Some (probably naïve and simplistic) early morning thoughts on statistics, and RNAseq.

Titus Brown

Sampling M&Ms from very large boxes of them.

Setup 1: you have two trunks of M&Ms

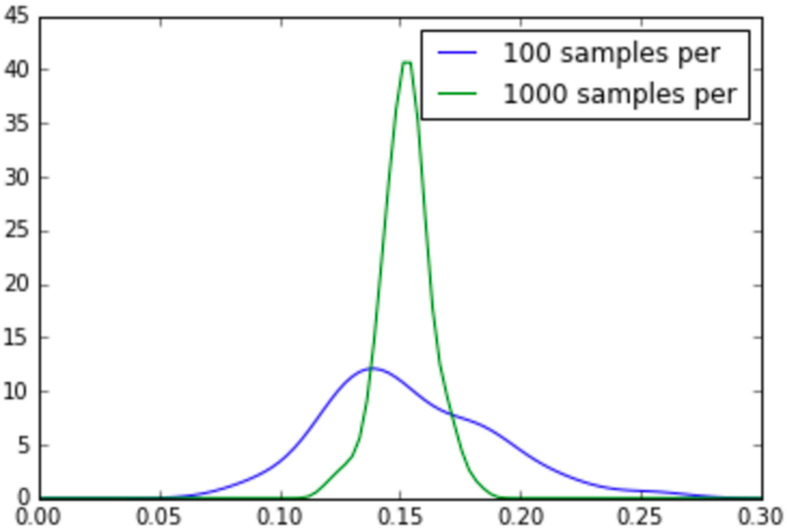
- You want to know which trunk has more blue M&Ms
- …but it costs you money/time to sample from each trunk.

Assumptions:

- Trunks are big enough that you're sampling with replacement
- Trunks are well mixed.

Conclusion from 1st set up --

The deeper you sample, the lower your relative variation across replicates.



Setup 2: you want to know which M&Ms are more or less prevalent between trunks

Now ***multiple testing*** becomes a problem –

If you have 10 colors you're looking at,
And a false-positive rate of 0.2 on your statistical test,

roughly 2 of your "different prevalence" calls will be wrong.
(Similar reasoning applies to false negative rates.)

Setup 3: different trunk-producing factories.

- You can order trunks of M&Ms from two different factories.
- You believe that the factories are producing different color M&Ms at different rates.
- You want to know which M&Ms are being produced at different rates.

Each factory has a different ”true” rate;

Each trunk is produced according to that “true” rate but each trunk is a somewhat inaccurate sampling of that true rate;

Setup 3 complicates your sampling strategy!

How deeply do you sample M&Ms from each trunk, vs how many different trunks do you sample? (since it costs extra money to get each trunk – shipping charges!)

(This is separate from how many times you sample M&Ms from each trunk! => technical replicates)

Only in the last case, are you doing science…

- First case, you have an n of 1 (you’re looking at only one trunk).
  - What if the factory was having a weird day? How would you know?

- Second case, you’re ***still*** only looking at one trunk.

- Third case, you can look at ***technical*** and ***experimental*** replicates both, and make a statistical argument.

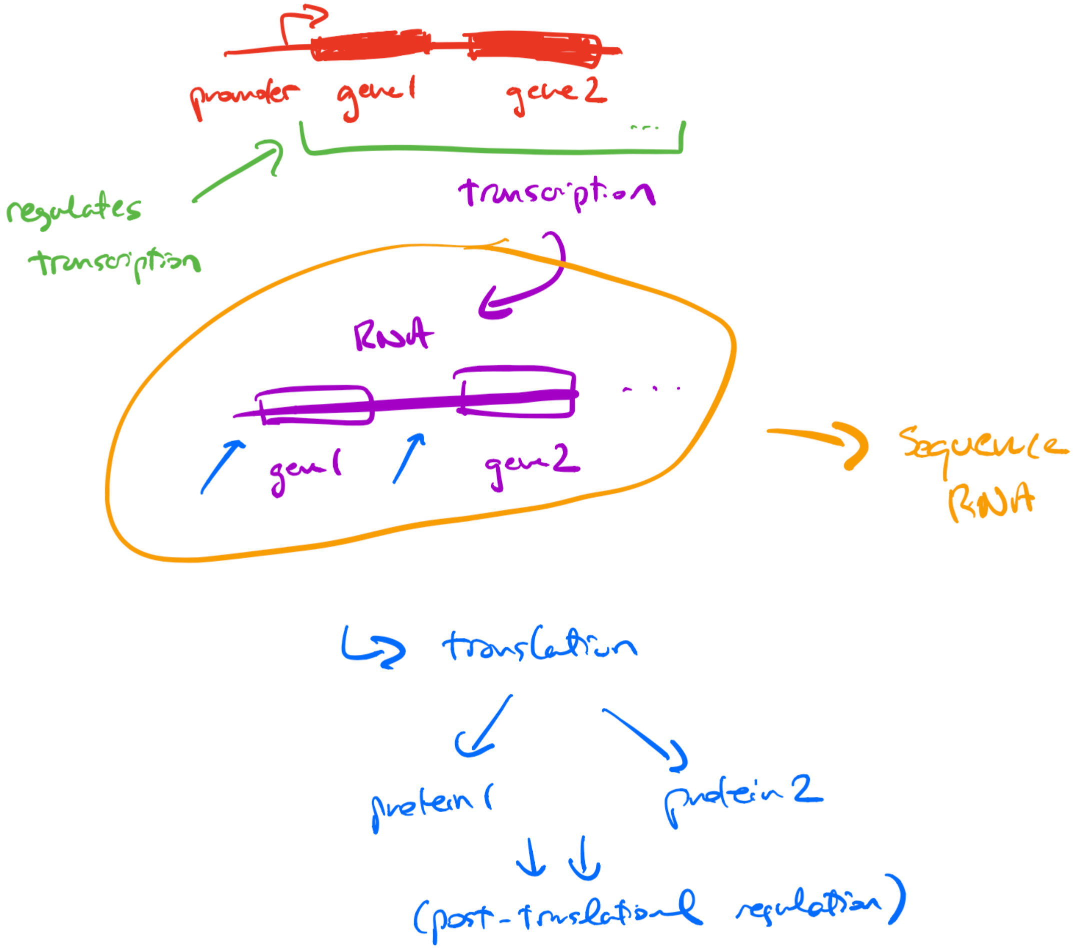
You still have to worry about ***batch effects.***

- E.g. Maybe you have different people sampling the trunks on different days, and those people have biases in how they’re sampling.

Hypothesis testing vs exploratory analysis.

- Are you doing reaching conclusions, or do you plan on doing followup studies to understand your results?
- In the first case, you need to make a conclusive statistical argument (replicates, etc.) about the distributions of M&Ms being generated by the factory.
- In the second case, you only need to say “hey, it looks like white M&Ms are more abundant in factory 2 – let’s go visit and see if we can understand why.”

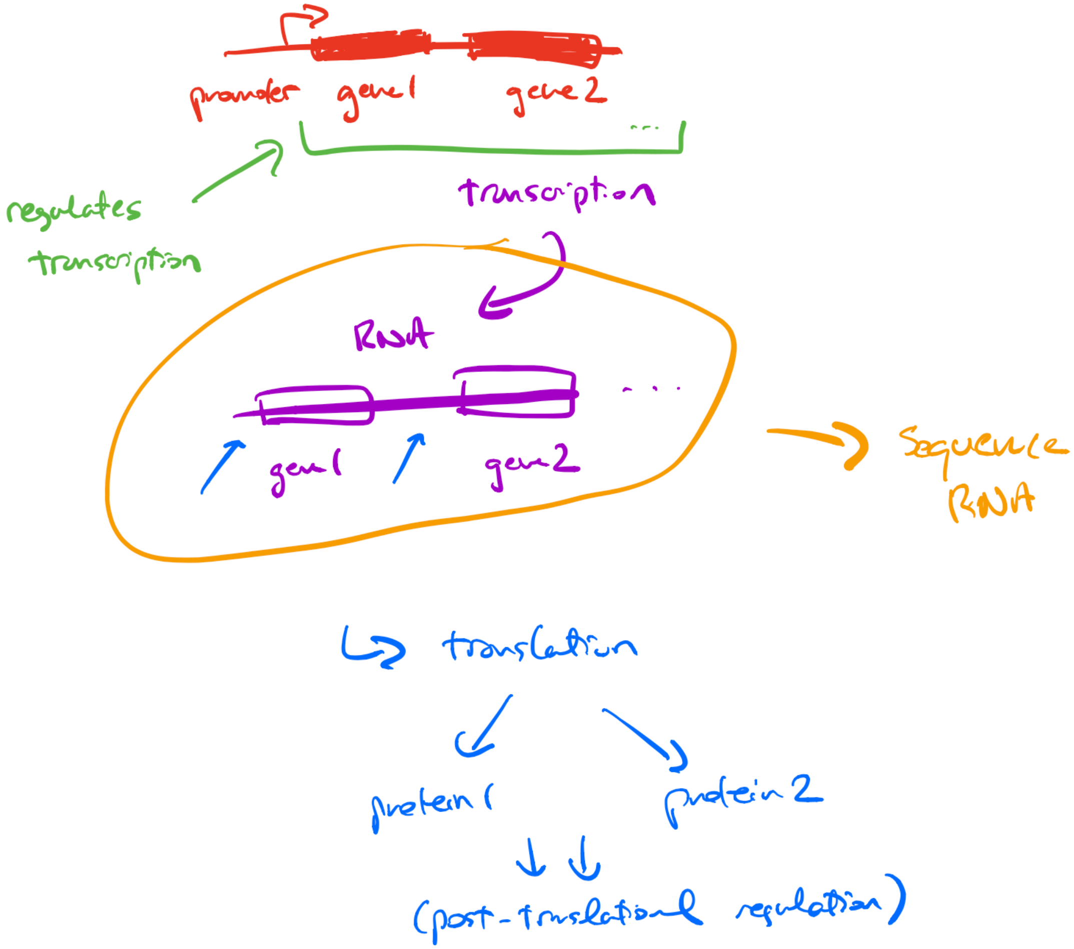
(Most statisticians speak about the *former* in papers.)


promoter
gene 1
gene 2
...
regulates transcription
transcription
RNA
gene 1
gene 2
...
Sequence RNA
translation
protein 1
protein 2
(post-translational regulation)

Making the analogy:

- Each M&M color is a gene.
- Each M&M count is a gene count.
- Each trunk of M&Ms is a collection of cells.
  - & each sampling of a trunk is a technical replicate.
- Each factory is a different experiment (biological replicate).

- Batch effects: different days; different “hands”; different water; different kits; different sequencing core; …anything *systematic.*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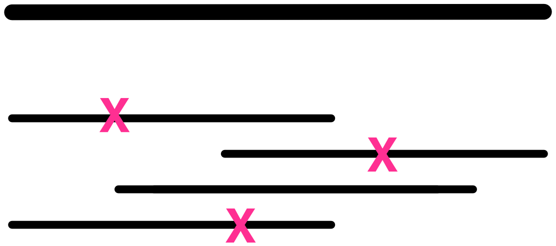
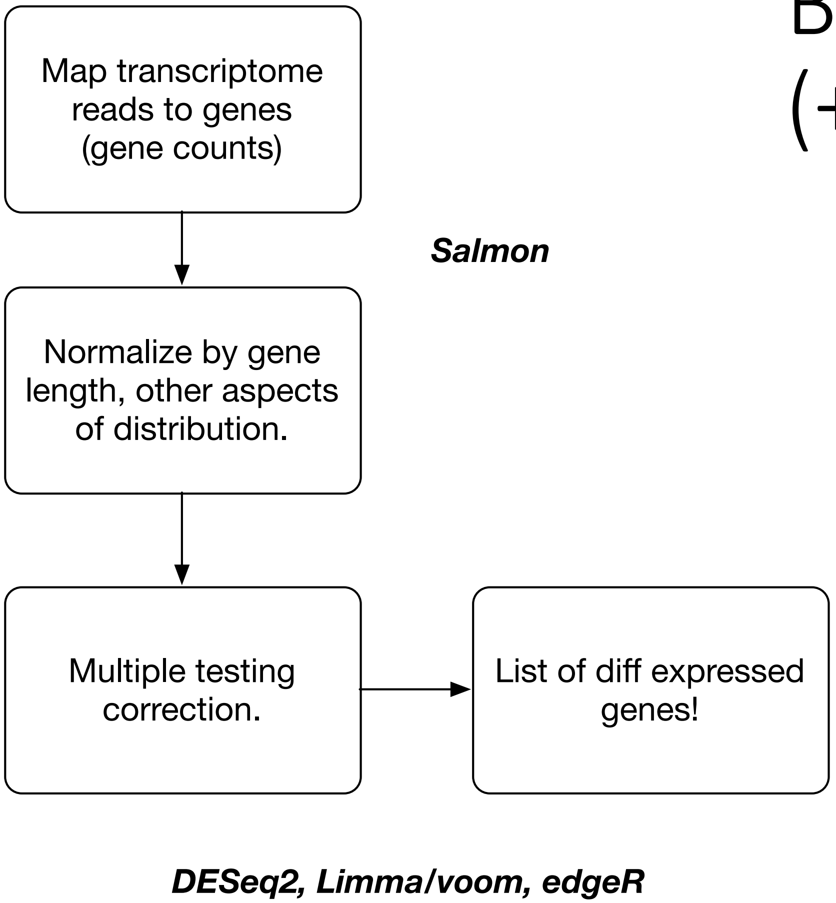
Counting with sequencing

We have a reference gene set and want to know how *much* we have. So, you "map" reads back to reference and count.



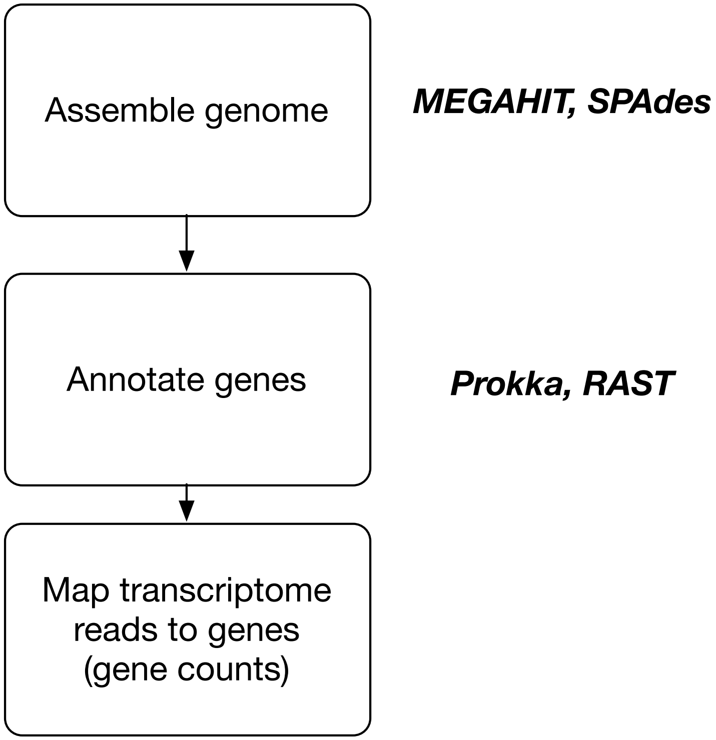
Basic RNAseq procedure (+ tools)


Map transcriptome reads to genes (gene counts)
Normalize by gene length, other aspects of distribution.
Multiple testing correction.
List of diff expressed genes!

Salmon

DESeq2, Limma/voom, edgeR

…doing RNAseq on a new genome:



Assemble genome

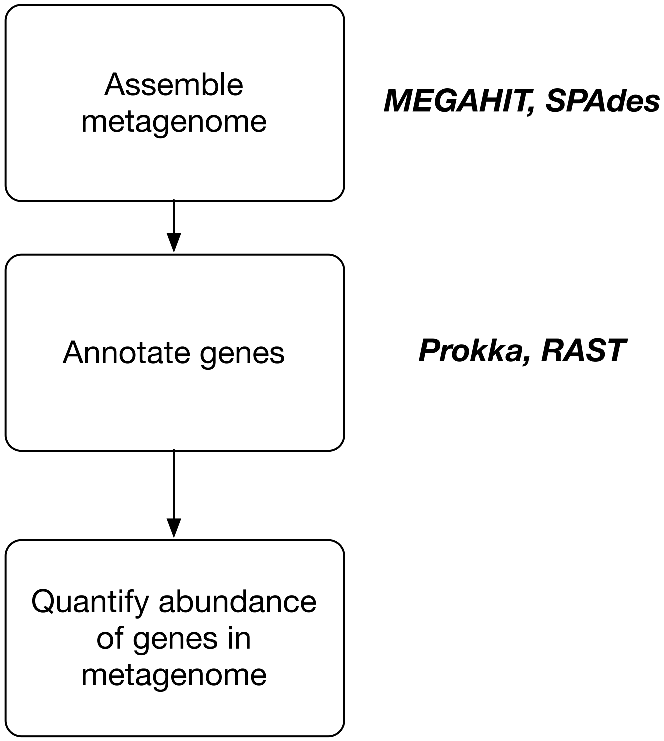
MEGAHIT, SPAdes

Annotate genes

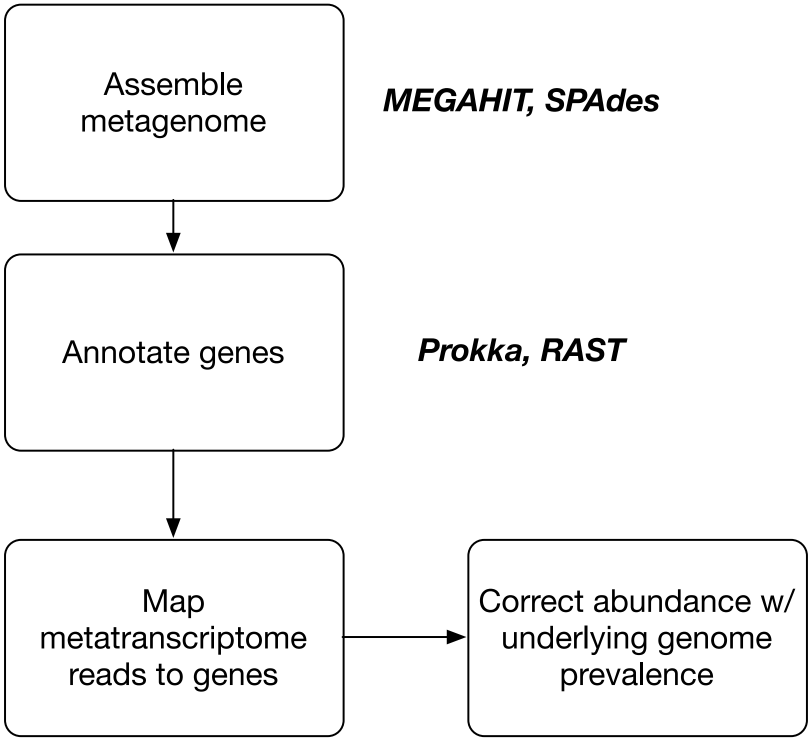
Prokka, RAST

Map transcriptome reads to genes (gene counts)

…counting genes in a metagenome:



…doing RNAseq on a metatranscriptome:



Assemble metagenome

MEGAHIT, SPAdes

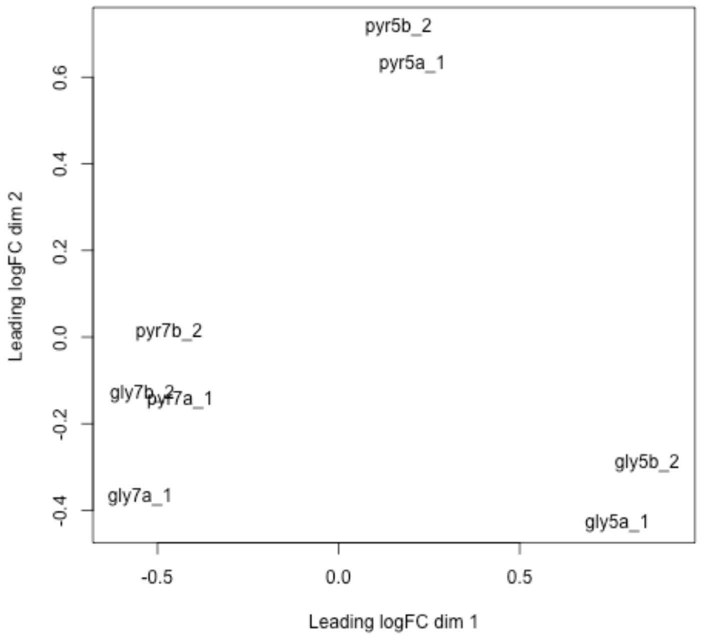
Annotate genes

Prokka, RAST

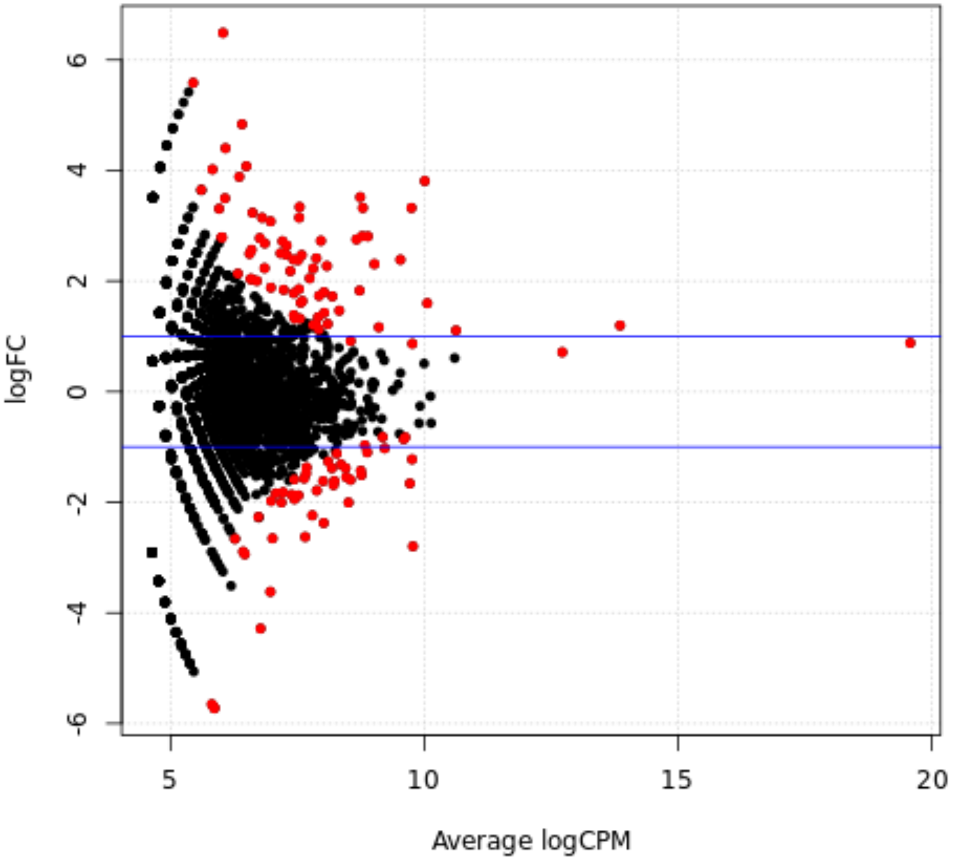
Map metatranscriptome reads to genes

Correct abundance w/ underlying genome prevalence

Examine your sample groupings – do an MDS plot (~PCA, tSNE, etc – "ordination")



https://2015-sep-microbial.readthedocs.io/en/latest/diffexp.html



Standard plot: “MA plot”

https://2015-sep-microbial.readthedocs.io/en/latest/diffexp.html

Interpreting differential expression spreadsheets

- Pick an FDR cutoff and live with it; don’t cherry-pick “low” FDR genes.

- Don’t sort by magnitude of differential expression unless you’re pretty sure it matters biologically for the gene(s) you’re looking for.

Normalization considerations

Have to take into account many things when comparing:

- Usually end up with different read amounts per sample.
- Changes in shape of distribution, # of expressed genes, etc.
  - Most differential expression toolkits assume less than ~20% of genes changing… probably NOT true in many microbiology situations.
- re QPCR – housekeeping genes are less reliable than ALL the genes…

Other thoughts

- Unless doing single-cell RNAseq, you are averaging across multiple cells. But we know that gene expression is often punctate (ref DKN).

- Assumption that RNA ~ protein abundance which is manifestly not true in at least some situations.

- Your computing is one part of the whole workflow – think about your experimental design and your controls :)

References

- “How many biological replicates are needed in an RNAseq experiment…?” – Schurch et al., 2016 – PMC 4878611.
- An older pipeline:

  https://2015-sep-microbial.readthedocs.io/en/latest/
- A more modern (but not microbial) set of tutorials:

  https://angus.readthedocs.io/en/2017/
- Metagenomics tutorials:

  http://2017-dibsi-metagenomics.readthedocs.io