4.83      1  12.5 F     Muta… A          42452222
##  5 0610010K14Rik Sample_…       5.96      1  12.5 F     Muta… A          42452222
##  6 0610011F06Rik Sample_…       4.62      1  12.5 F     Muta… A          42452222
##  7 0610012G03Rik Sample_…       4.03      1  12.5 F     Muta… A          42452222
##  8 0610030E20Rik Sample_…       3.22      1  12.5 F     Muta… A          42452222
##  9 0610031J06Rik Sample_…       4.38      1  12.5 F     Muta… A          42452222
## 10 0610037L13Rik Sample_…       4.23      1  12.5 F     Muta… A          42452222
## # … with 534,942 more rows, and 1 more variable: FeatureCounts <dbl>
```

Organizing data: Option 3 - Bioconductor way

The Bioconductor way - combine main data & metadata into one specially formatted object

Pros:

- Unambiguous - keeps all data in one object with special slots that can be accessed with handy functions
- Plays nice with Bioconductor tools
- Efficient storage (no duplication of information like tidy way)

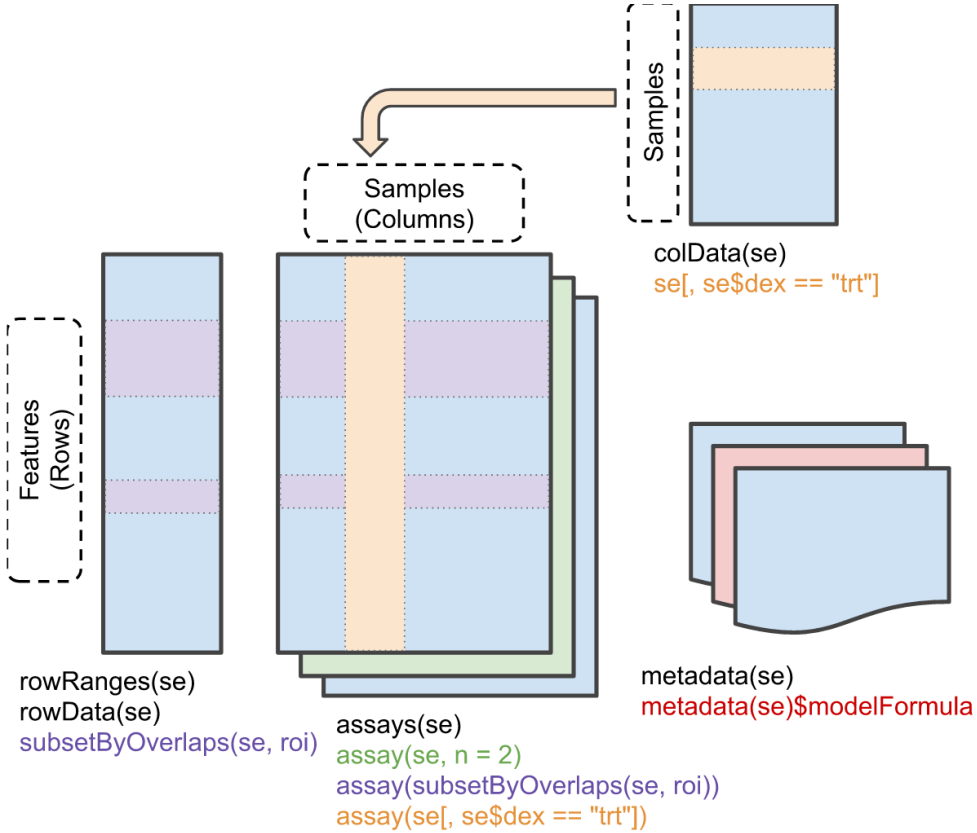
Cons:

- Specific to Bioconductor
- Not a compatible format for visualization (e.g. ggplot2)

Overall: *recommended for downstream analysis (e.g. Differential Expression)*

The Bioconductor way: SummarizedExperiment

- `SummarizedExperiment`: A special object format that is designed to contain data & metadata
- Comes along with handy accessor functions
- Many related / similar types of objects for specialized data types (e.g. `RangedSummarizedExperiment`, `SingleCellExperiment`, `DGEList`)



Anatomy of a SummarizedExperiment object

Now what? Time to explore!

- Understand / get a feel for the data
- Formulate hypotheses / develop models
- Identify problems

First questions - sanity checks

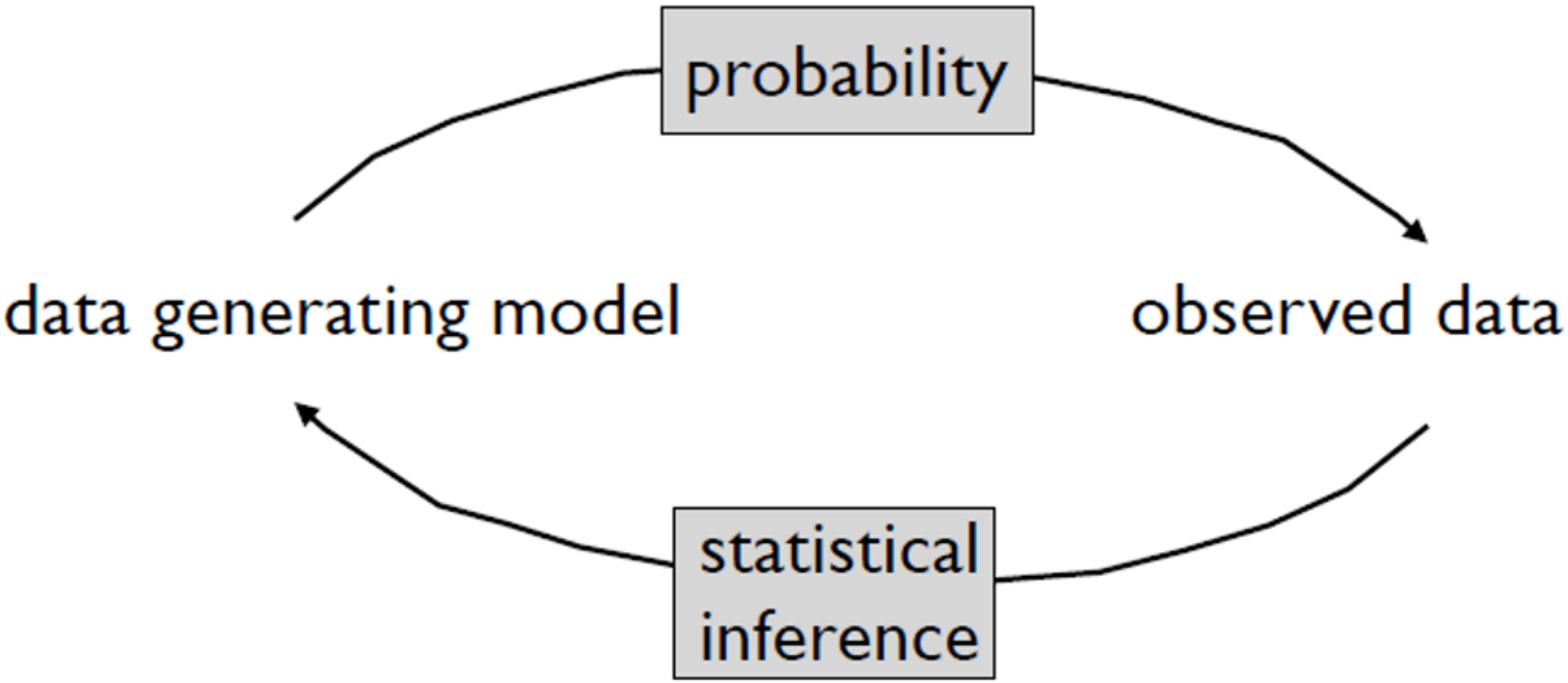
- Is the file the expected size? Format?
- Do we have the expected number of samples?
- Do the sample names in both files match? (Do not assume same ordering!)
- Are sample / feature names formatted correctly (e.g. no Excel conversion errors)?
- What do features represent? (e.g. Gene names, probe identifiers, etc.)
- Is the data numeric? Integers or decimal? Ratios (to what?)
- Are the data on a log scale? If so what is the base?
- Are there missing data points? What do they mean?
- Do factors have the expected number of levels?
- Do we have all the sample information we need?

These questions might lead to concerns

- If you are the one generating the data, save yourself grief by paying attention to these issues up front. Document what you did and be consistent!
- If you are the analyst, hopefully you were involved in the design stage so there will be fewer surprises.

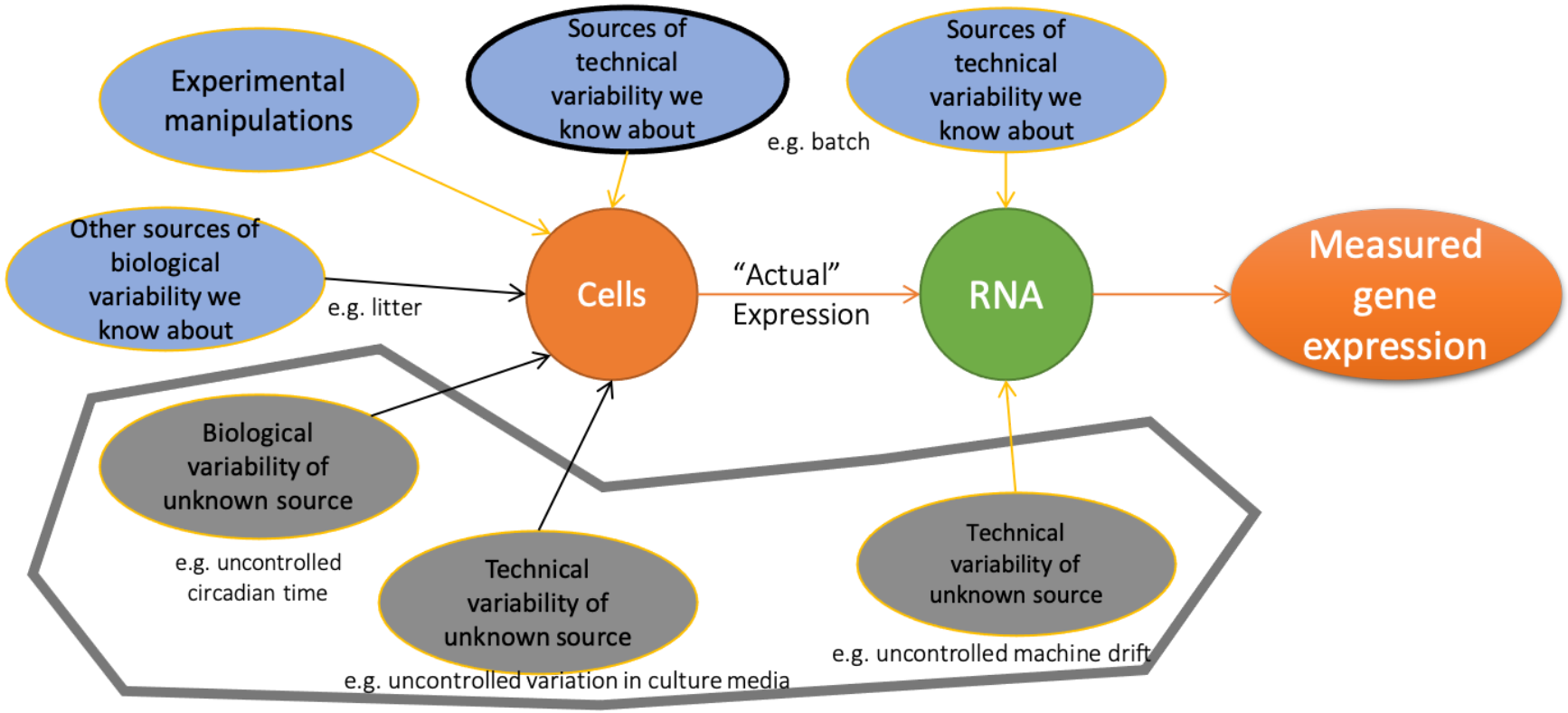
Making sense of the data

Data is what we observe, we want to infer something about “where it came from”



Model of gene expression data generation

- The measured expression level of gene g is the combination of many effects
- Analysis goal is often to determine relative role of effects - separate signal from “noise”



Variability: friend and foe

- If there is no variability, you don't have any information. The key is controlling/understanding sources of *wanted vs. unwanted* variability. One person's noise may be another's signal.
- First line of defense: Know the enemy
  - You can only "correct" for things you know about
  - Keep track of potential sources of variance: Batches of reagents, slides, personnel, processing dates, etc.
- Design experiments to minimize impact of technical variability
  - Don't process all control samples on day 1 and all treated samples on day 10
- Ensure appropriate replication
  - Biological (important)
  - Technical (usually less important but might need to convince yourself)

Biggest pitfall in (high-dimensional) data analysis

If you don't look at the data, you are likely going to miss important things

- Not just at the beginning, but at every stage
- That could mean making plots, or examining numerical patterns - probably both
- "Sanity checks" should make up a lot of your early effort
- Blindly following recipes/pipelines/vignettes/seminar code → trouble.

First looks at the CHD8 expression data

- What is the size of the data?
- What is the range of the data?
- Are there any missing values?
- Are the data transformed in any way?

```
se
```

```
## class: SummarizedExperiment
## dim: 12158 44
## metadata(0):
## assays(1): logrpkm
## rownames(12158): 0610007P14Rik 0610009B22Rik ... Zzef1 Zzz3
## rowData names(0):
## colnames(44): Sample_ANAN001A Sample_ANAN001B ... Chd8.adult.S29
##   Chd8.adult.S31
## colData names(8): Number Sample ... MappedReads FeatureCounts
```

```
range(assays(se)$logrpkm)
```

```
## [1] -7.87235 13.26689
```

Examining the metadata

Sample info:

- Age (Days Post-conception, DPC)
- Sex
- Group (genotype)
- Sequencing run (batch)
- Number of mapped reads
- feature counts

```
table(se$DPC)
```

```
##
## 12.5 14.5 17.5   21   77
##    8    9   10   11    6
```

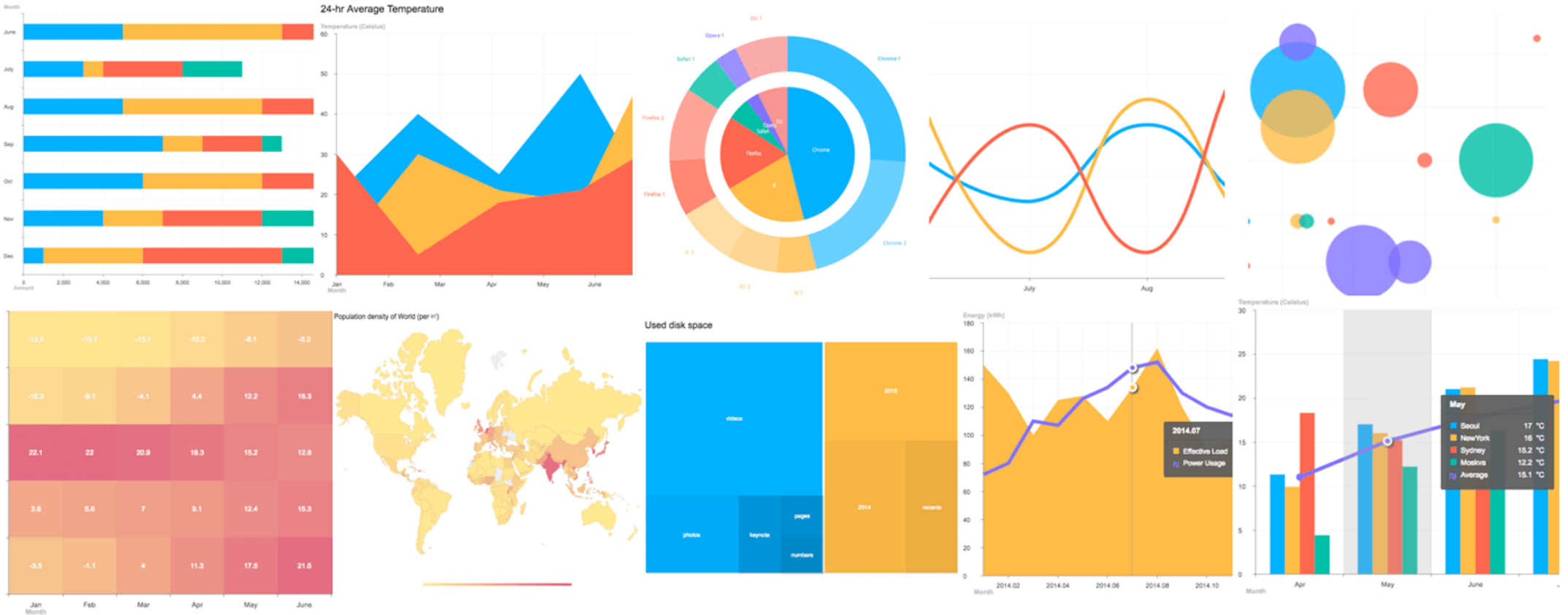
```
table(se$Sex)
```

```
##
##  M  F
## 25 19
```

```
table(se$SeqRun)
```

```
##
##  A  B  C  D  E  H
##  8  9  5  5 11  6
```

How to look at the rest of the data?

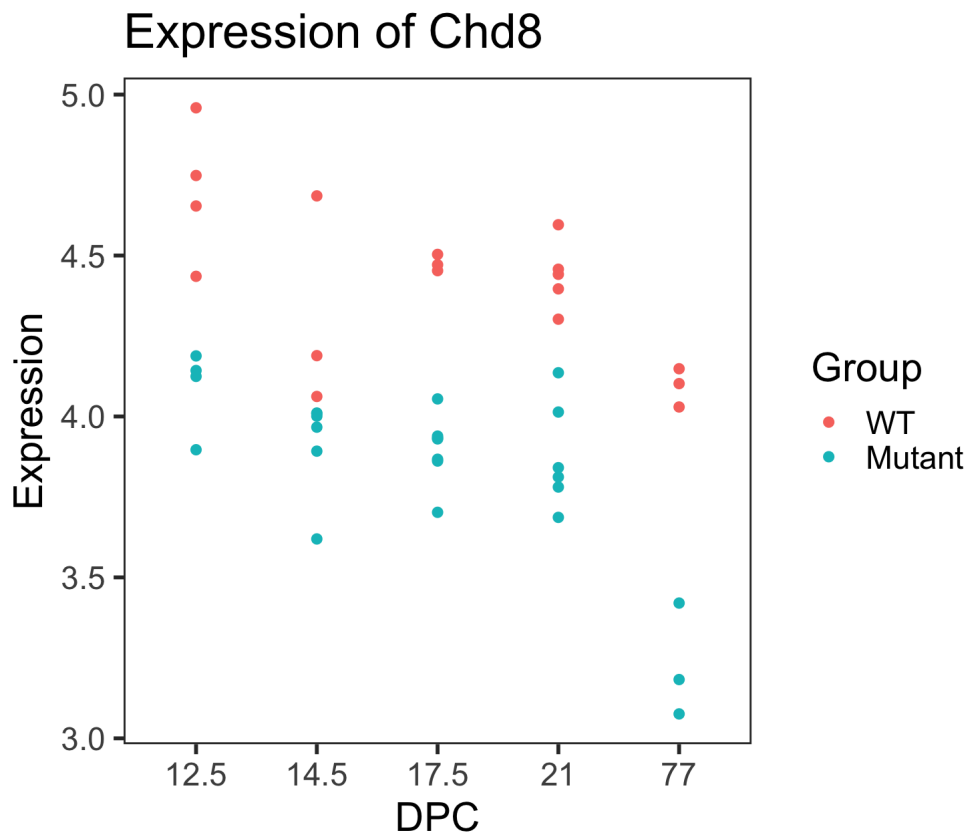


Exploratory Data Analysis (EDA)

- EDA is "compute a little and graph a lot"
- Exploratory plots can be quick and dirty - making them publication quality takes a lot of time and effort
- I'll show a few simple approaches that are common in genomics
- Reminder that code to generate the plots you see here is posted in the notes linked on slide 3

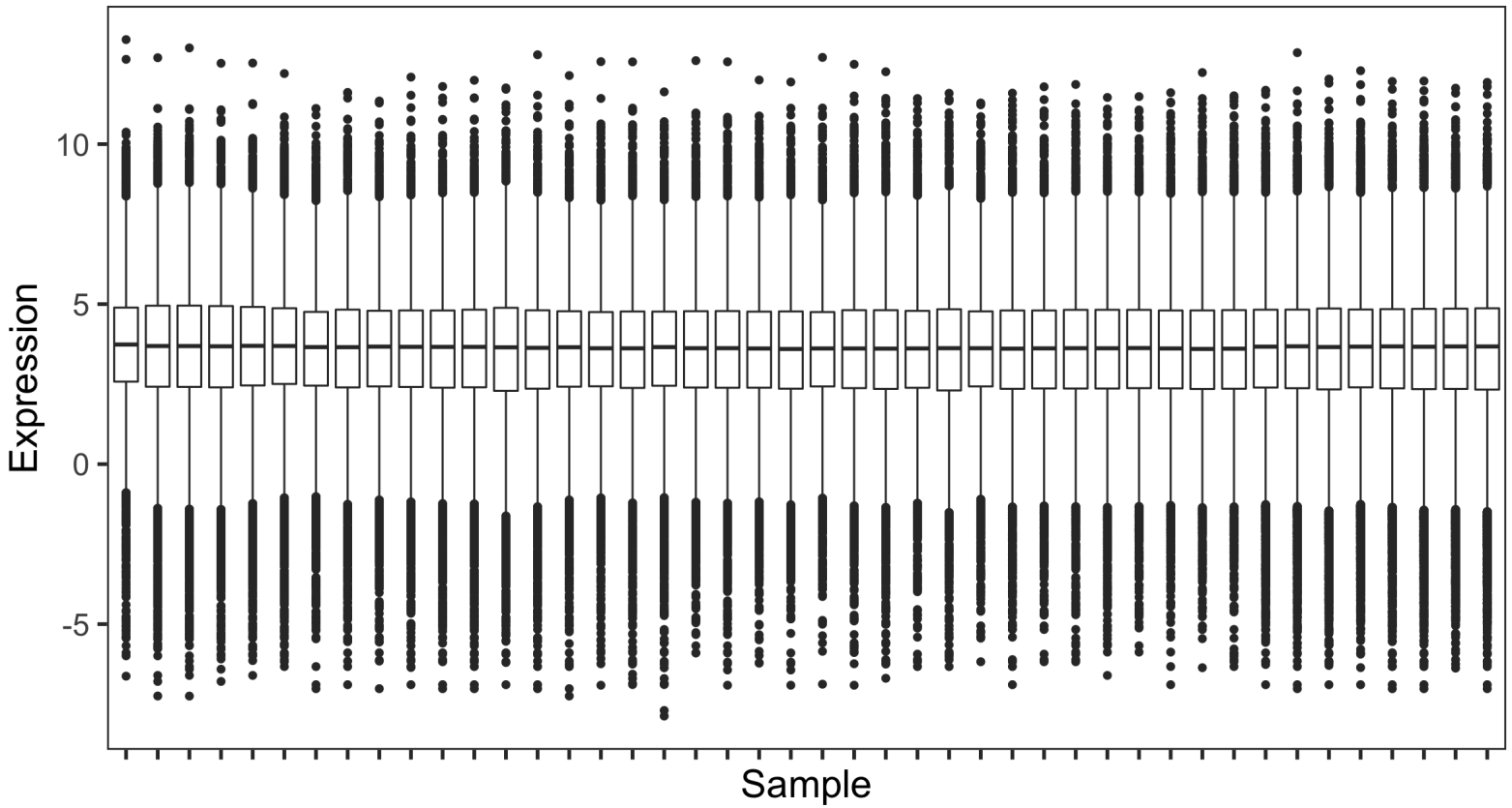
Sanity check: expression of CHD8

- Paper reported that CHD8 went down over time, and is lower in the mutant: confirmed!
- Note that we are not doing any formal "analysis" here nor trying to make this plot beautiful - keeping it very simple for our exploration

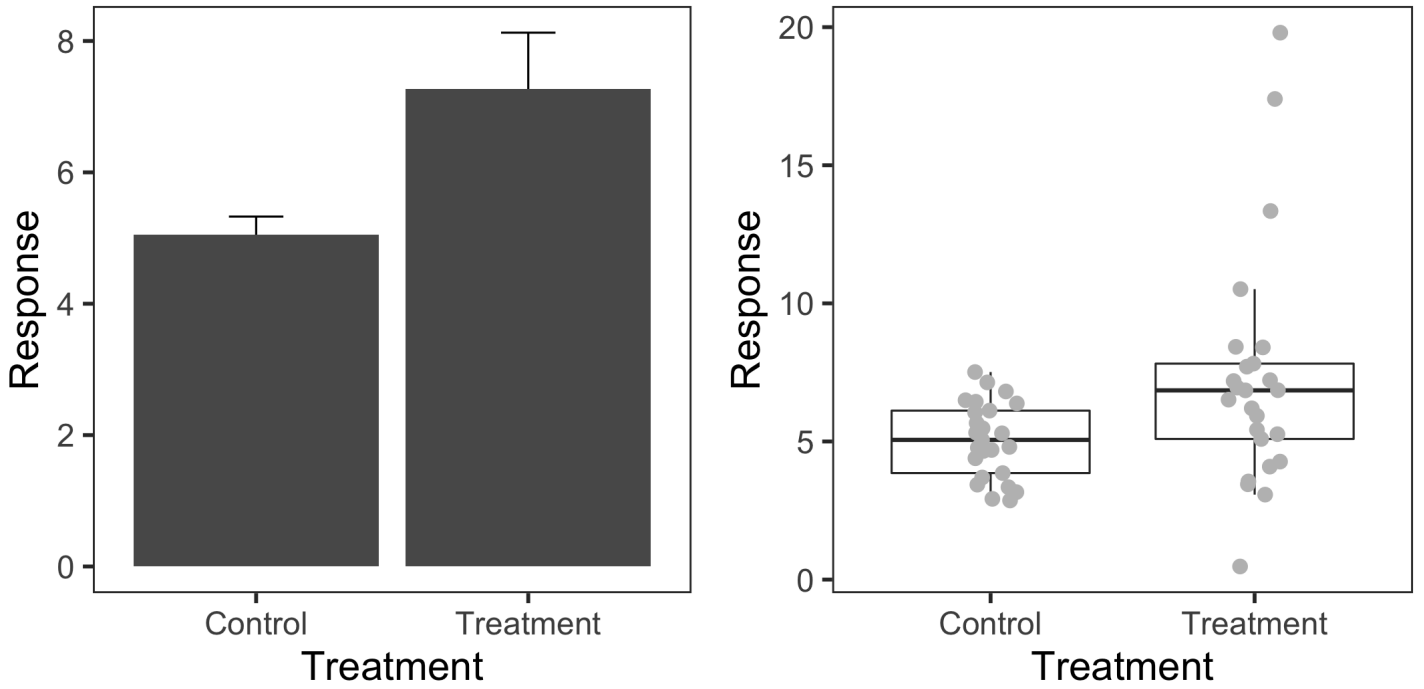


Boxplots to compare samples

- Quick and dirty; reasonable tool to summarize large amounts of data
- Not ideal if the distribution is multimodal
- Don't use box plots (alone) when you have small numbers of points; show the points instead!

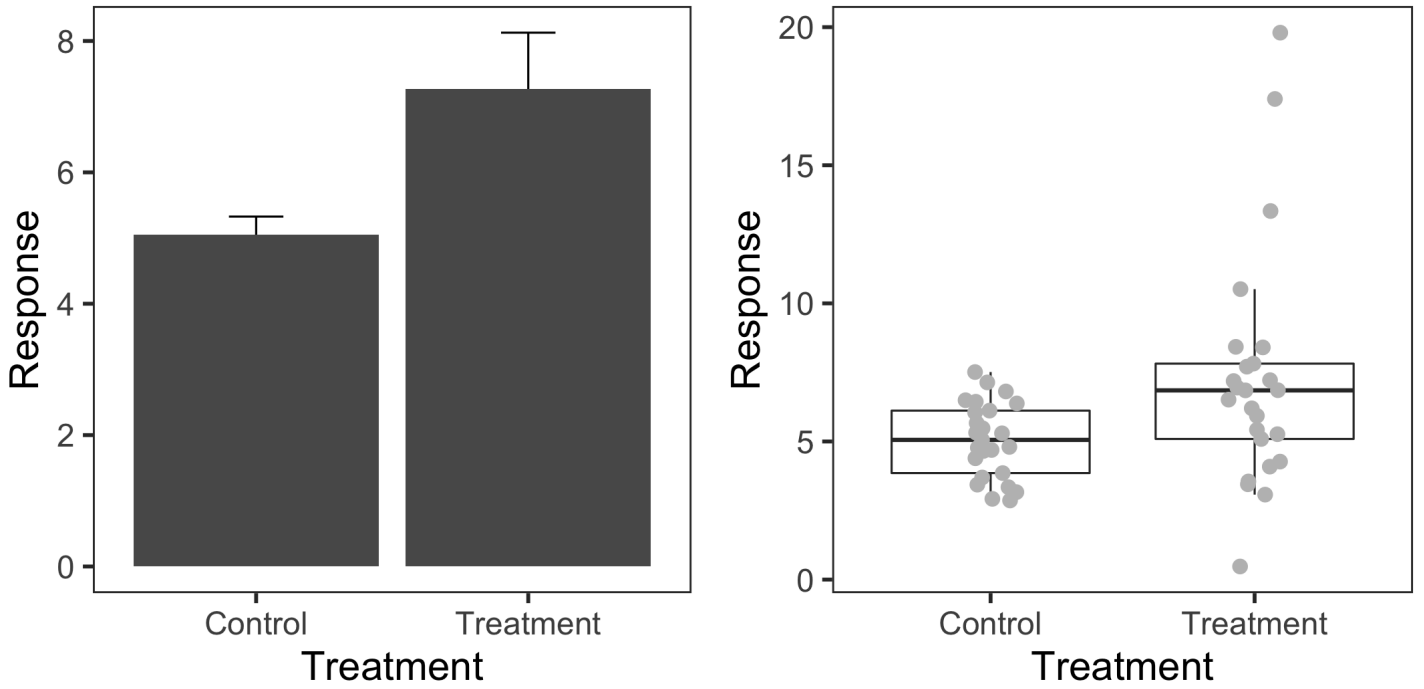


Two views of the same data: Which do you prefer?

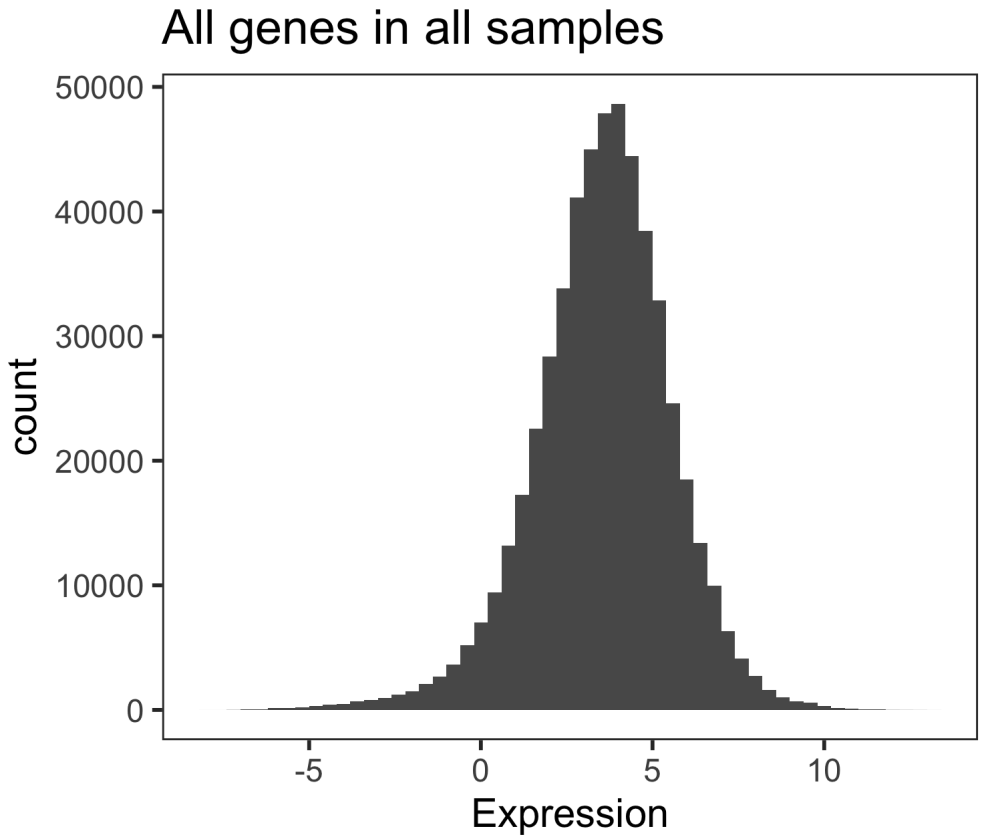


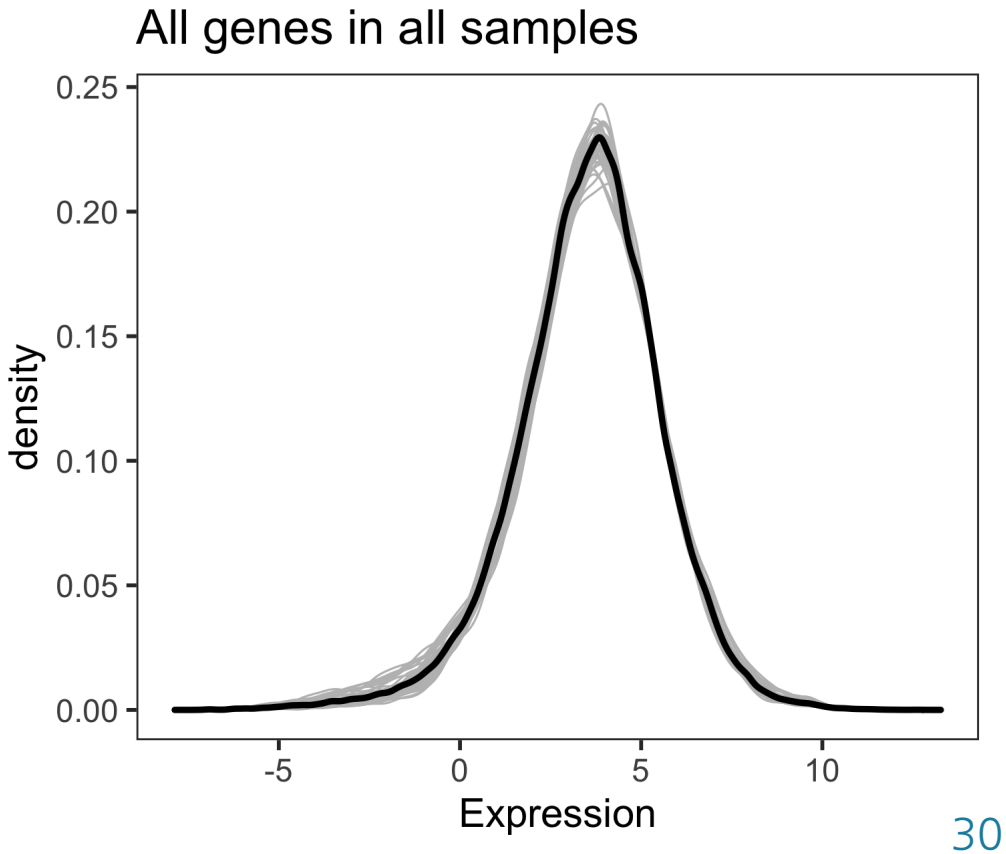
Two views of the same data: Let the data speak for itself!

- What is the sample size?
- Is the distribution symmetrical, or skewed?
- Are there any outliers?

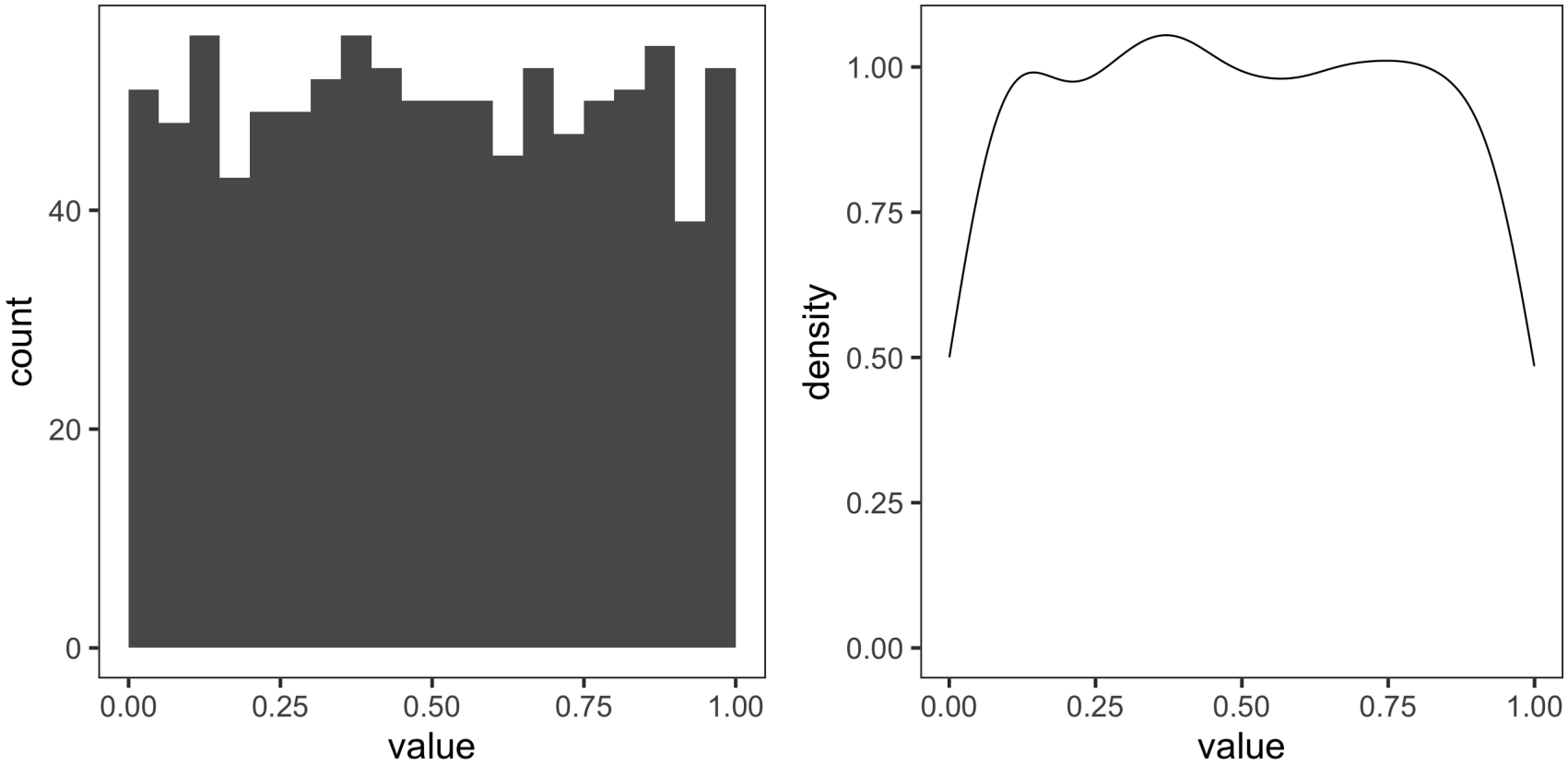


Examining distributions: Histograms and Density plots



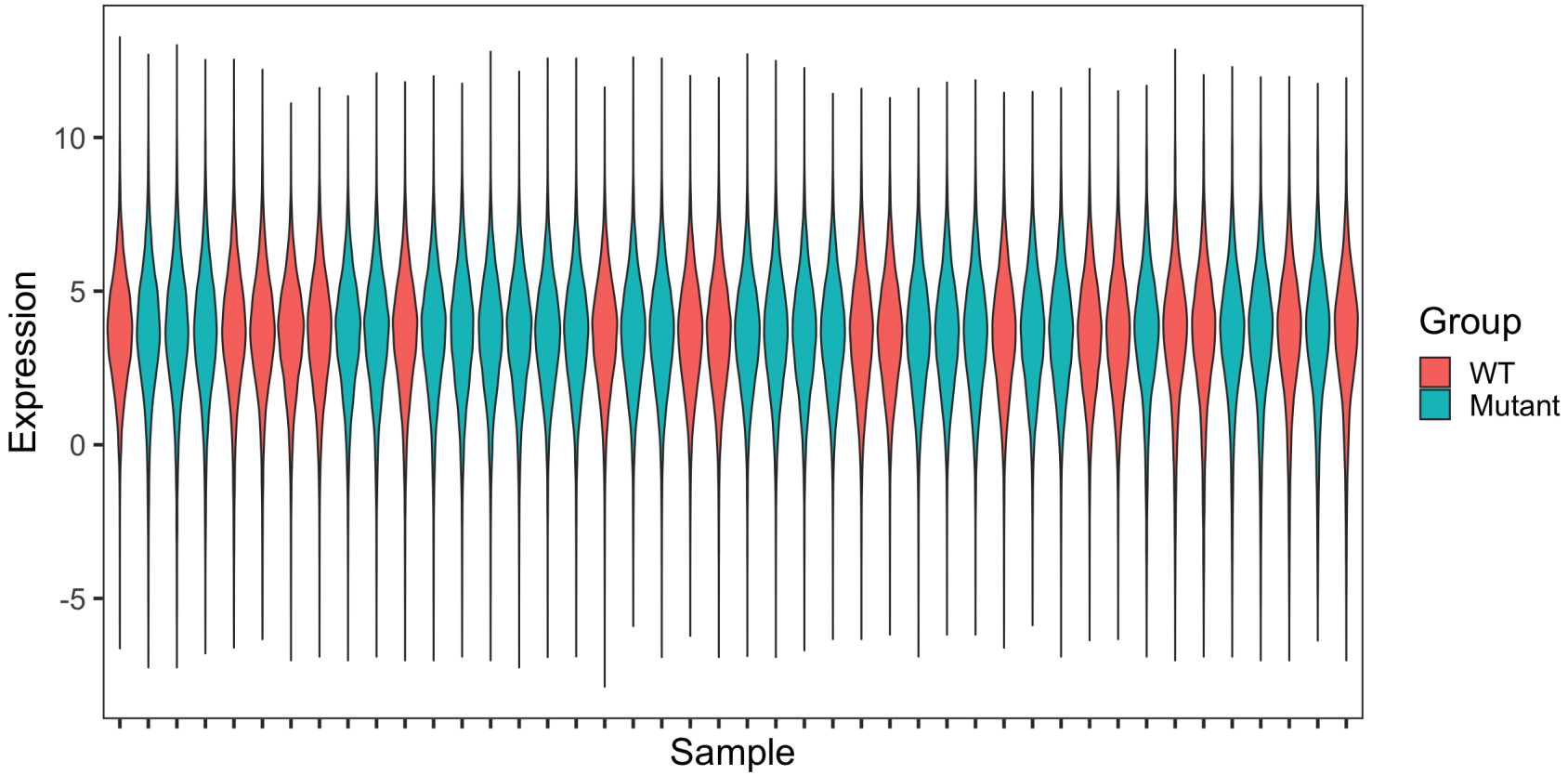


Don't use density plots for bounded data



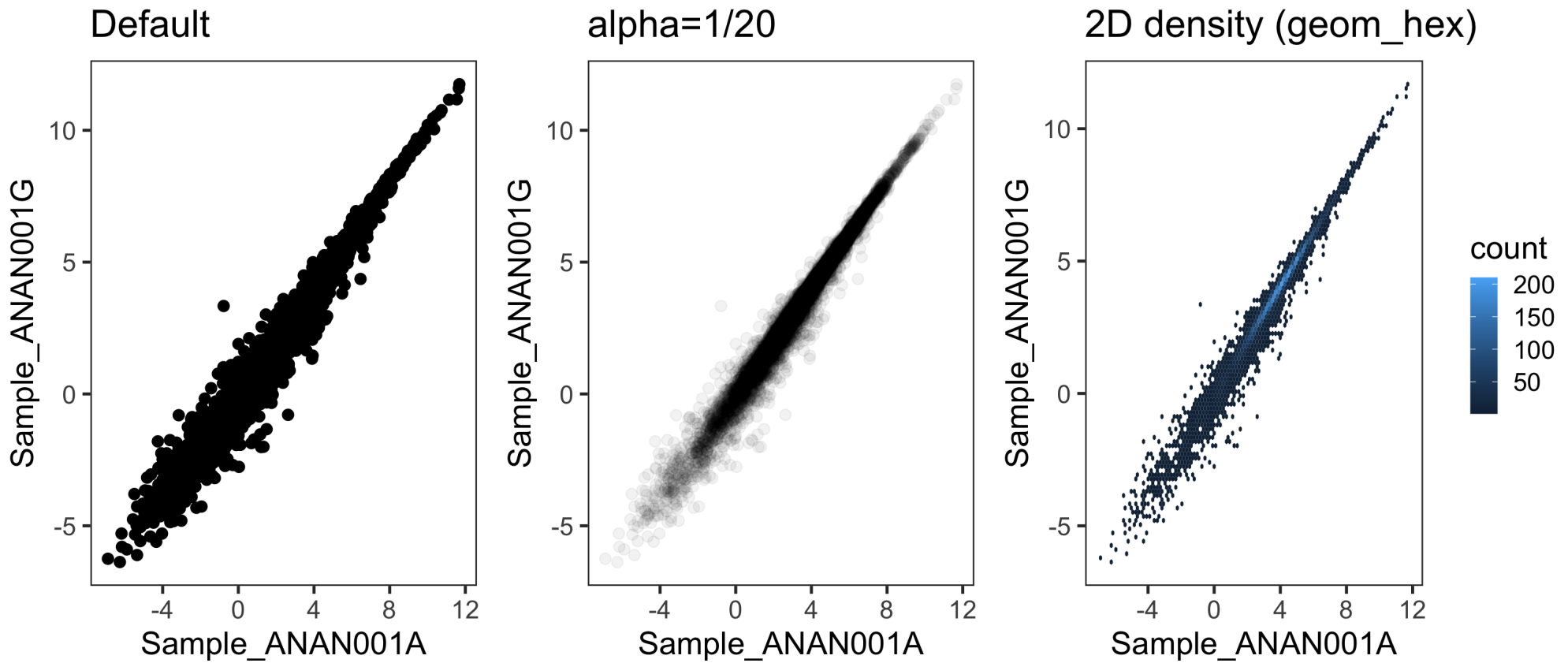
Violin plots

A hybrid of density plots and box plots



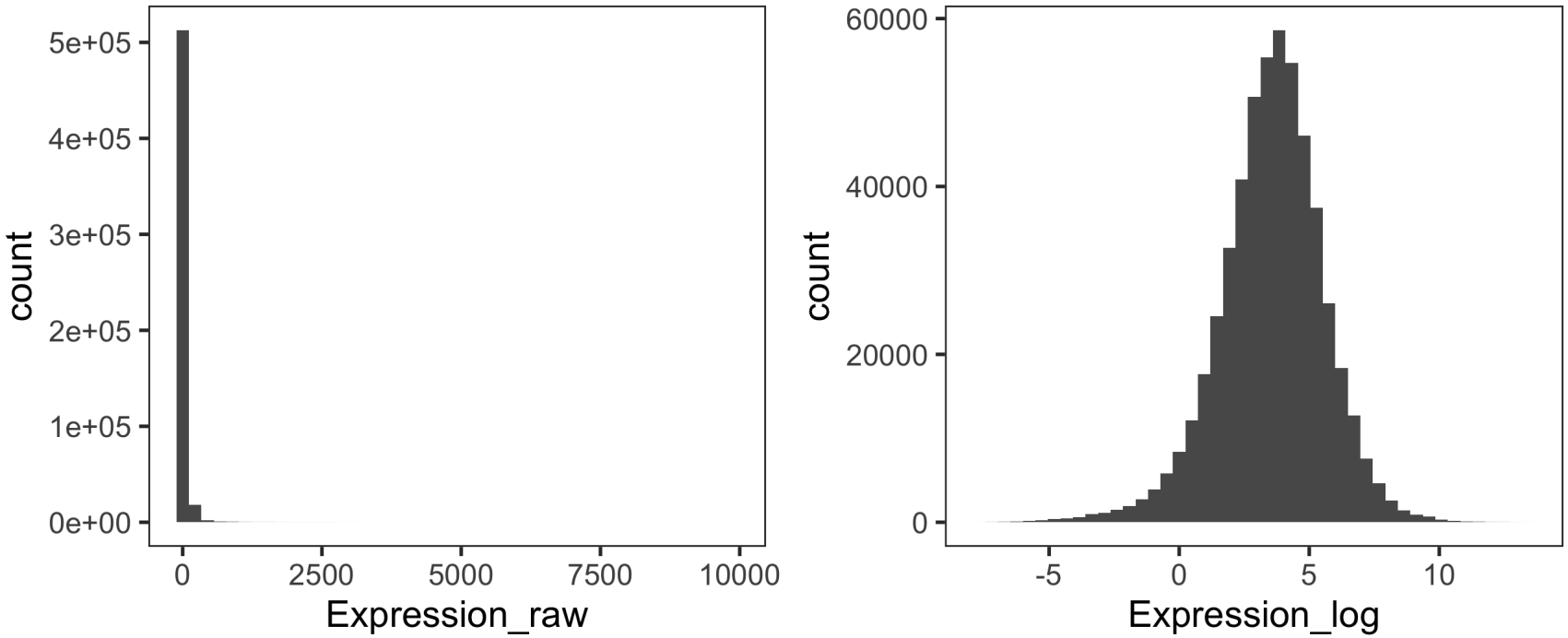
Scatter plots

Major problem is 'over-plotting' - use transparency or 2D density



Transformations

- Many real data have very skewed distributions - in this case, most values are <500, but there are a smattering up to 10,000
- If your plots look like this, try taking the logarithm
- If have non-positive values (typically 0) add a small constant "pseudocount"

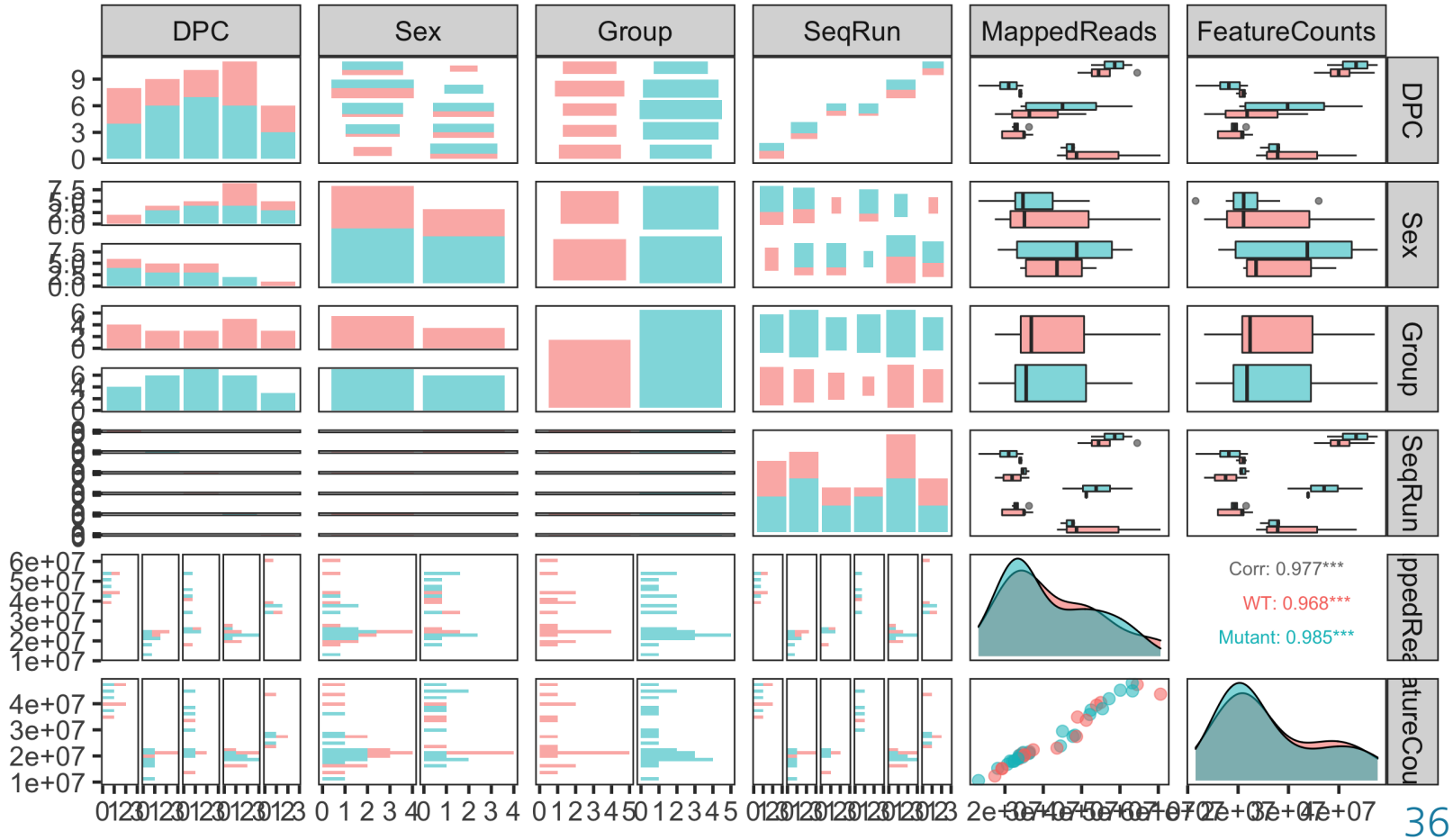


Pairwise (scatter) plots to compare samples

This is nice but unwieldy (or won't work) for large data sets

Examining the metadata more globally

- Sex is not that well balanced; all but one of the adults is male
- There is a batch confound: The stages were run in different batches (except 17.5 was split in two)
- Mapped reads varies with the batches (SeqRun)



EDA Summary

Purpose:

- Sanity checks
- Visualization to spot patterns and/or oddities

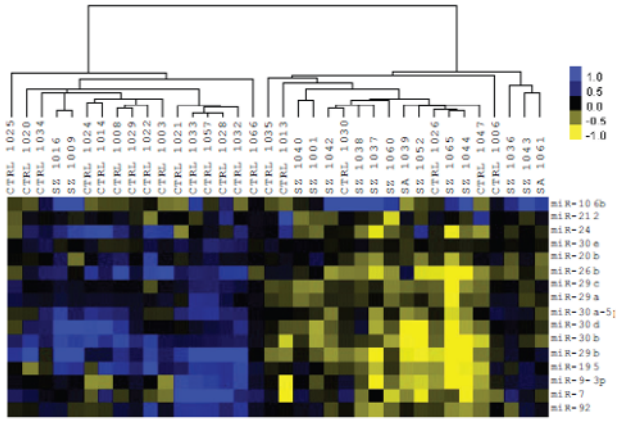
Three principles:

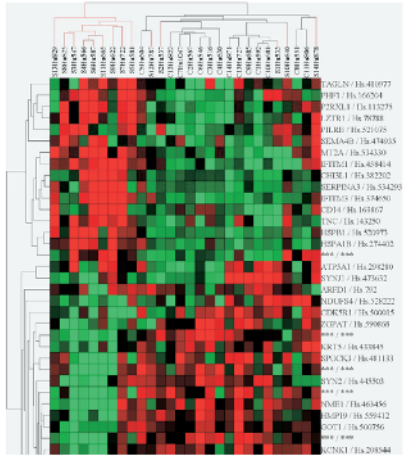
- Let the data speak for itself - avoid dynamite plots in favor of boxplots, overlayed with points if feasible number
- Avoid over-plotting points with transparency and/or 2D density plots
- Consider transformations (e.g. log) for skewed distributions

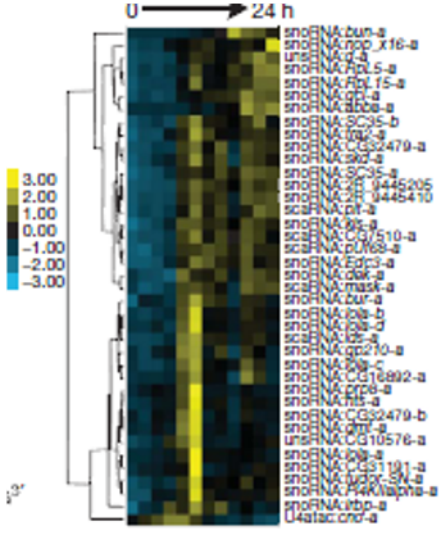
Additional exploratory techniques will be discussed later in the course

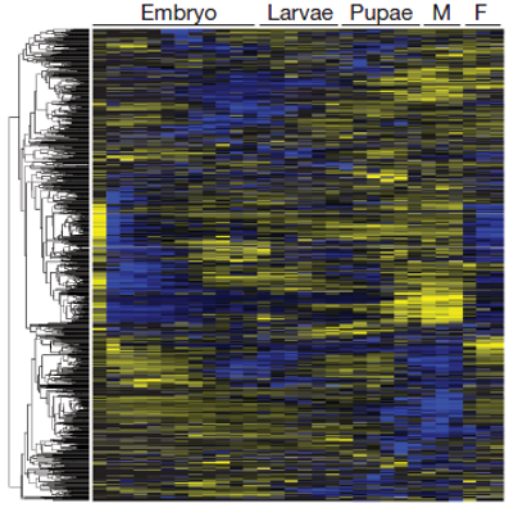
- Clustering
- PCA

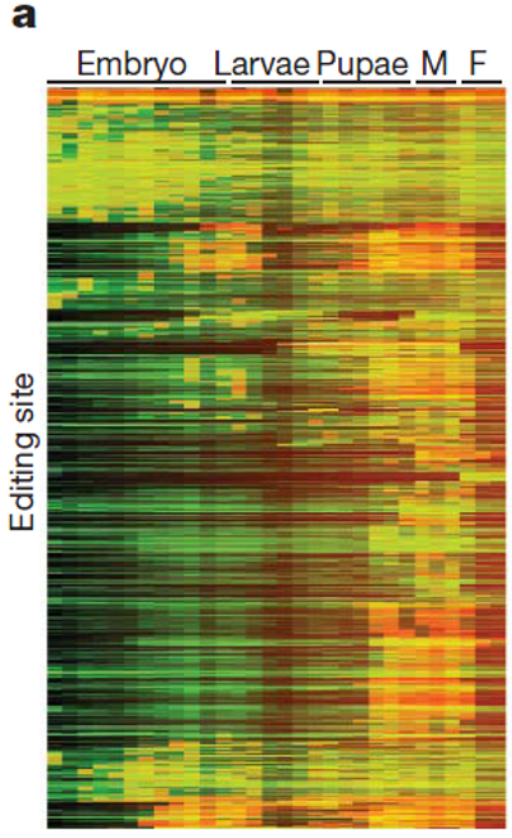
Heatmaps


1.0
0.5
0.0
-0.5
-1.0
miR-10 6b
miR-21 2
miR-24
miR-30 e
miR-20 b
miR-26 b
miR-29 c
miR-29 a
miR-30 a-5
miR-30 d
miR-30 b
miR-29 b
miR-19 5
miR-9- 3p
miR-7
miR-92




0
24 h
3.00
2.00
1.00
0.00
-1.00
-2.00
-3.00

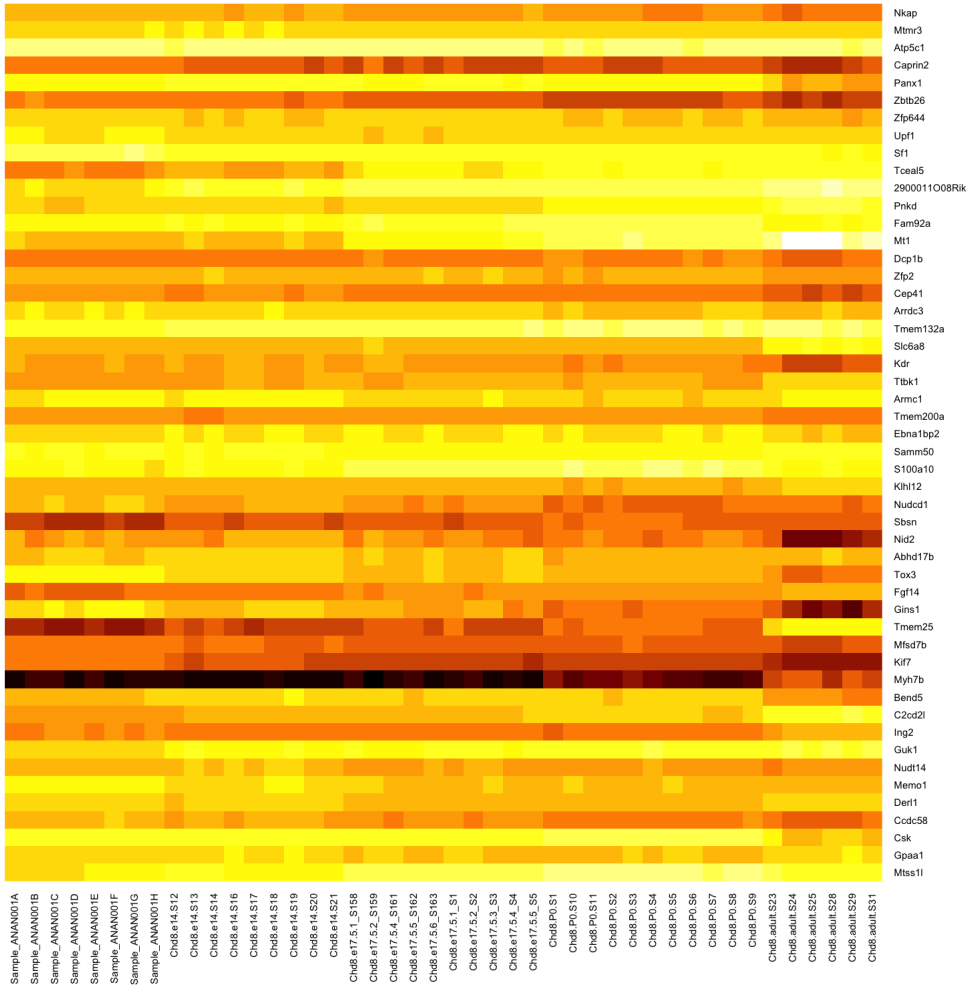

Embryo
Larvae
Pupae
M
F


a
Embryo
Larvae
Pupae
M
F
Editing site

R options for making heatmaps

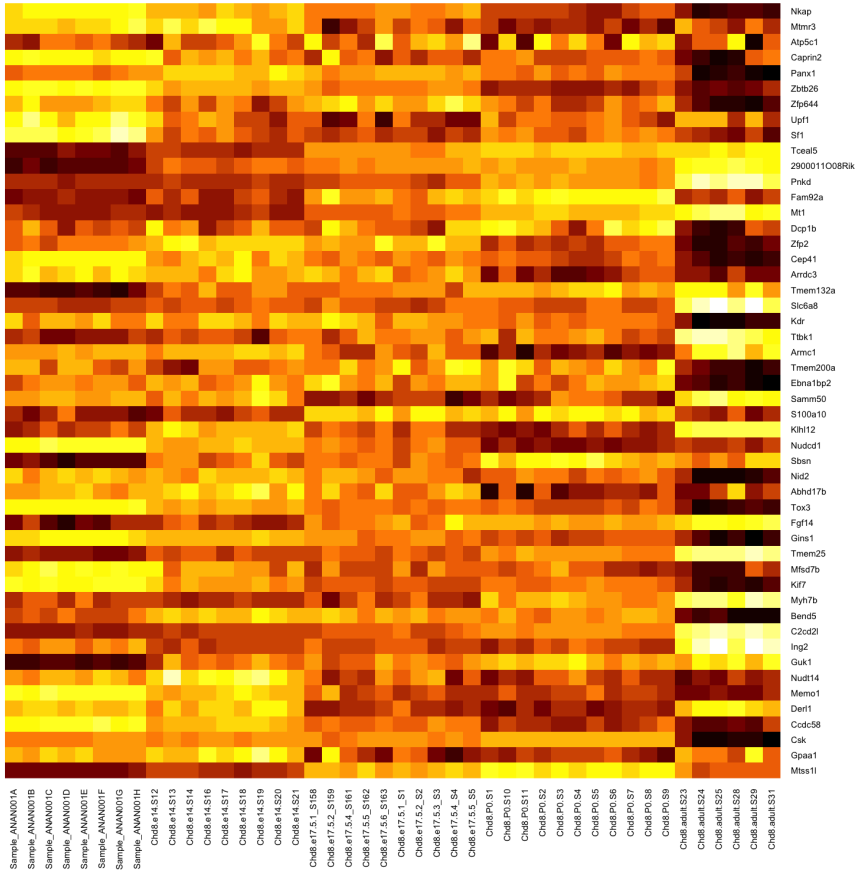
- Desired features
  - Doesn't complain when handed slightly messy data and has sensible default behaviour
  - Allow easy control of layout such as annotations and scale bar
  - Allow easy control over clustering behaviour (row/column ordering)
- There are *many* functions/packages for making heatmaps; here are three
  - base::heatmap - ok for quick and dirty but otherwise very limited
  - pheatmap - Default colour schemes not ideal, otherwise good option (used in STAT 540 seminars)
  - ComplexHeatmaps - most powerful/flexible, but a bit more complex to learn

Heatmap of 50 random rows



Revision 1: Same input data; rows are scaled

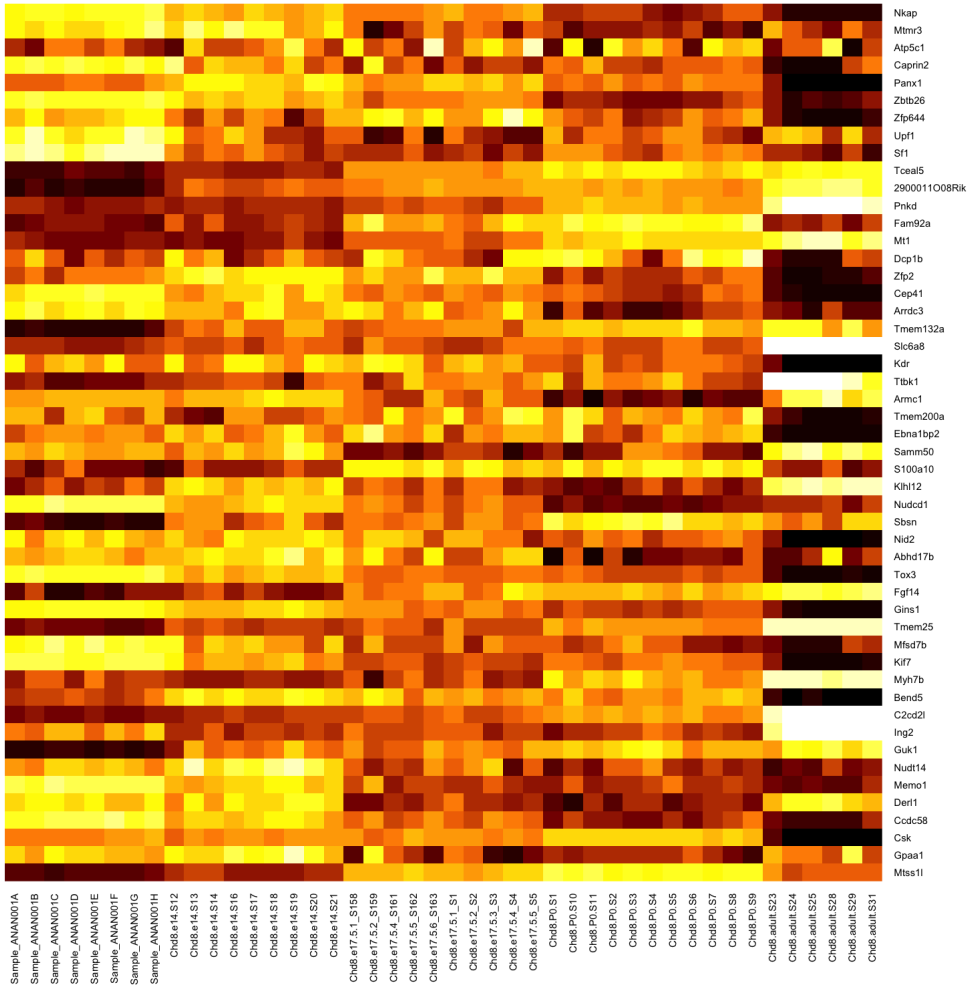
- Rows are scaled to have mean 0 and variance 1 (z-scores)
- Subtract the mean; divide by the standard deviation - use `scale()` on the data rows (*some packages will do this by default*)
- It is now easier to compare the rows and see a bit of structure



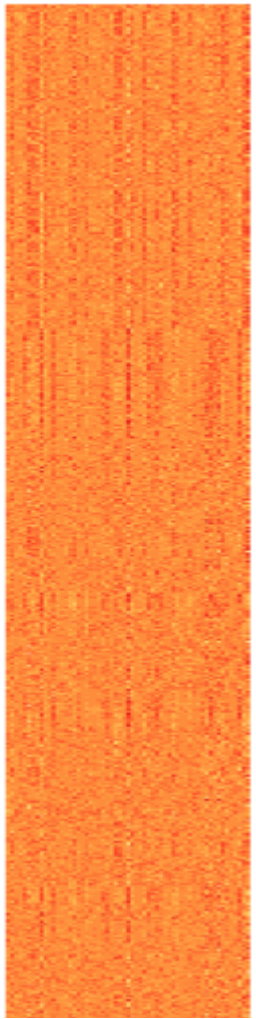
Revision 2: adjusting contrast

- Range of values is clipped to (-2,2): aything more than two SDs from the row mean is set to 2
- Limit values of 2 or 3 SDs are common

Clipping hides outliers but allows us to see variation in the bulk of the data

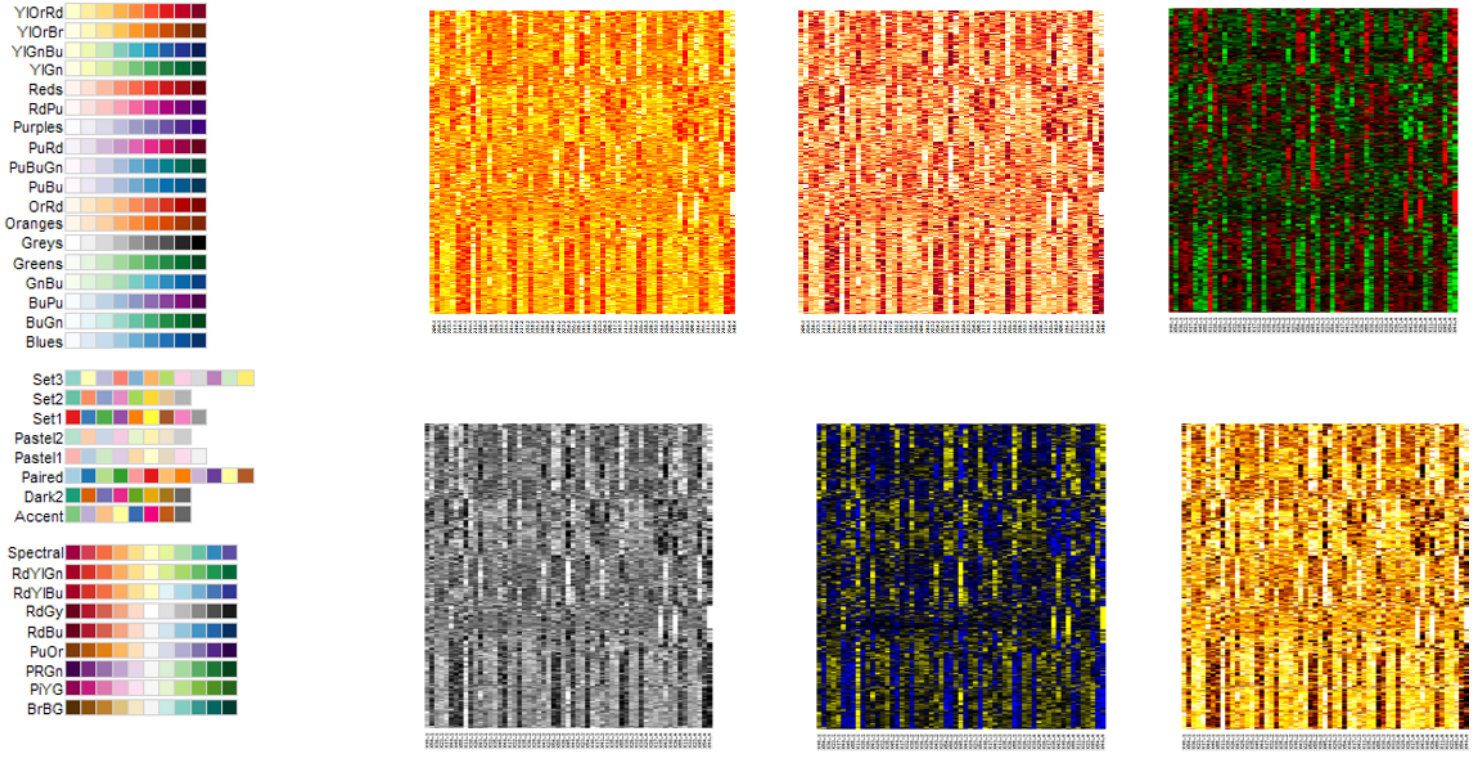


Plotting too much data



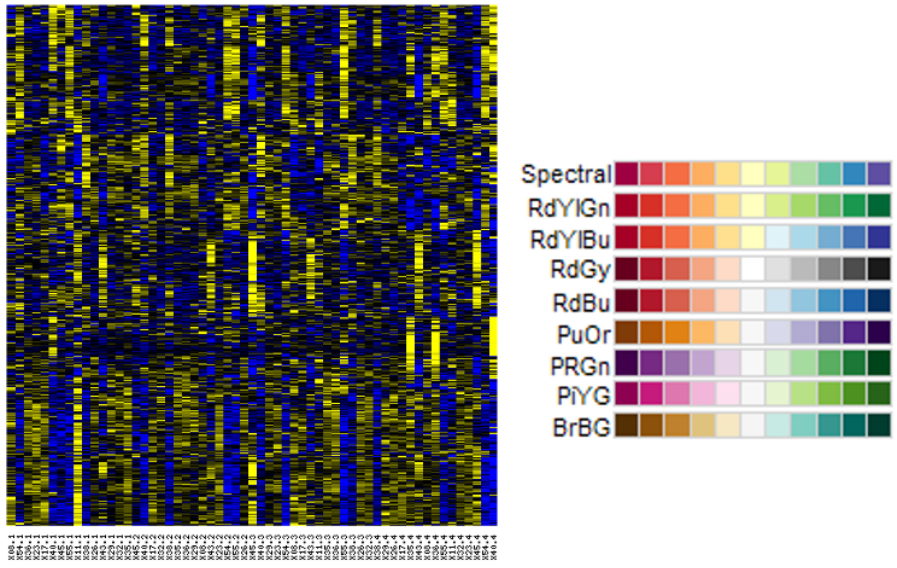
- An entire data set (>10k rows)
- If the cells are less than 1 pixel, everything starts to turn to mush and can even be misleading
- If your heatmap has too many rows to see labels (genes), make sure it is conveying useful information (what are you trying to show?)

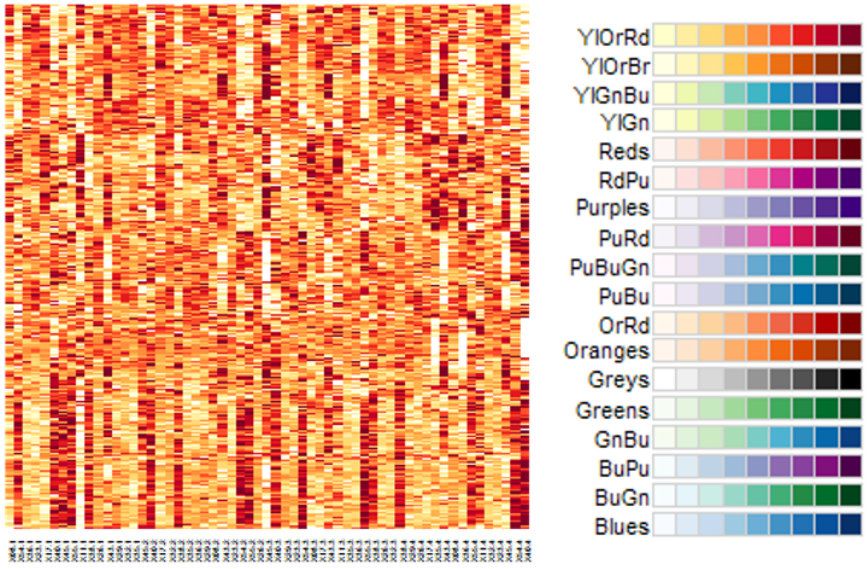
Choice of colours



- Humans can't really tell the difference between a 8- and 16-colour scale
- Many R defaults, other common scales (green/red) *not colourblind friendly*
- `RColorBrewer`: scales based on work of visualization experts
- Greyscale: loss of dynamic range, but cheaper to publish!

Divergent vs. sequential maps





Divergent: colours pass through black or white at the midpoint (e.g. mean). Ideal if your original data are naturally "symmetric" around zero (or some other value) - Otherwise it might just be confusing

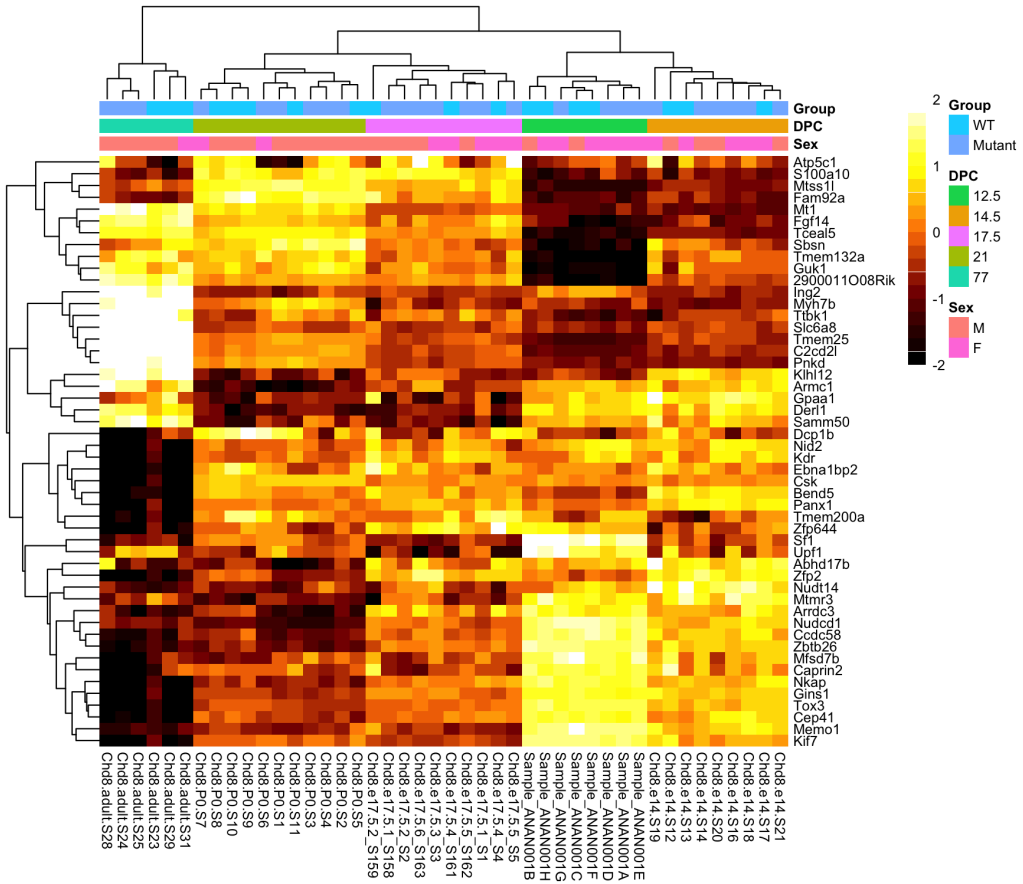
Sequential: colours go from light to dark. Darker colours mean "higher" by default in `RColorBrewer`. No defined midpoint.

A confusing heat map (Matlab)

- Too many different colours to readily interpret relative ordering of values
- Not recommended to use these types of scales for continuous values
- Rainbow or other scales with many distinct colours are better for factors/categorical variables

Heatmap recommendations

- Pick an appropriate colour scale
- Show a scale bar (so don't use `base::heatmap`)
- Either cluster rows / columns, or order by something meaningful (e.g. sample info)
- Add annotation bars of meaningful covariates
- If you have missing data points, make sure it is obvious where they are (e.g. different colour)



Experimental design and quality

Experimental design considerations

- Will the experiment answer the question?
  - Consider properties of the measurement technology as well as the biology
- How many individuals should I study?
  - For many experiments, this is hard to answer (*even though your grant reviewer might demand it (power calculations)!*)
  - Often settle for "enough power to find something useful" rather than "find everything"
- Biological replicates are essential and more important than technical replicates (usually)
  - Doing a study with too few replicates is arguably worse than not doing the study
- Beware of (and try to control for) unwanted variation
  - "Pooling" samples is not a substitute for replication
  - E.g. if your question is "do people respond to a drug", the unit of analysis must be Person, not Group of people

Data quality considerations

General types of issues:

- High technical variability
- Outliers
- Batch artifacts (or other systematic trends)

Effects on the data:

- Some will yield false positives (confounds, "false signals")
- Others will yield false negatives (by decreasing signal-to-noise)

Consistency over perfection

- Assuming your focus is on comparing samples to each other in some way, the variance of a QC measure is at least as important as its mean
- Be prepared to remove samples to tighten up the spread in quality (removing outliers)
- Though, if most samples are poor by objective criteria, you might want to start over
- Signal to noise ratio is more important than amount of noise

Filtering your data

- Here I mean "Removing part of the data from a sample" and doing that to all samples
- In many studies, especially gene expression, it is common to remove genes that have no or very low signal ("not expressed")
- Similarly: Common to remove data that have "too many missing values" compared to other samples (if you actually have missing values)
- Deciding what to remove is often not straighforward, but make a principled decision and stick with it (see next slide)

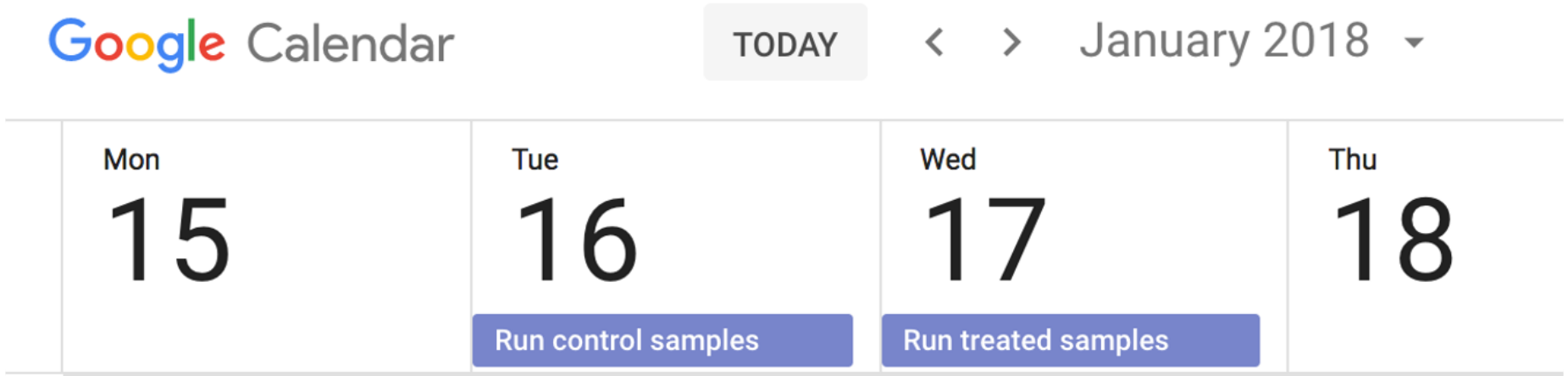
Filters must be “unsupervised”

- Filtering strategy should treat all samples the same
  - Don't apply one filtering strategy to treated samples and another to controls
  - This could bias towards retention of genes that have differences between conditions
- Filter strategy should be decided up front; do not apply iterative filtering
  - Once you filter the data, you are stuck with it. Don’t be tempted to go back and change it based on downstream results
  - For example: "I filtered out 30% of the mostly lowly experessed genes, but then I didn’t get any cool results in my analysis, so I was worried I may have filtered out some good stuff, so I went back and filtered at a less stringent 10%" = Data Dredging or p-hacking

Batch effects

"Batch effects are sub-groups of measurements that have qualitatively different behaviour across conditions and are unrelated to the biological or scientific variables in a study"

Leek et al. 2010 Nature Rev. Genetics 11:733



- Magnitude of batch effects vary
- Consider correcting for them if possible - batch artifact detection and correction will be covered in more detail in a later lecture

Avoiding batch artifacts

- Don't samples run in batches - Confounding (can be hard to avoid)
- Balance or randomize design with respect to batches
- Avoid (or at least record) obvious potential sources of artifacts, such as a new tube of a reagent, or a different person doing the bench work
- Run some technical replicates across your batches

```
table(se$DPC, se$SeqRun)
```

```
##
##         A  B  C  D  E  H
##   12.5  8  0  0  0  0  0
##   14.5  0  9  0  0  0  0
##   17.5  0  0  5  5  0  0
##   21    0  0  0  0 11  0
##   77    0  0  0  0  0  6
```

Outliers

Hard to define. To some extent, you'll know it when you see it.

- "A sample that deviates significantly from the rest of the samples in its class"
- "An observation differing widely from the rest of the data."
- "A data point notably further out from the central value than the others. Outliers invite explanation as observational errors, or intrusions from another set, or something of that sort."
- "... a value that lies 1.5 IQR beyond the upper or lower quartile"

Features (e.g. genes) are not usually called “outliers” in the sense of “should remove from the data”

Recommendation for outliers

- Make sample sizes large enough that your study won't fail if you have a couple of outliers to remove
- Don't freak out: a mild outlier in a well-powered study probably won't cause a lot of problems
- Develop criteria for deciding what is an outlier and stick with it
- Once outliers are removed, they stay removed

Identifying outlier samples

- Relative vs. absolute quality is important
- Usually, we might consider a sample an outlier if (relative to others):
  - It has "very low" signals
  - "High" background
  - "Low" correlation with most (or all) other samples from the same group
  - There may be technology-specific factors
- If a sample is questionable, we might ask: Is there anything in the notebook that would make us suspect it? (e.g. "Sample dropped on floor") - this will help justify decisions to remove a sample beyond supposedly objective criteria like ">1.5 IQR"

A basic but effective outlier detection method

A heatmap of the sample-sample correlation matrix (generally a useful diagnostic plot)

Expect correlations to be tighter within experimental groups than across groups

- There are no firm guidelines for evaluating this … but you could apply a rule like “out of >1.5 IQR”
- In practice, outliers are often pretty obvious