Conducting studies of the microbiota

Matthew Stoll MD,PhD,MSCS

MIC741

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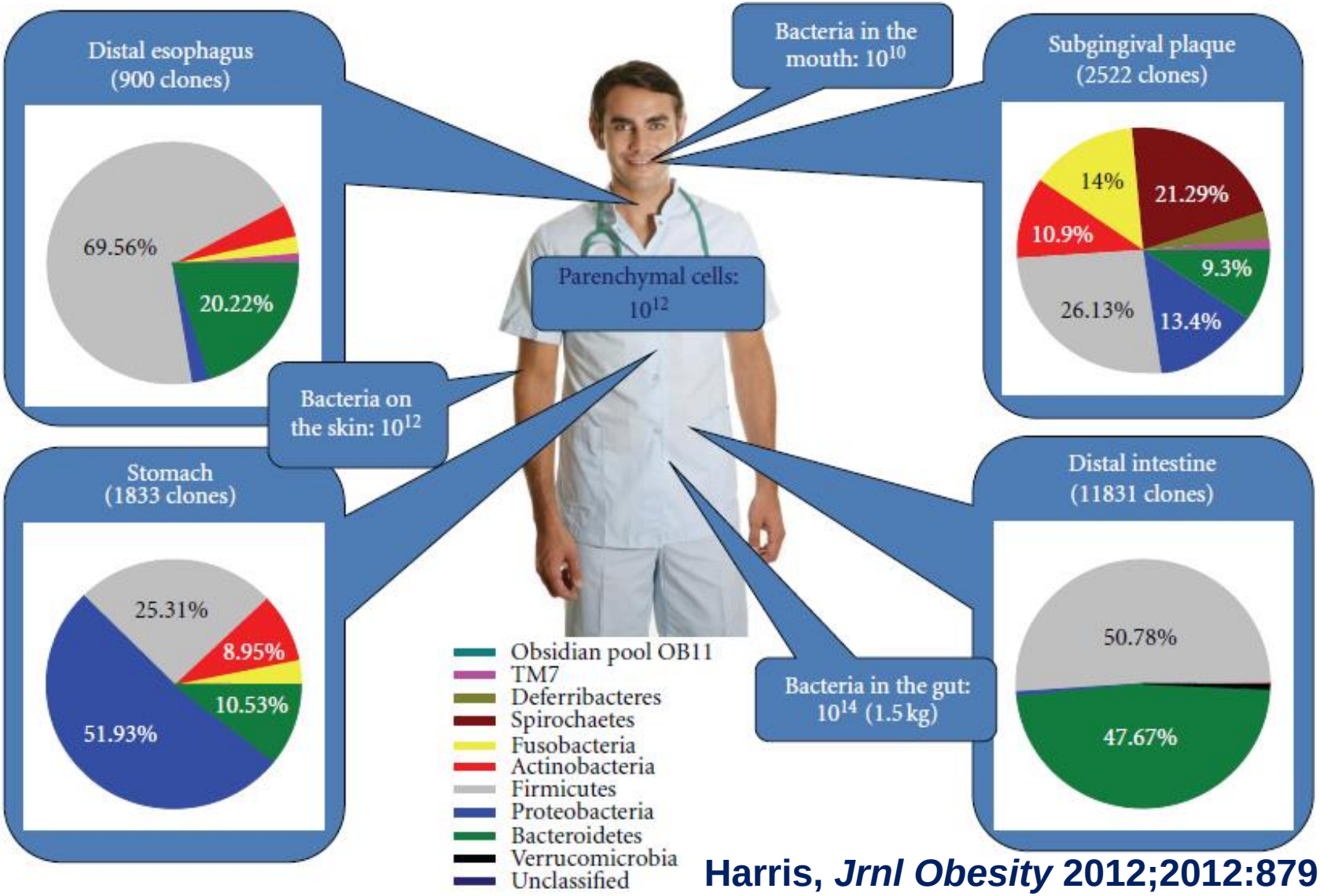
Outline

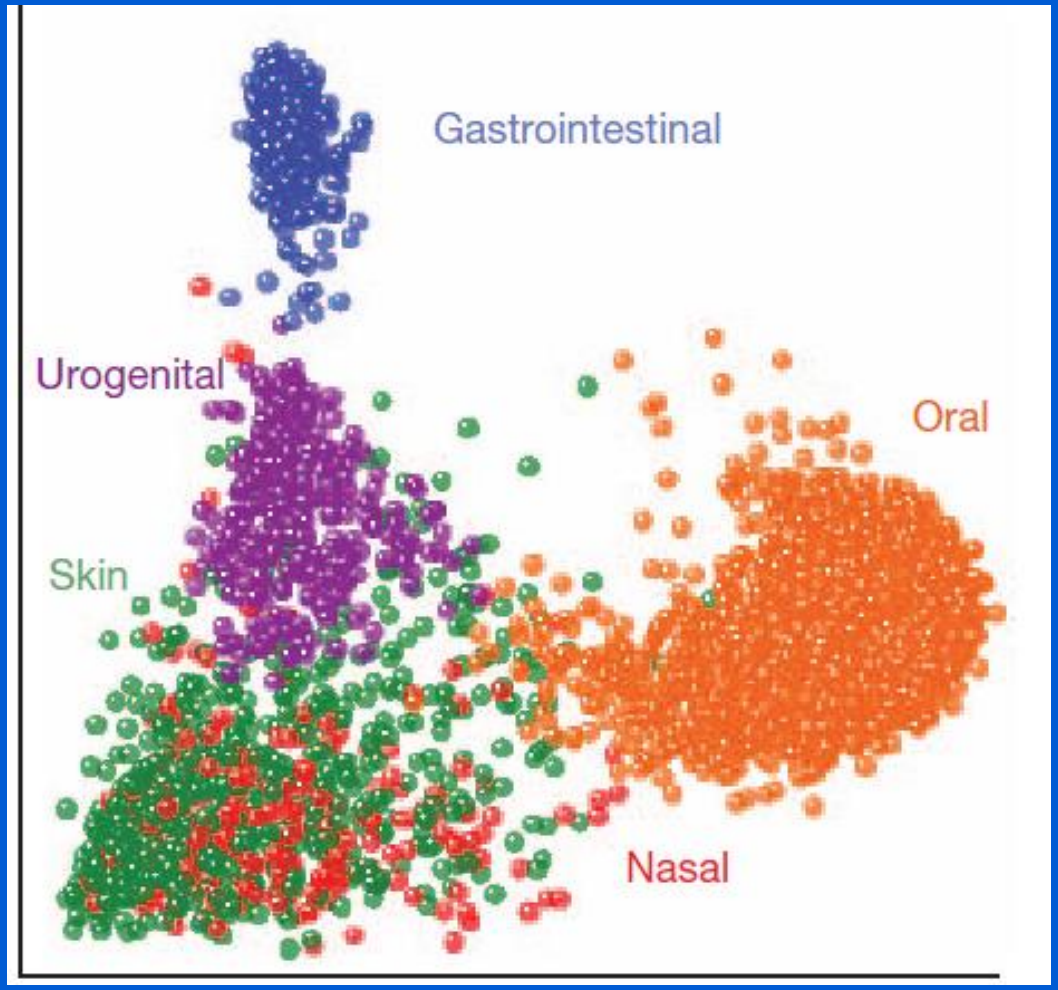
- **Where and how to sample**
- **Sequencing**
- **Data analysis**

Multiple habitats



Harris, *Jrnl Obesity* 2012;2012:879151

Bacteria composition differs by site



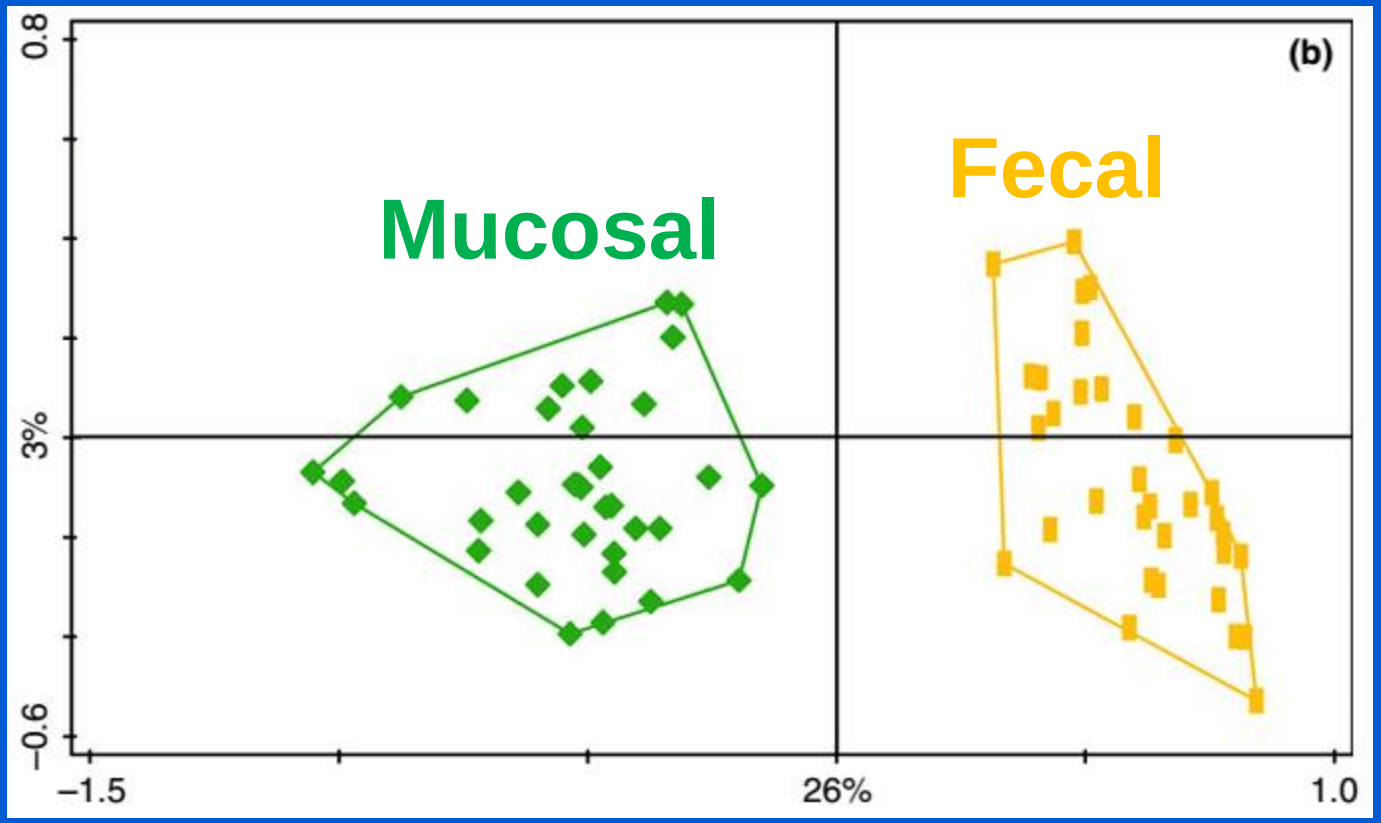
HMP Consortium, *Nature* 2012;486:207

Heterogeneity within habitats

- Gut
- Skin
- Mouth

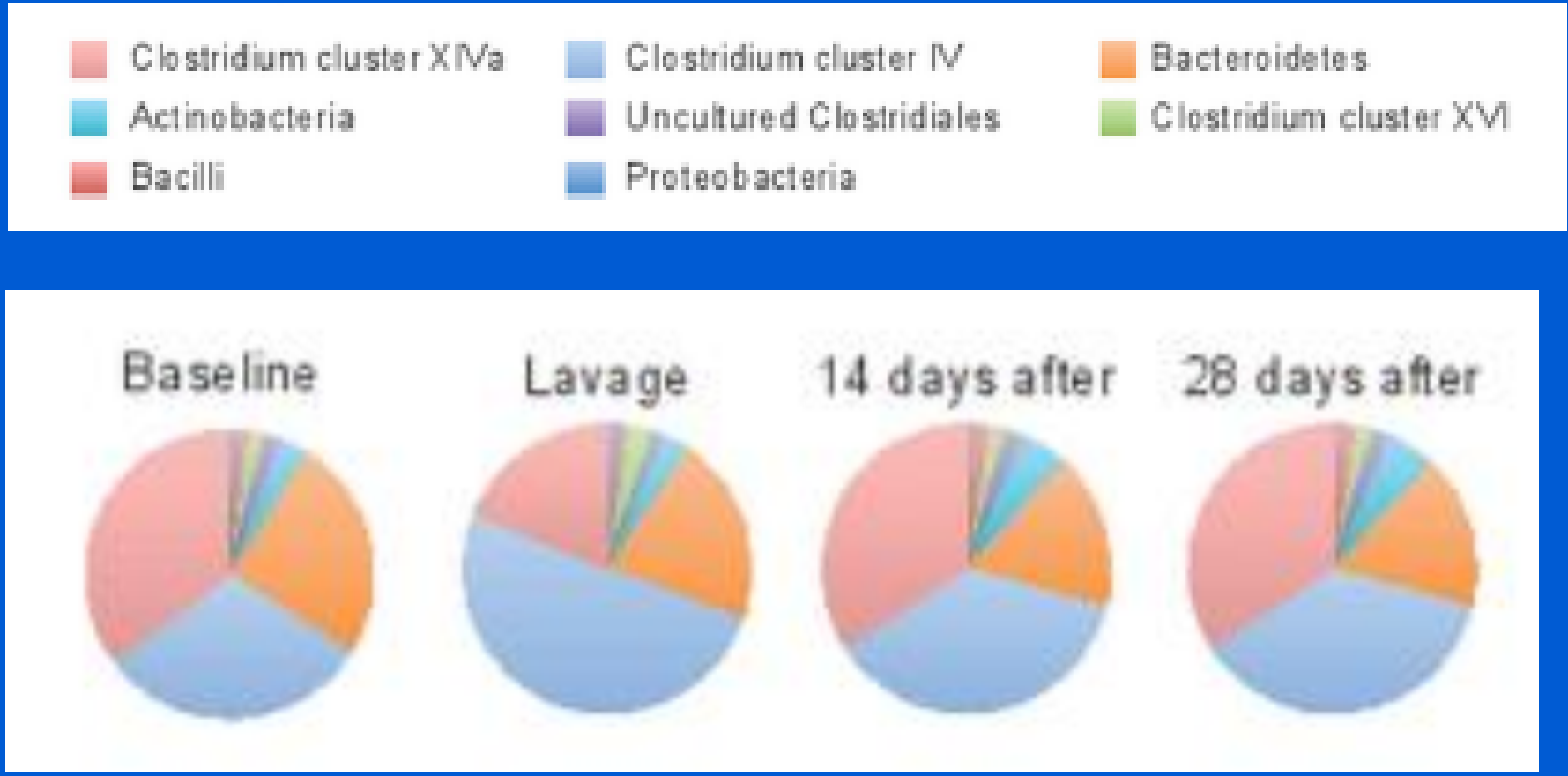
Fecal vs mucosal microbiota

Unprepped sigmoid biopsies



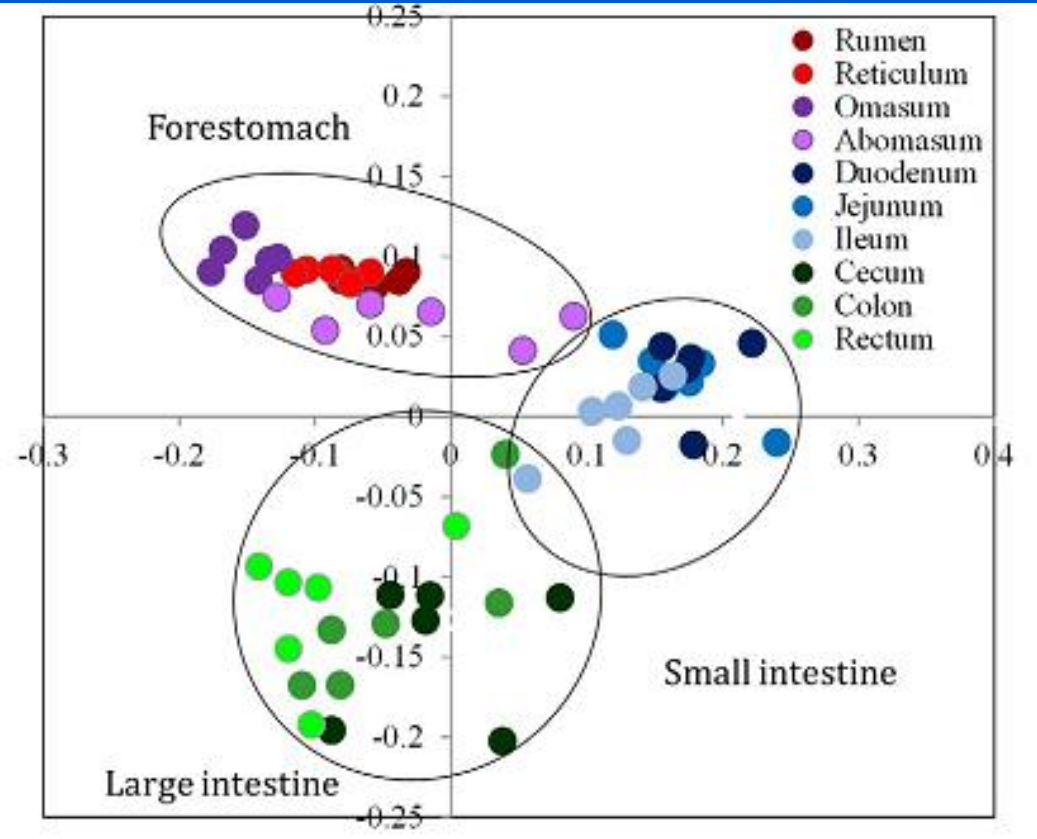
Rangel, *Aliment Pharmacol Ther* 2015;42:1211

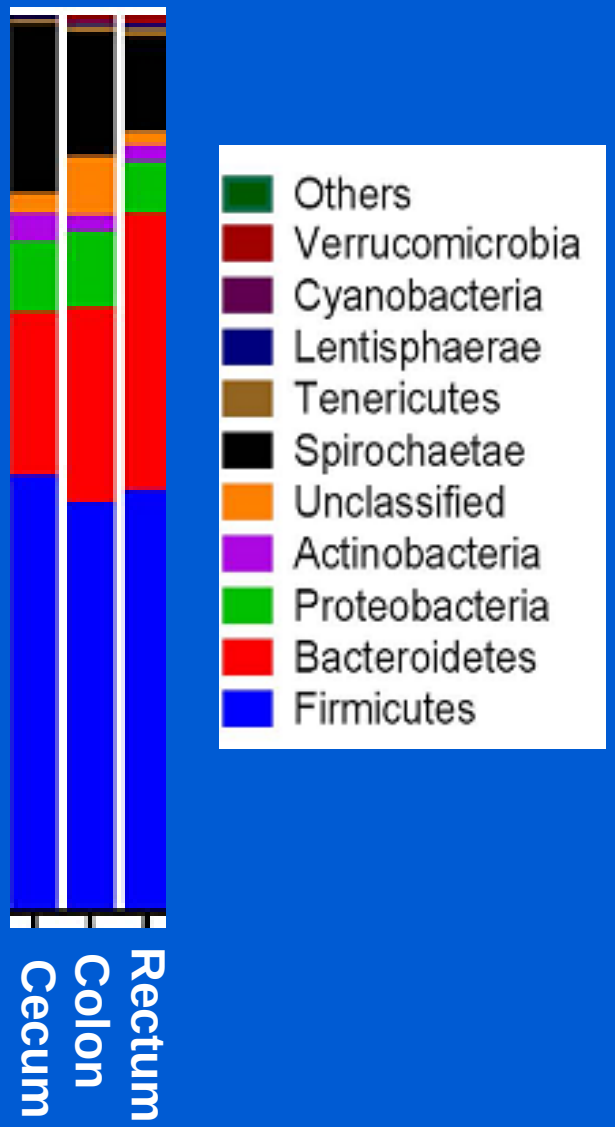
Affect of washout



Jalanka, *Gut* 2015;64:1562

Spatial heterogeneity in intestines





Mao, *Scientific Reports* 2015;5:16116

Fecal microbiota

- Patient prepares own sample
  - Usually at home
- Shipment

Money helps

	$25 payment	$50 payment
Received / Promised	27 / 62 (44%)	128 / 178 (72%)

Sample preparation

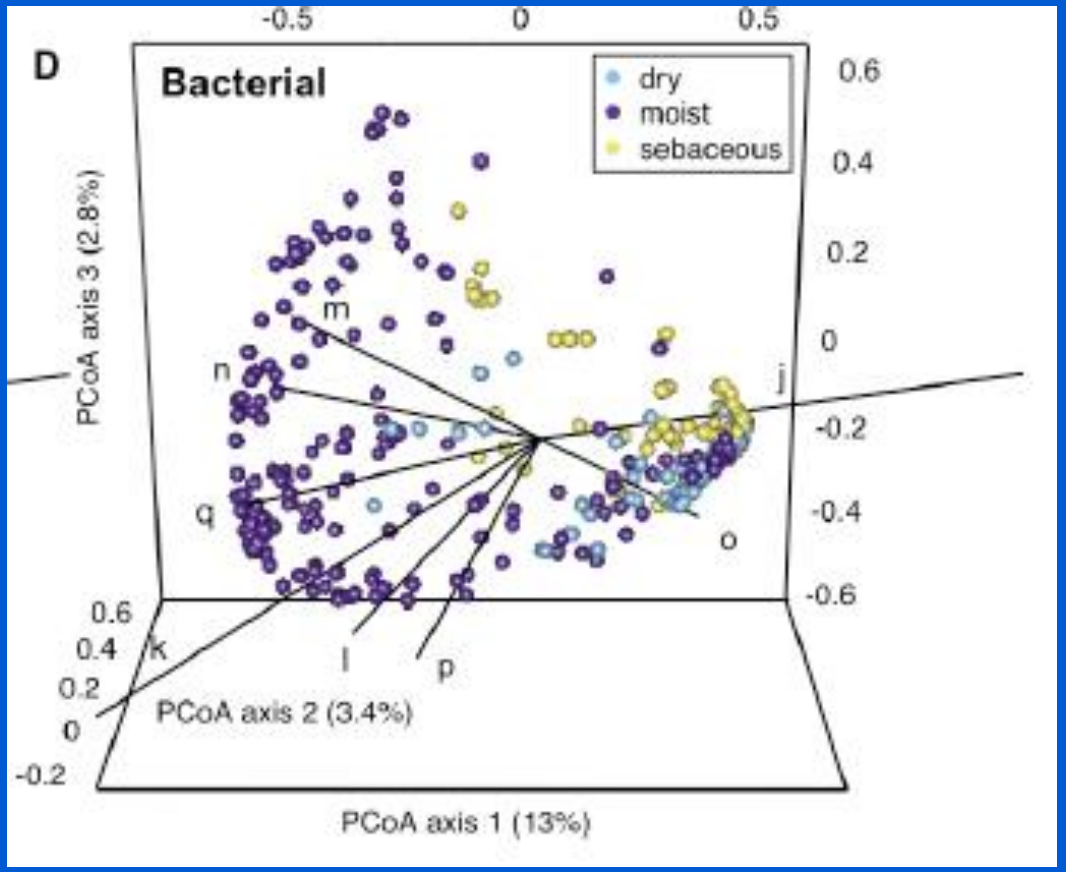
- Can get small amounts with used toilet paper
- I prefer to use a stool collection device (“hat”), subject transfers to collection vial
- Carey-Blair media (permits growth of live bacteria), if functional studies are planned
- Send overnight via commercial carrier

Skin has topographically distinct microbiota

Heel

Elbow

Back



Findley, *Nature* 2013;498:367

Skin collection

- Consistency with respect to personal hygeine measures (bathing, lotions, perfumes, topical antibiotics, etc.)
- Two methods
  - Cotton swab x 15 seconds (easier)
  - Skin scrapings using sterile blade (higher yield)

Mouth also has topographically distinct microbiota

Buccal mucosa
Keratinized gingiva
Hard palate

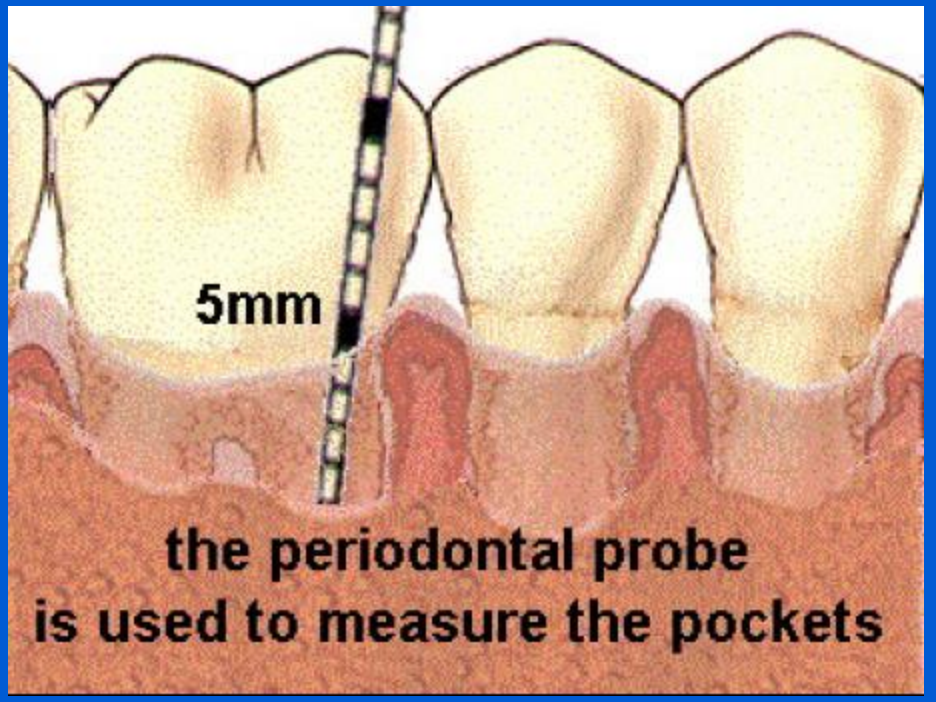
Throat
Tonsils
Tongue Dorsum
Saliva

Supragingival plaque
Subgingival plaque

Segata, *Genome Biol* 2012;13:R42

Oral cavity collection

- Saliva
- Cotton swab
- Gingival plaque



Genitourinary

- **Sample collection swab**
  - **HMP: Collected by study team**
  - **Alternative: self-collection**

Useful information to collect

- Diet (fecal microbiota)
- Antibiotics
- Skin and oral hygeine products
- Menstrual cycle and contraceptive use (reproductive tract microbiota)

DNA preparation

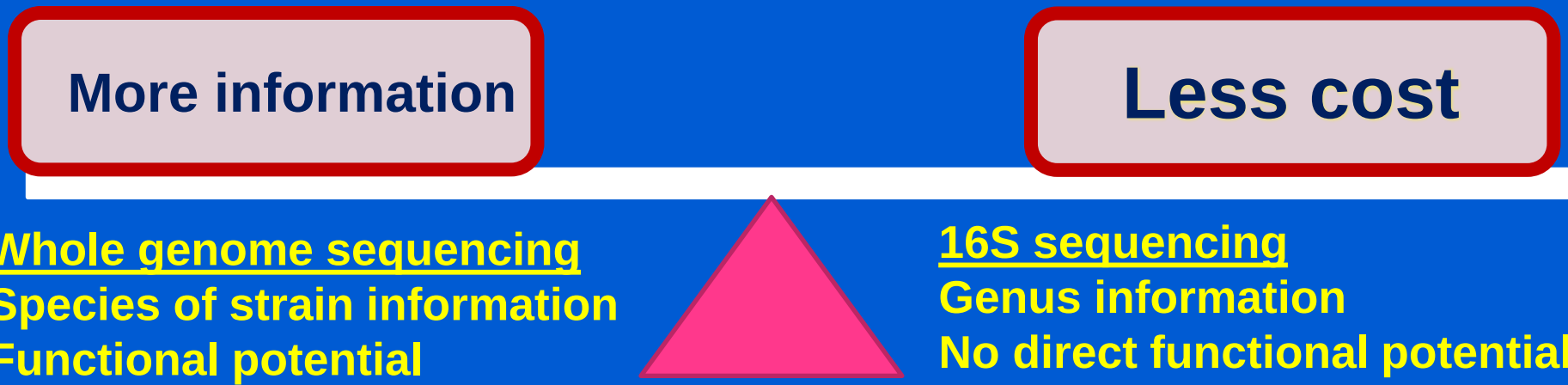
- Various kits used for DNA purification
  - MoBio tubes preferred by HMP
  - We used Zymo for fecal collection
- Key is that you need conditions harsh enough to lyse the microbes

Outline

- Where and how to sample
- Sequencing
- Data analysis

Type of sequencing

- Amplification of 16S ribosomal DNA
- Whole genome sequencing



Cost comparison of 16S and WGS

- An Illumina flow cell costs about $1000
- Can handle 100 16S samples
  - Avg cost of $10; ~ $15 including PCR
  - Currently subsidized by UAB
- The same flow cell runs only 3 WGS samples
  - Creating the library and additional expenses bring it close to $900 / sample
- Higher informatics costs

Sequencing prep in one slide

Starting with purified DNA

16S (Peter Eipers PhD)

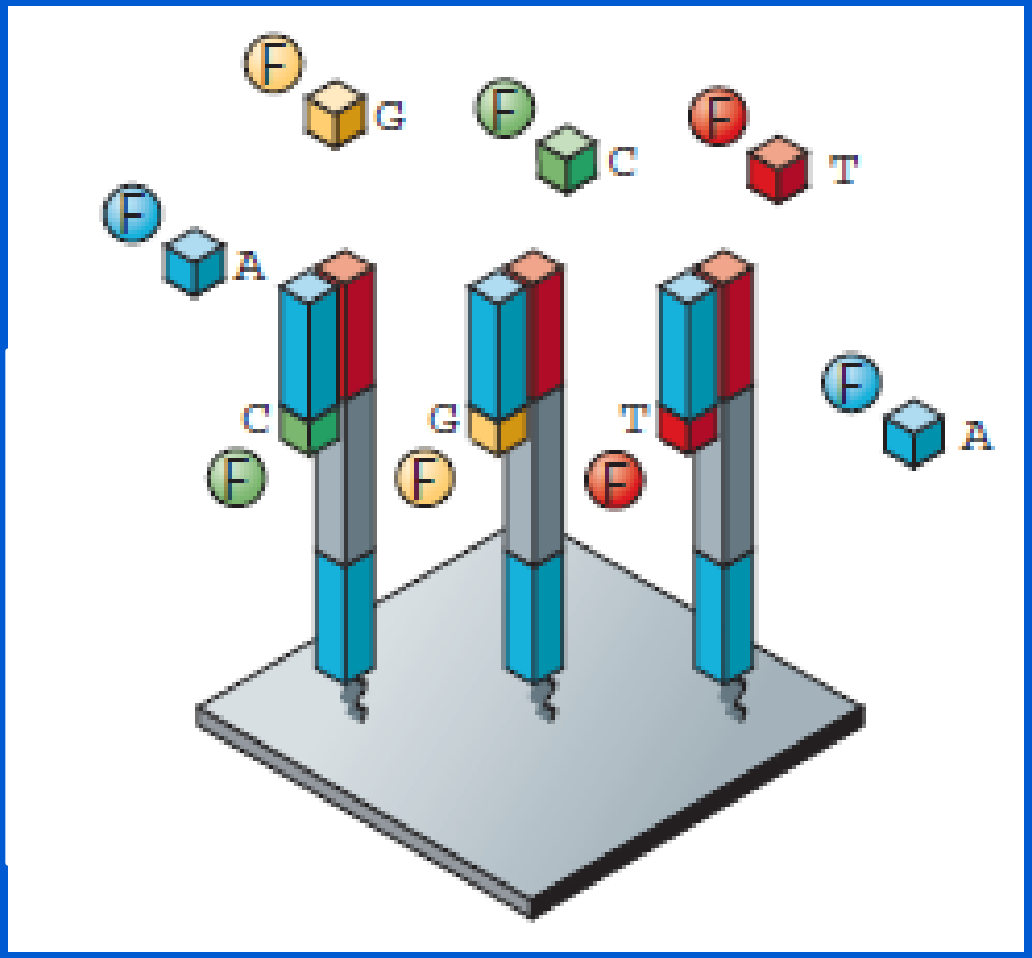
- PCR of 16S region
- Special primers
  - Barcode at one end
  - Adaptor at other

WGS (Mike Crowley PhD)

- Shear DNA
- Ligate adaptors to each end
  - Includes barcodes
- Short PCR
- Optional size selection

Cyclic reversible termination

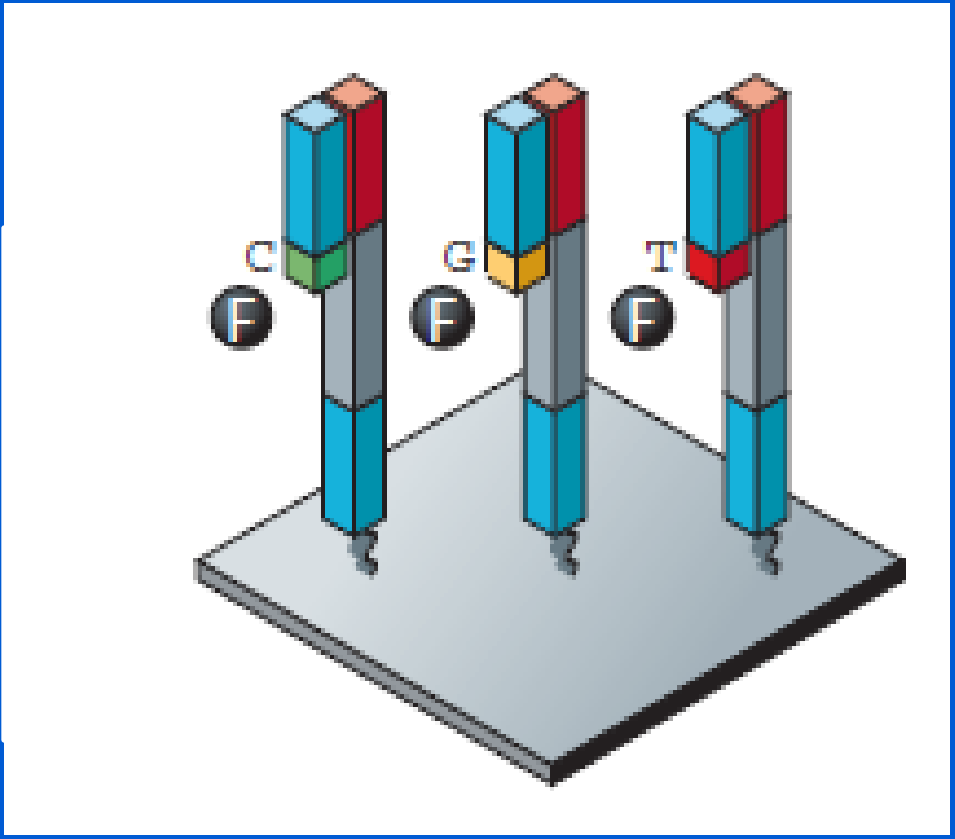
Metzker, *Nature Rev Genet* 2010;11:31



Incorporate all four nucleotides, each with different dye

Cyclic reversible termination

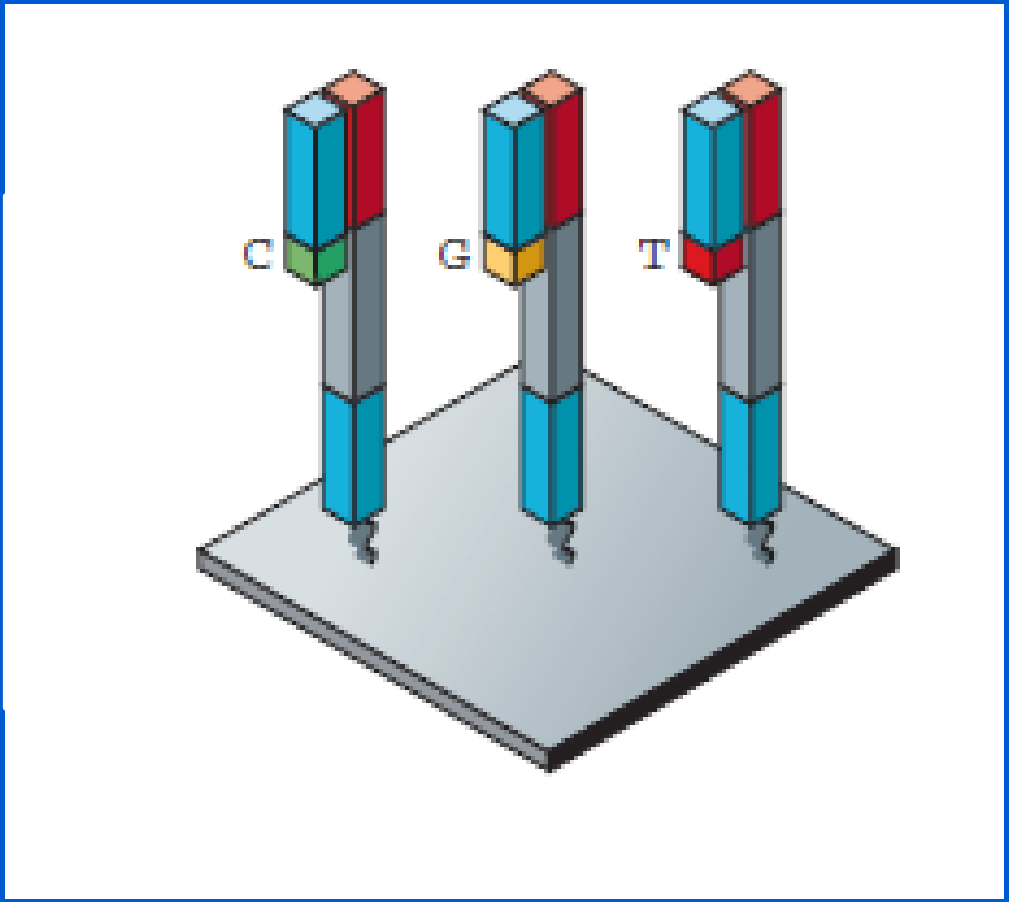
Metzker, *Nature Rev Genet* 2010;11:31



Wash out unused nucleotides; image

Cyclic reversible termination

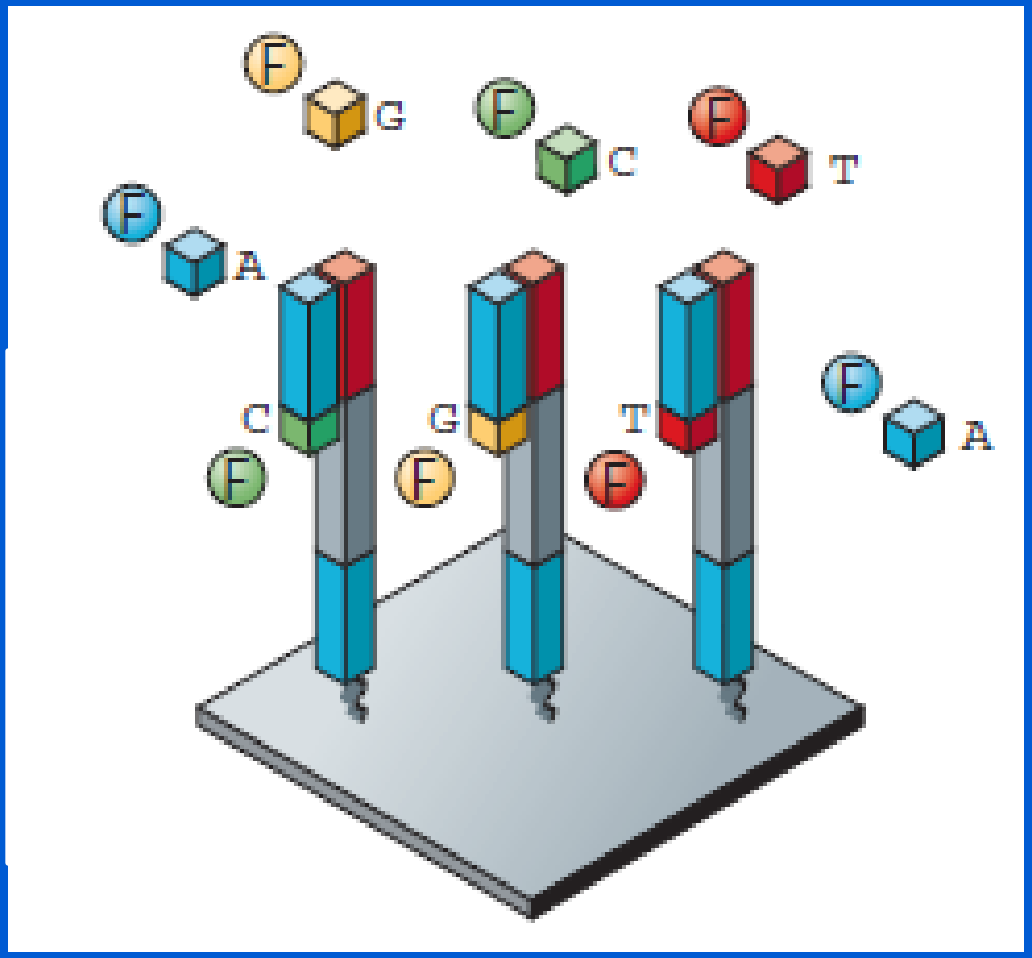
Metzker, *Nature Rev Genet* 2010;11:31



Cleave dye and terminating groups

Cyclic reversible termination

Metzker, *Nature Rev Genet* 2010;11:31

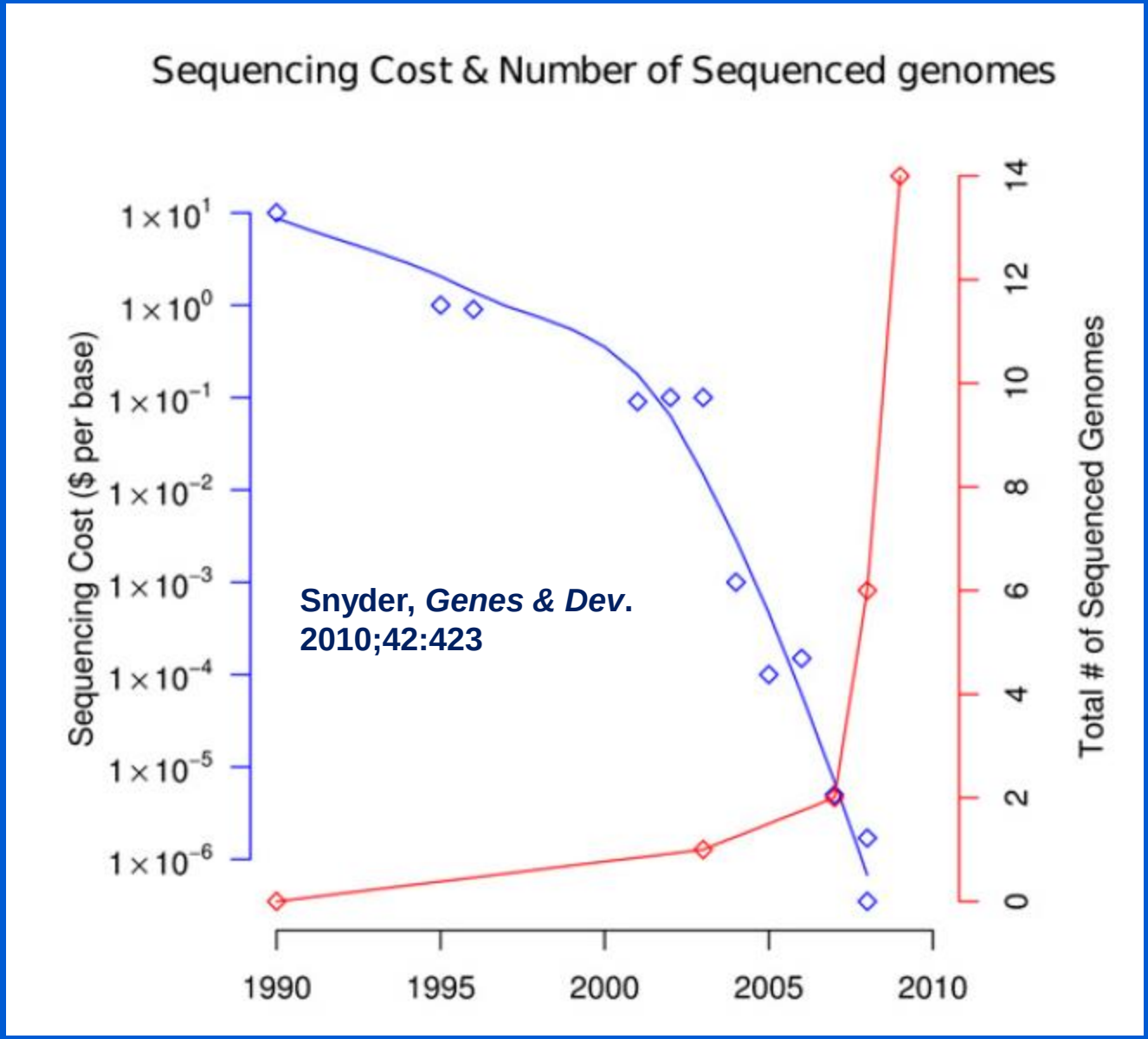


Back to step one

Barcodes to sort out samples

Sample 1: AGGTTCCA
Sample 2: GGCAATTT
Sample 3: TTGGAAAC

Trends in sequencing cost



Outline

- Where and how to sample
- Sequencing
- Data analysis

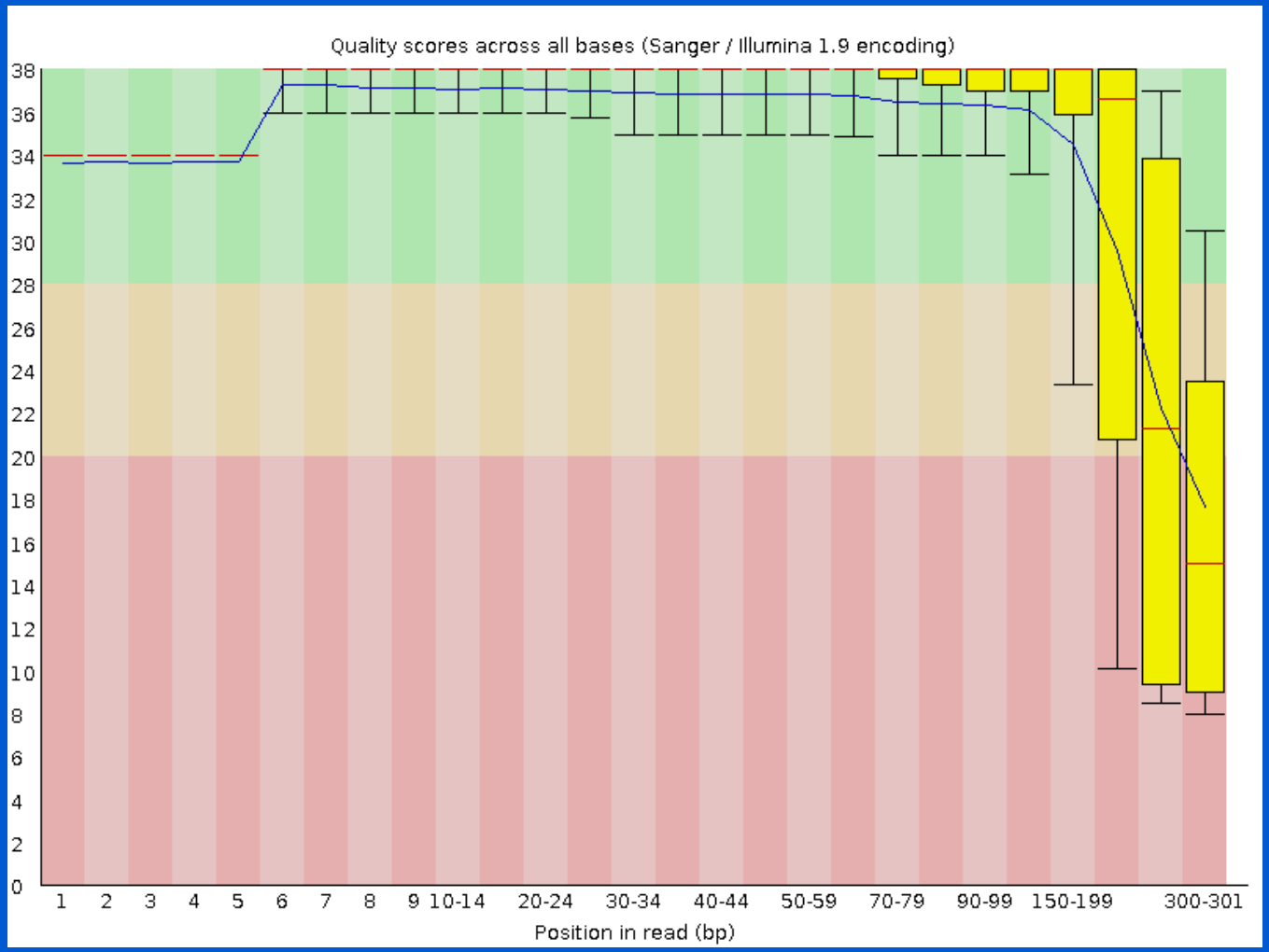
Output: fastq files

Sample Fastq output (two DNA strands)

Header: @M02079:147:000000000-AK0J5:1:1101:15736:1676 1:N:0:49
Sequence: TACAGAGGTCTCAAGCGTTGTTCGGAATCACTGGGCGTAA
Additional line: +
Quality: >//>>EEGGFFE/////</>-<0>DBF1<F<1.<<-<GD

Header: @M02079:147:000000000-AK0J5:1:1101:15989:1722 1:N:0:49
Sequence: TACGGAGGATGCGAGCGTTATCCGGATTTATTGGGTTTAAAG
Additional line: +
Quality: B3EHFGGHF3FB43/E?EFGGFFGH3/B4?//B/FG?122FB

Assess quality of reads



Position in read (BP)

Managing paired reads

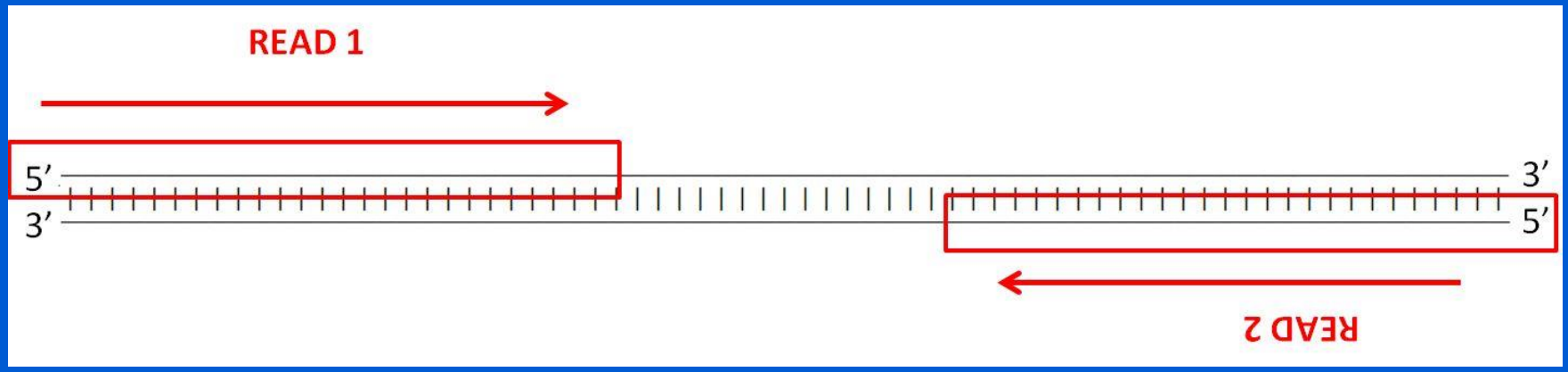
16S sequencing

- PCR output is 250 – 300 bp in length
- Illumina MiSeq produces 250 bp paired-end reads

Paired reads do not always overlap

WGS sequencing

- **Fragments may be 400 – 500 bp**
- **Sequence output may be shorter, and may not overlap**



Quality control and merger of paired end reads

- If there is substantial overlap, the merging itself is a QC step
- If the reads disagree on a base call, the program accepts the base with a higher associated quality score
- User can input minimal amount of overlap, number of permissible errors

Quality control and merger of paired end reads

- If there is NOT substantial overlap, the paired reads cannot be merged
- Need to use programs that apply the QC steps to both the forward and reverse reads
  - If one is removed, its mate must also be removed

Quality filtering options

- Trim the low-quality tails
  - Option: remove sequence if more than a set percentage of bases are trimmed
- Remove sequences with a certain number of ambiguous bases
- Remove sequences which have quality scores below a threshold
  - Can permit a set percentage (e.g. remove if 5% have q-scores < 30).

Convert to fasta

- Fasta files do not have quality information
- Are used for most analyses

Fasta sequences

Header: >M02079:147:000000000-AK0J5:1:1101:15736:1676 1:N:0:49
Sequence: TACAGAGGTCTCAAGCGTTGTTCGGAATCACTGGGCGTAA

Header: >M02079:147:000000000-AK0J5:1:1101:15989:1722 1:N:0:49
Sequence: TACGGAGGATGCGAGCGTTATCCGGATTTATTGGGTTTAAAG

16S data analysis

- Different programs exist for 16S analysis
  - Mothur
  - Quantitative Insight into Microbial Ecology (QIIME)

QIIME

- Open-source bioinformatics pipeline
- Designed for 16S sequence analysis
- Every step from fastq processing through data analysis
- Coming soon: QIIME 2
  - GUI
  - Better support for whole genome sequencing

www.QIIME.org

The OTU table

- Analyses are performed on the "operational taxonomic unit (OTU)" table, not the fasta sequence file
- OTUs are groups of similar sequences
  - User can set the similarity; 97% is standard
- Matter of efficiency (may have 2 million sequences, just 3000 OTUs)
- Clearly, OTU picking is essential step

Biom format of OTU table

- Information contained in OTU table
  - List of sample IDs (subj 1, subj 2, etc)
  - List of OTU IDs
  - Frequency of each OTU in each sample
  - Optional: taxonomy
- Earlier versions of QIIME had OTU tables in .txt format
- Biom format has same information, but can store more data
- Not particularly intuitive to look at

Closed vs open reference OTU picking strategies

OTU picking strategy	Compare with existing database	Speed	Inclusion of new taxa
CLOSED REFERENCE	YES	Faster	No
OPEN REFERENCE	NO	Slower	Yes

QIIME offers a hybrid approach

- Script: pick_open_reference_otus.py
- User inputs database; default option is the latest greengenes release
  - Curation of all the 16S reference sequences
  - Has associated taxonomy file

Greengenes fasta and taxa files

Greengenes fasta

>1111881
GCTGGCGGCGTGCCTAACACATGTAAGTCGAACGGGAC
TGGGGGCAACTCCAGTTCAGTGGCAGACGGGTGCGT
>1111882
AGAGTTTGATCATGGCTCAGGATGAACGCTAGCGGCAG
GCCTAACACATGCAAGTCGAGGGGTAGAGGCTTTCG

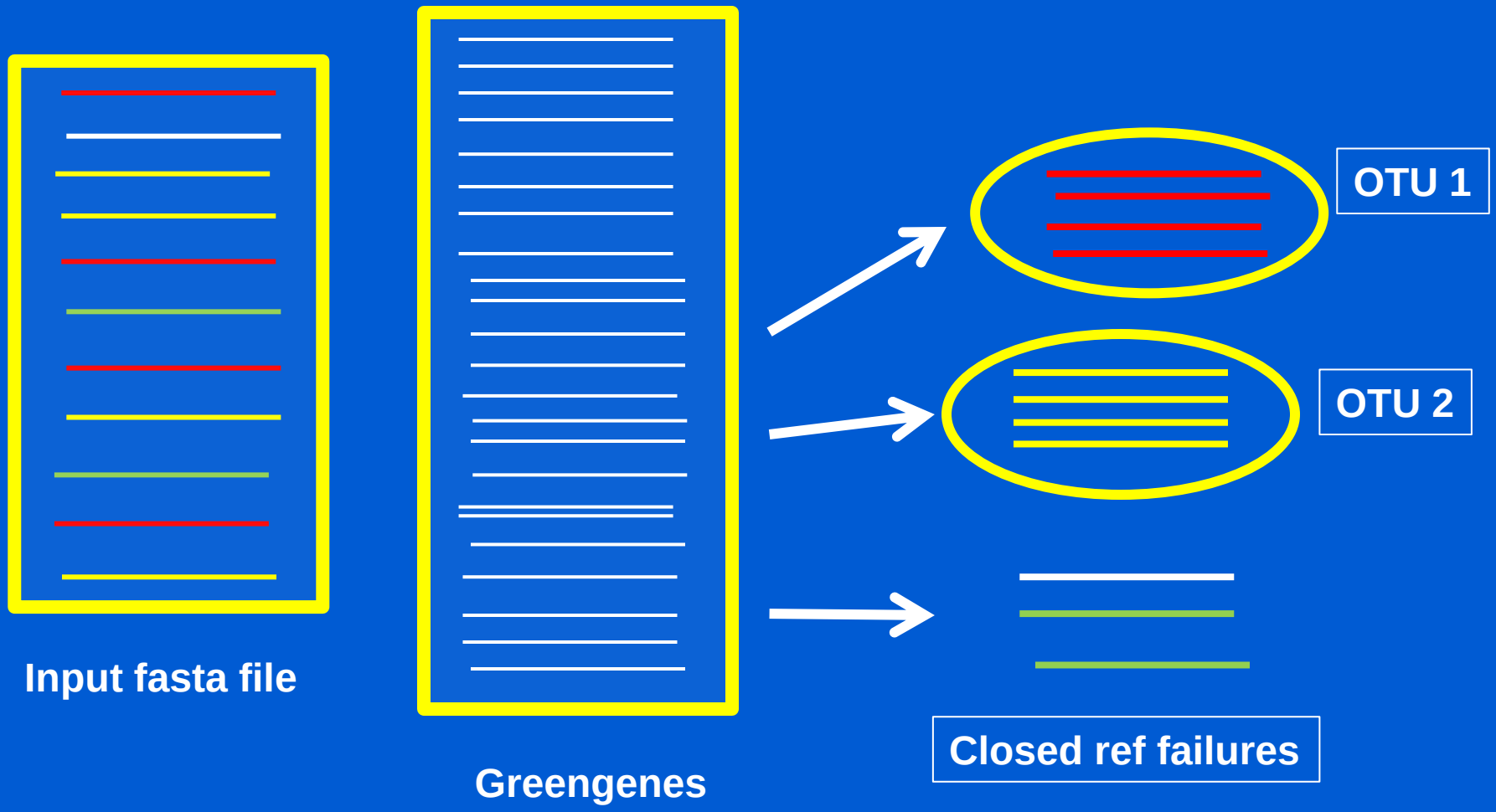
Greengenes taxonomy

1111881 k__Bacteria; p__Proteobacteria; c__Epsilonproteobacteria; o__Campylobacterales; f__Helicobacteraceae; g__; s__

1111882 k__Bacteria; p__Bacteroidetes; c__Flavobacteriia; o__Flavobacteriales; f__Flavobacteriaceae; g__Flavobacterium; s__

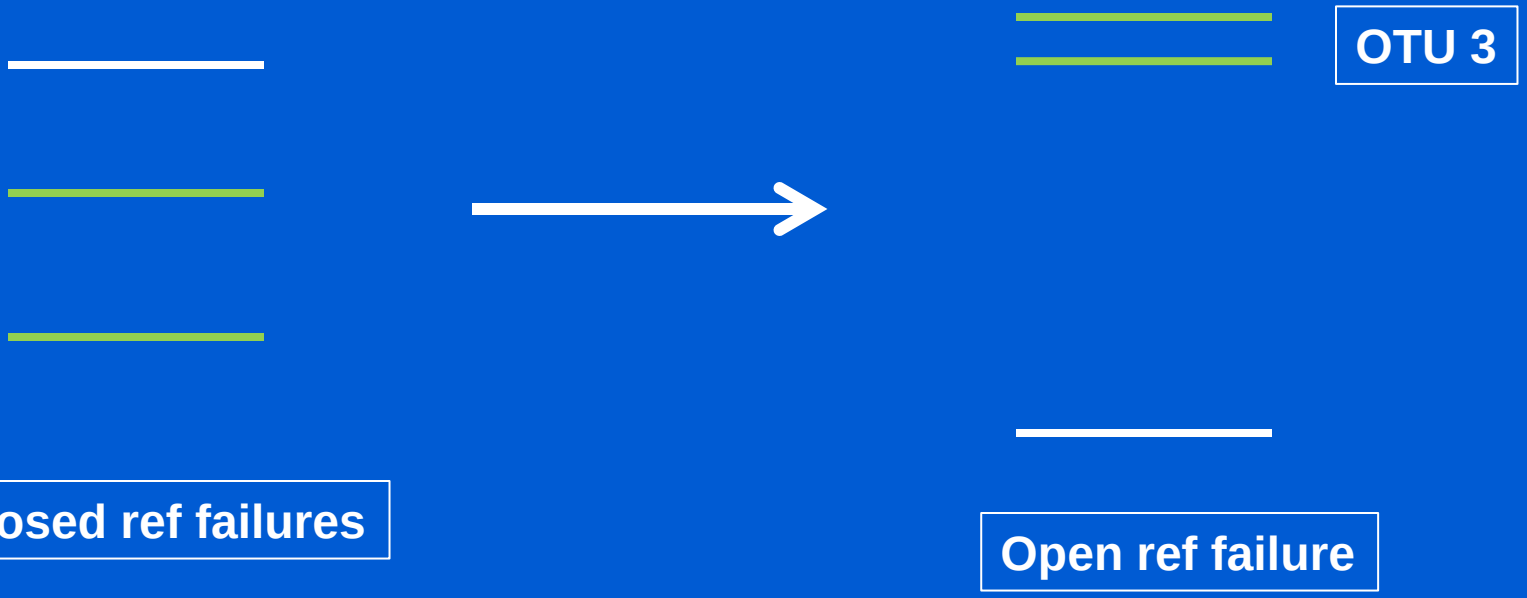
pick_open_reference_otus.py

Closed ref OTU picking against greengenes



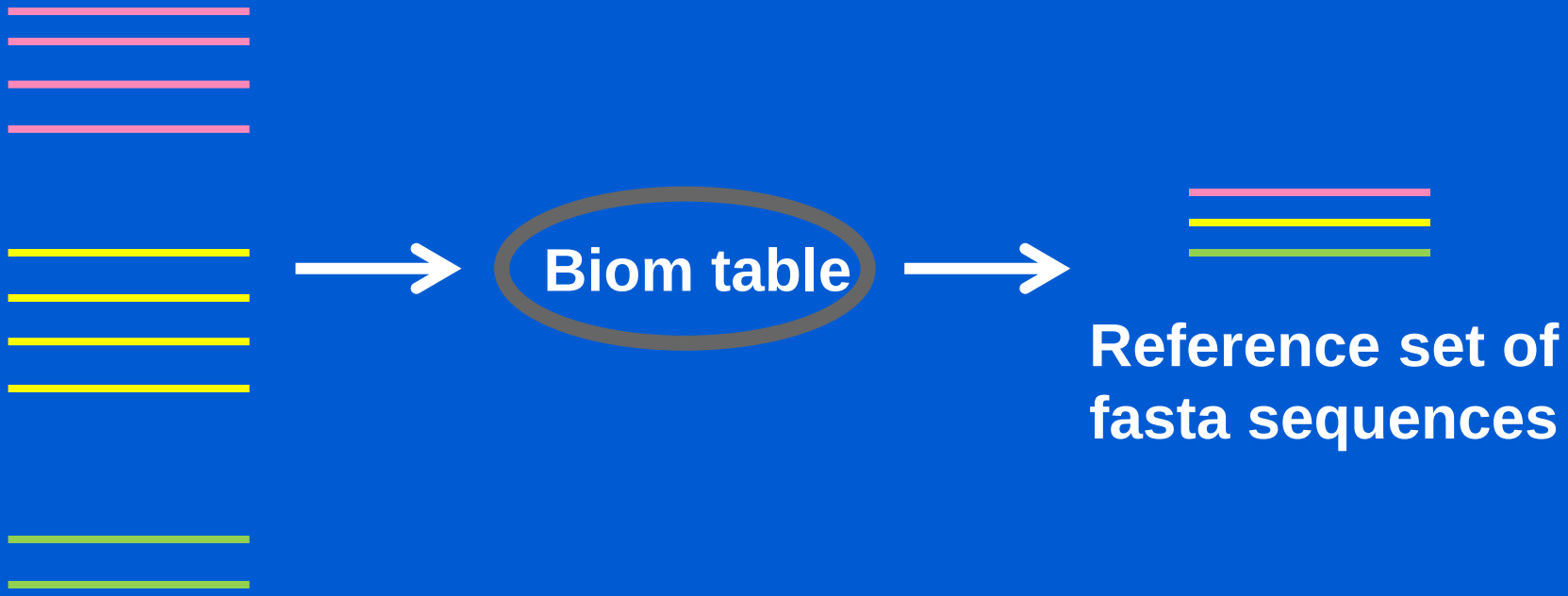
pick_open_reference_otus.py

Open ref OTU picking with the failures



pick_open_reference_otus.py

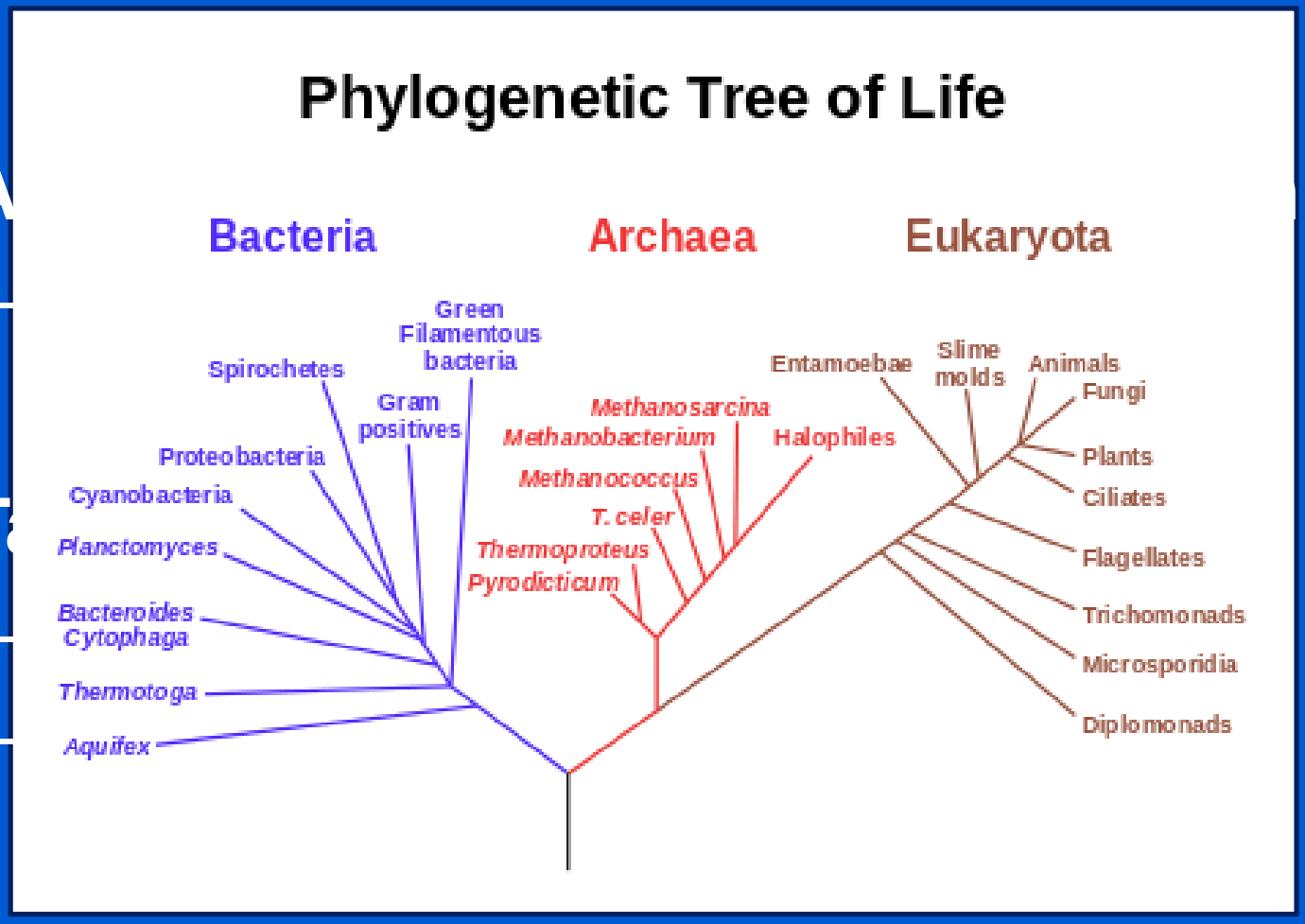
Make OTU table and pick reference set



pick_open_reference_otus.py

Final steps

- A… table
- T…



What do you do with the biom table?

Not much, if you don’t have a metadata file

Contents of metadata file

- **Subject IDs**
  - **Whatever is printed on tube with DNA**
  - **This will be name of output fastq file**
  - **Be sure to follow HIPAA**
- **Important metadata**
  - **Disease status**
  - **Treatment**
  - **Sex**
  - **etc.**

Metadata file

#SampleID	Disease	Sex	Antibiotics
Subj1	Arthritis	Male	No
Subj2	Arthritis	Male	No
Subj3	Arthritis	Female	Yes
Subj4	Control	Female	No
Subj5	Control	Female	No
Subj6	Control	Male	No

#SampleID should be header of the first column

What do you do with the biom table?

- Show taxonomy
- Alpha diversity (within group)
- Beta diversity (between group)
- Comparisons

First, get summary information

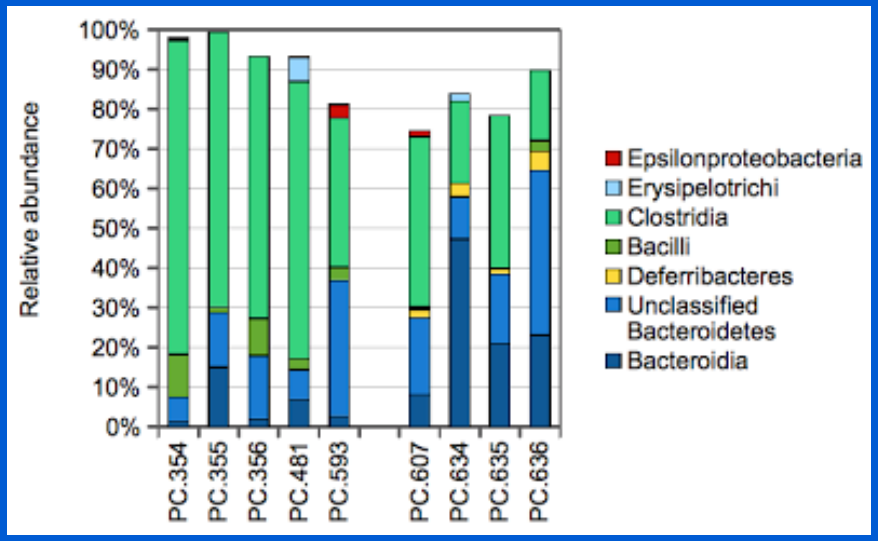
- biom summarize-table -i otu_table.biom -o summary.txt
- Creates a .txt file which includes:
  - Sample IDs included in file
  - Sequencing depth of each ID
  - Number of samples
  - Number of OTUs

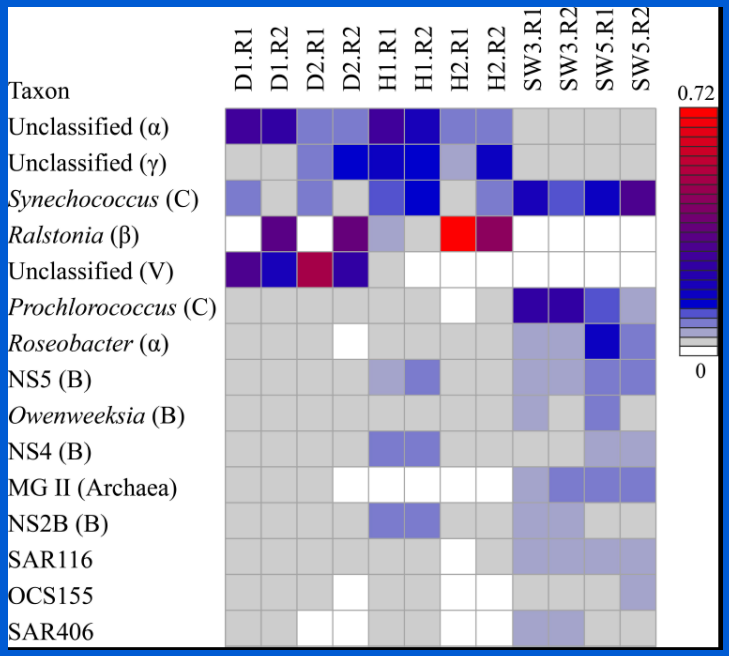
Assigning taxonomy

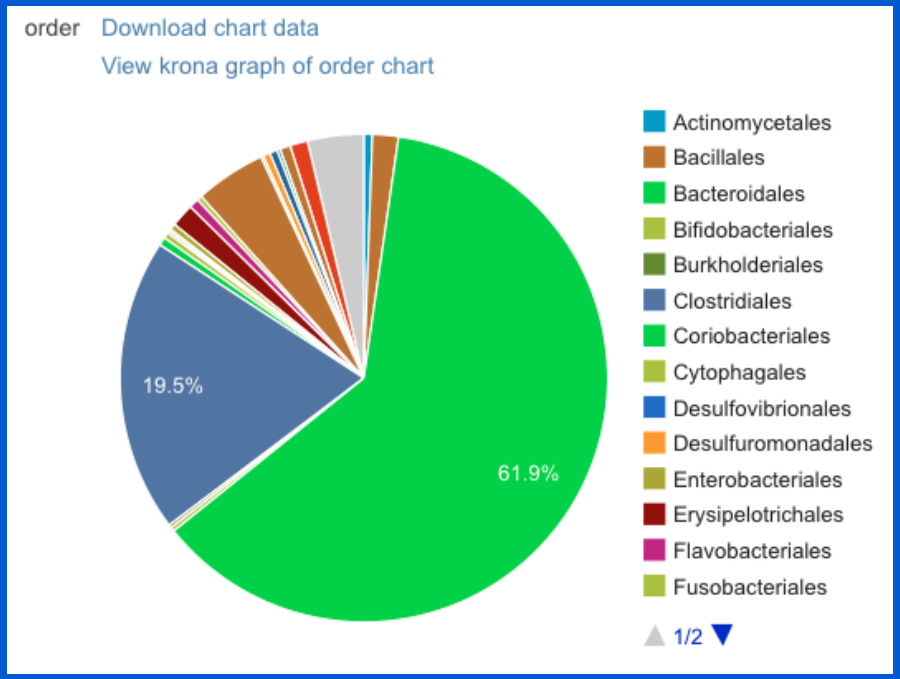
- **assign.taxonomy.py -i rep_set.fasta -t greengenes_97_otus.txt -r greengenes_97_otus.fasta -m uclust**
  - **Generates a .txt file with two columns: OTU and taxonomy**
  - **One row for each OTU**
  - **Info can be incorporated into OTU table**

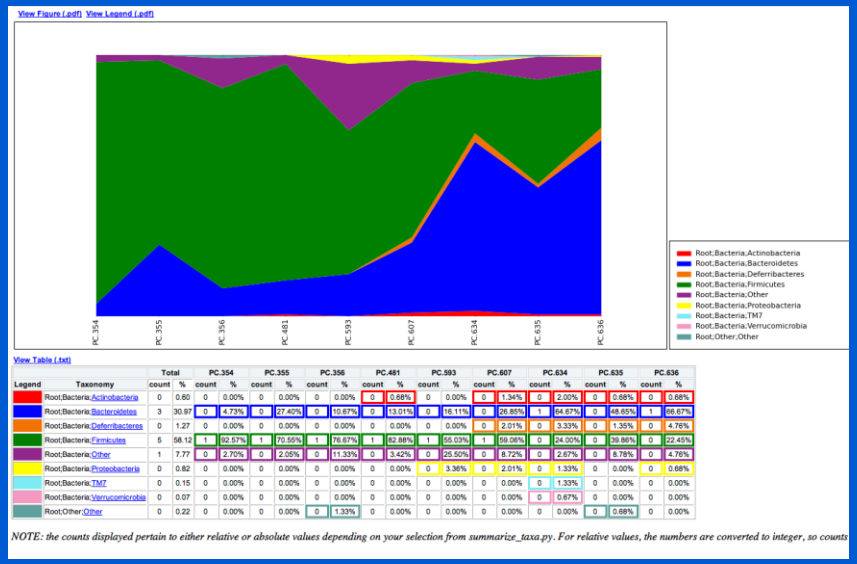
- **summarize_taxa.py -i otu_table.biom -o taxa/**
  - **Creates a set of .biom and .txt files for each taxonomy level (by default, L2 - L6)**

Displaying taxonomy







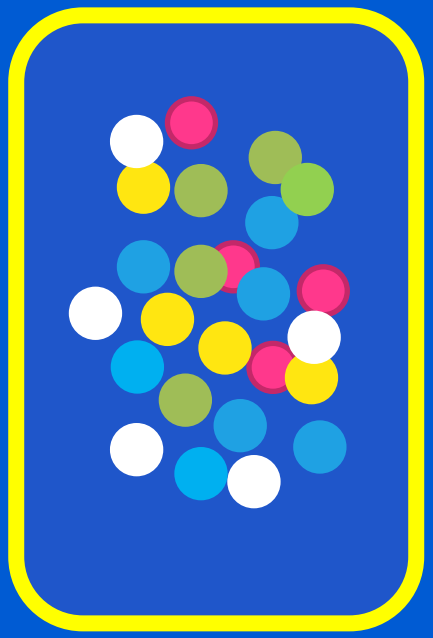


Alpha diversity

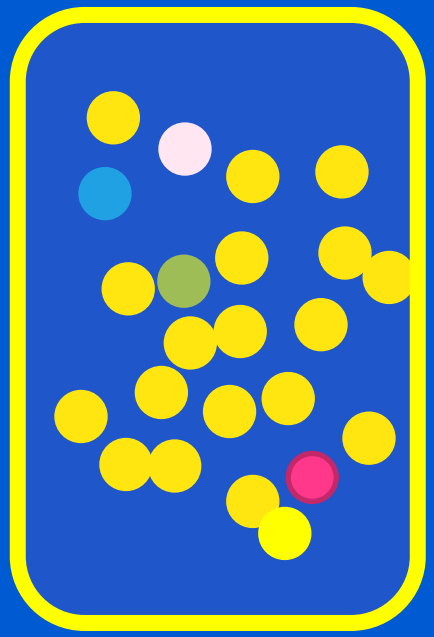
- **Richness: number of different species present in a sample**

- **Evenness: how evenly dispersed these species are**

Alpha diversity



Rich and even



Rich, not even

Not rich or even

Phylogenetic alpha diversity

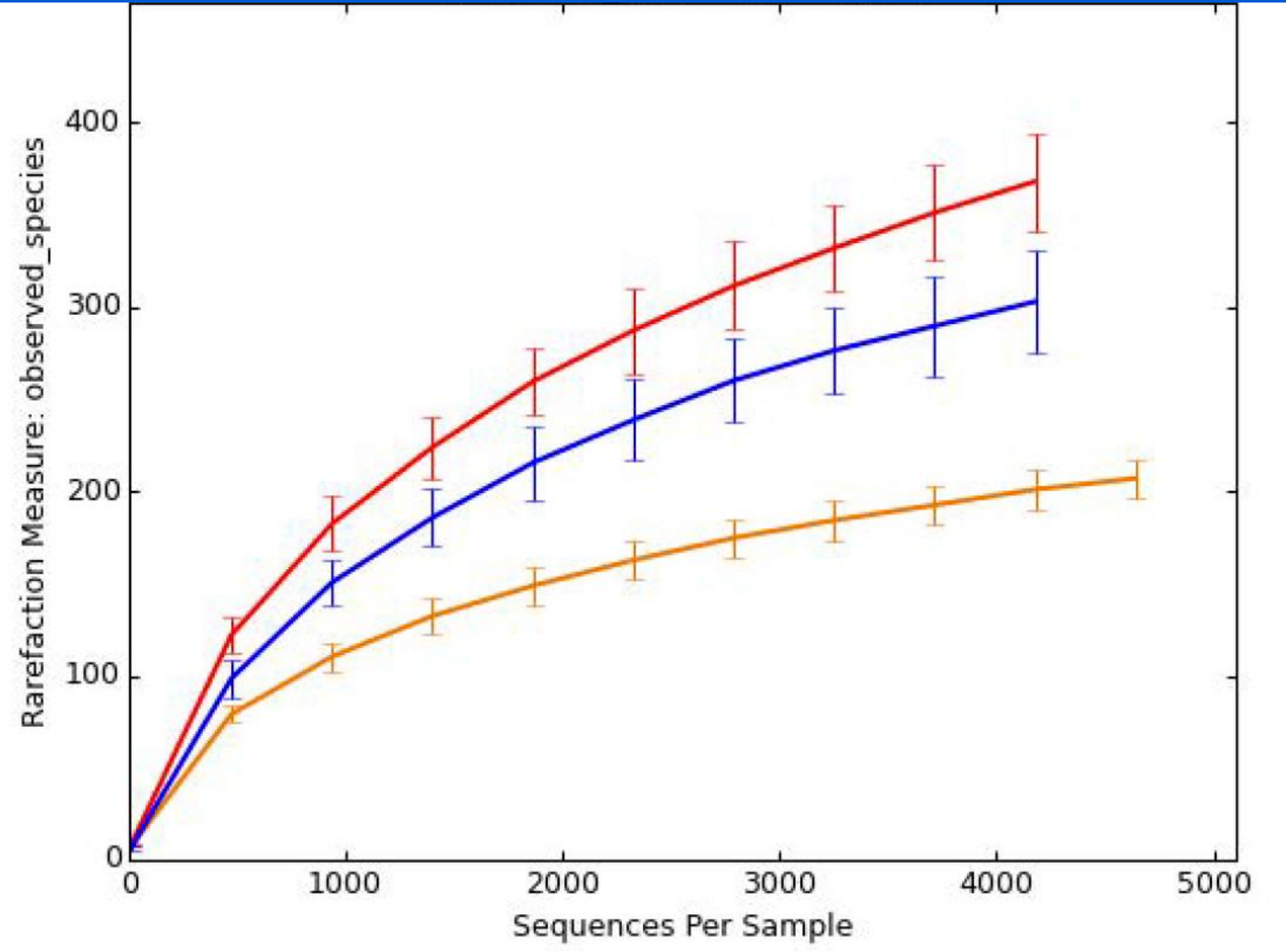
- Takes into account the phylogenetic tree and similarity between species

- A mixture of bacteria from different phyla is seen as more diverse than, say, 20 different species of staphylococcus

Cautionary note about measuring alpha diversity

- Must take into account sequencing depth
- Typically, at UAB, depth is 50K – 150K sequences per sample for 16S
- To a point, diversity increases with higher depth, as you pick up more rare species
- Rarefaction curves often are shown

Illustration of rarefaction curves



Measuring alpha diversity in QIIME

- Step 1. Perform rarefactions, selecting which sequencing depth or depths will be evaluated
- Generally the highest should be lower than the lowest sequencing depth of your samples
- multiple_rarefactions.py -m 10000 -x 70000 -s 10000 -n 10 -i otu_table.biom -o rarefactions/

Measuring alpha diversity in QIIME

- **Step 2. Run alpha diversity at each rarefaction**
- **alpha_diversity.py -i rarefactions/ -o alpha/ -t rep_set.tre -m shannon,simpson,PD_whole_tree,chao1**

Measuring alpha diversity in QIIME

- Step 3. Collate into individual files for each metric
- collate_alpha.py -i alpha/ -o alpha_collated/
- Output is multiple text files (one for each metric) consisting of tables listing the alpha diversity measurements for each subject at each rarefaction

Sample alpha diversity output

Sequence	Iteration	Subj1	Subj2	Subj3
10000	1	6.9	4.2	6.8
10000	2	7.1	3.8	8.0
10000	3	7.05	3.9	8.1
20000	1	8.4	6.1	8.5
20000	2	8.2	6.2	8.2
20000	3	8.1	6.0	8.0
30000	1	8.3	6.1	8.4
30000	2	8.4	6.3	8.2
30000	3	8.4	5.9	8.3

Beta diversity

- This speaks to the diversity between two different groups
- To analyze this, the metadata file is essential
- QIIME can present beta diversity in the form of a PCOA plot
- Again, consider rarefactions / sequencing depth

Beta diversity script

- beta_diversity_through_plots.py -i otu_table.biom -e 70000 -o beta/ -t rep_set.tre -m map.txt
- Note that the metrics were not specified here
- For QIIME workflow scripts (which do multiple steps at once), some options are specified by a separate parameter file (-p QIIME_parameters.txt)

Sample distance matrix

	C1	C2	C3	C4	T1	T2	T3
C1	0	0.35	0.31	0.39	0.88	0.79	0.91
C2	0.35	0	0.42	0.22	0.92	0.90	0.74
C3	0.31	0.42	0	0.35	0.74	0.79	0.91
C4	0.39	0.22	0.35	0	0.82	0.84	0.92
T1	0.88	0.92	0.74	0.82	0	0.29	0.21
T2	0.79	0.90	0.79	0.84	0.29	0	0.32
T3	0.91	0.74	0.91	0.92	0.21	0.32	0

Sample distance matrix

	C1	C2	C3	C4	T1	T2	T3
C1	0	0.35	0.31	0.39	0.88	0.79	0.91
C2		0	0.42	0.22	0.92	0.90	0.74
C3			0	0.35	0.74	0.79	0.91
C4				0	0.82	0.84	0.92
T1					0	0.29	0.21
T2						0	0.32
T3							0

Comparisons

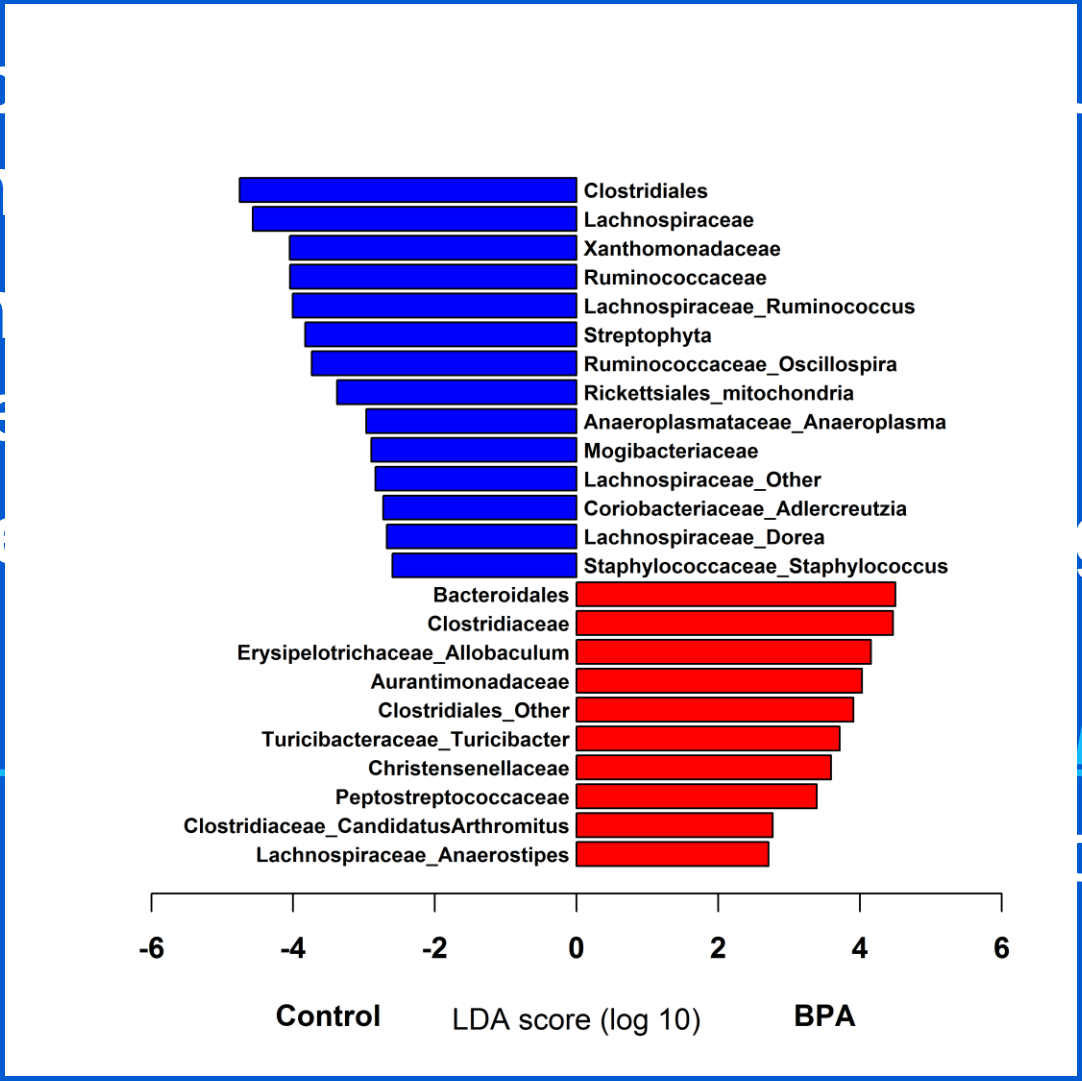
- QIIME can perform statistical comparisons
- Input files
  - Metadata file
  - Distance matrix or OTU table
- Two basic flavors
  - Global
  - Pairwise for each OTU
- Both have multiple options for statistical tests
  - Parametric or non-parametric
  - Dichotomous or continuous

Comparisons

- **Global** compare_categories.py -i weighted_unifrac_dm.txt -m map.txt -c Disease -o Results/ --method permanova
  - Provides a single p-value as to whether overall, the distance matrix shows differences based upon the selected metadata category
- **Pairwise** group_significance.py -i otu_table.biom -m map.txt -c Disease -s kruskal_wallis -o kruskal_wallis_output.txt
  - Performs pairwise testing of each OTU present in the biom table, using the selected metadata category
  - Outputs p-values, plus corrected (FDR and Bonferroni)

LEfSe: widely used tool for pairwise comparisons

- Can be p… U or taxonom…
- Uses Lin… to calculate…
- Available… galaxy page
  - https://h… /root
- Outputs … cal form



Whole genome sequencing

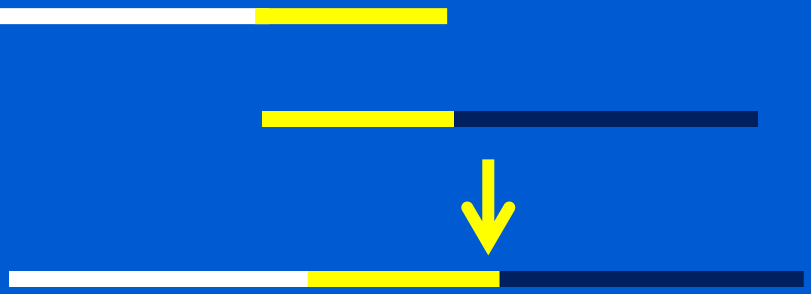
- Shotgun sequencing of all the DNA present in a sample
- May not include viral particles
- Will include human contaminant DNA

Removal of host DNA sequences

- **Not required with 16S analysis**
  - **Host DNA should not be amplified**
- **Contamination can occur with WGS**
  - **Variable with fecal microbes**
  - **High likelihood with other habitats**

Assembly

- Most packages not designed for microbiota
  - Thousands of species
- Unclear if needed with large reads
- Generally de novo
- Consider metAmos
  - Assembles with multiple packages
  - Determines optimal parameters for each



Options for host DNA removal

- Reference database of microbial organisms
  - Include sequences that align with dominant bacteria
  - Output will be limited to these bacteria
- Reference database of host DNA
  - Filter out alignments
  - BLAST or Bowtie2 / BWA

Assigning taxonomy

- **Comparison-based**
  - **Compare to database of sequences**
- **Composition-based**
  - **Internal structure**

Comparison based

- Align each sequence with reference gene or protein databases
- Gold standard is BLAST
- Derivative programs (BLAT, mega-BLAST and RAPSearch) increase efficiency, with acceptable loss of accuracy
- At UAB, we use RAPSearch, then MEGAN to assign taxonomy to the alignment files

Metaphlan for taxonomy assignment

- Assigns taxonomy based upon marker genes (in essence, polymorphisms)
- Metaphlan 2 includes 17000 reference genomes with 1 million marker genes
- Includes bacterial, viral, and fungal genomes
- Fast!
- Outputs .biom tables or .txt files

Composition based taxonomy

- **Bacteria have unique sequence features**
  - **GC content**
  - **Nucleotide repeats**
  - **Codon usage**

Functional annotation

- Alignment with BLAST
- Annotation with KEGG or other databases that link proteins to metabolic functions and pathways
- HUMAnN is a popular program that can tabulate the BLAST results

Functional annotation with 16S data

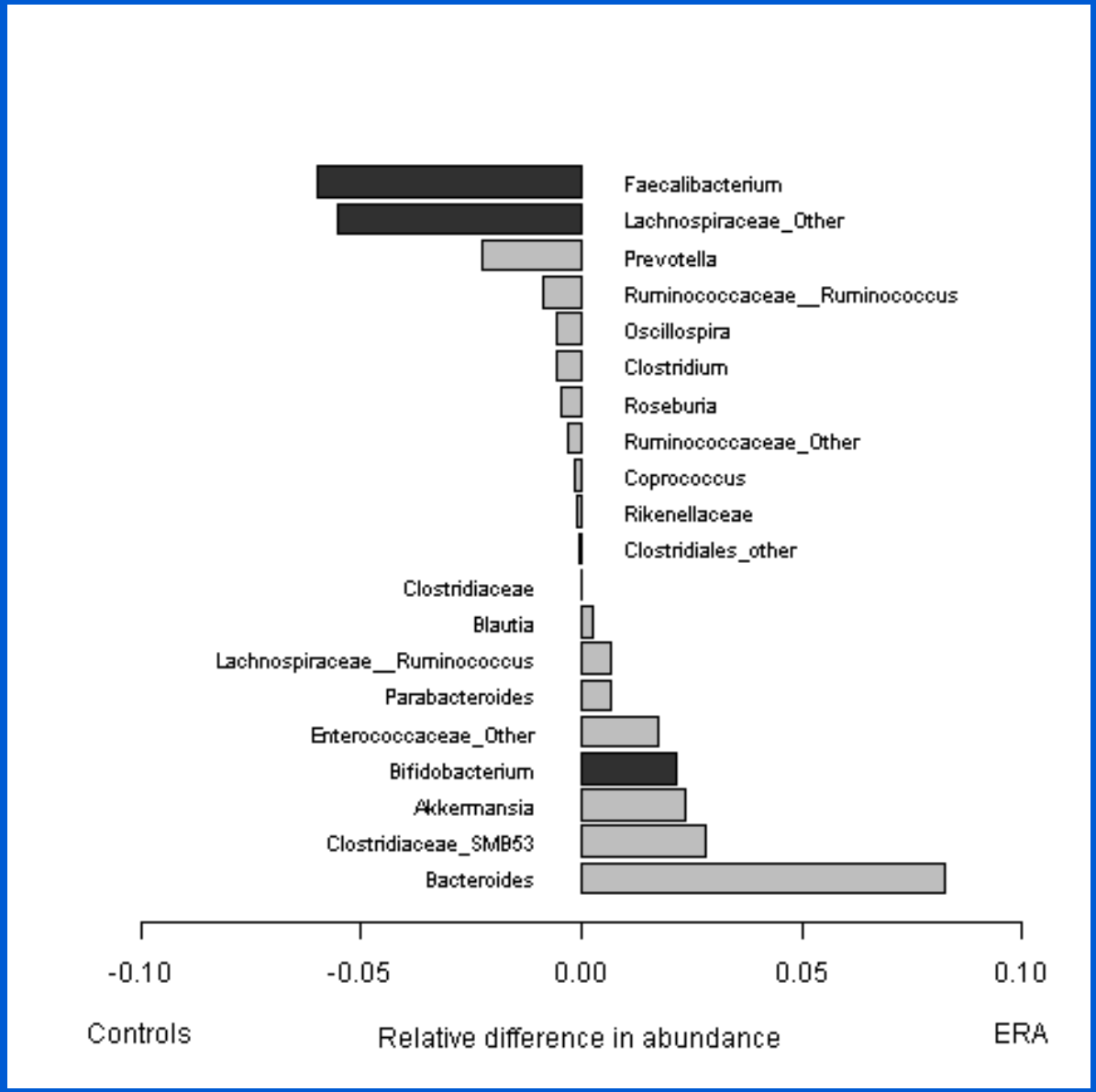
- 16S data provides taxonomic information
- Can infer function through taxonomy
- Phylogenetic Investigation of Communities by Reconstruction of Unobserved States (PICRUSt)
- This and other programs are available at Curtis Huttenhower's Harvard Galaxy site

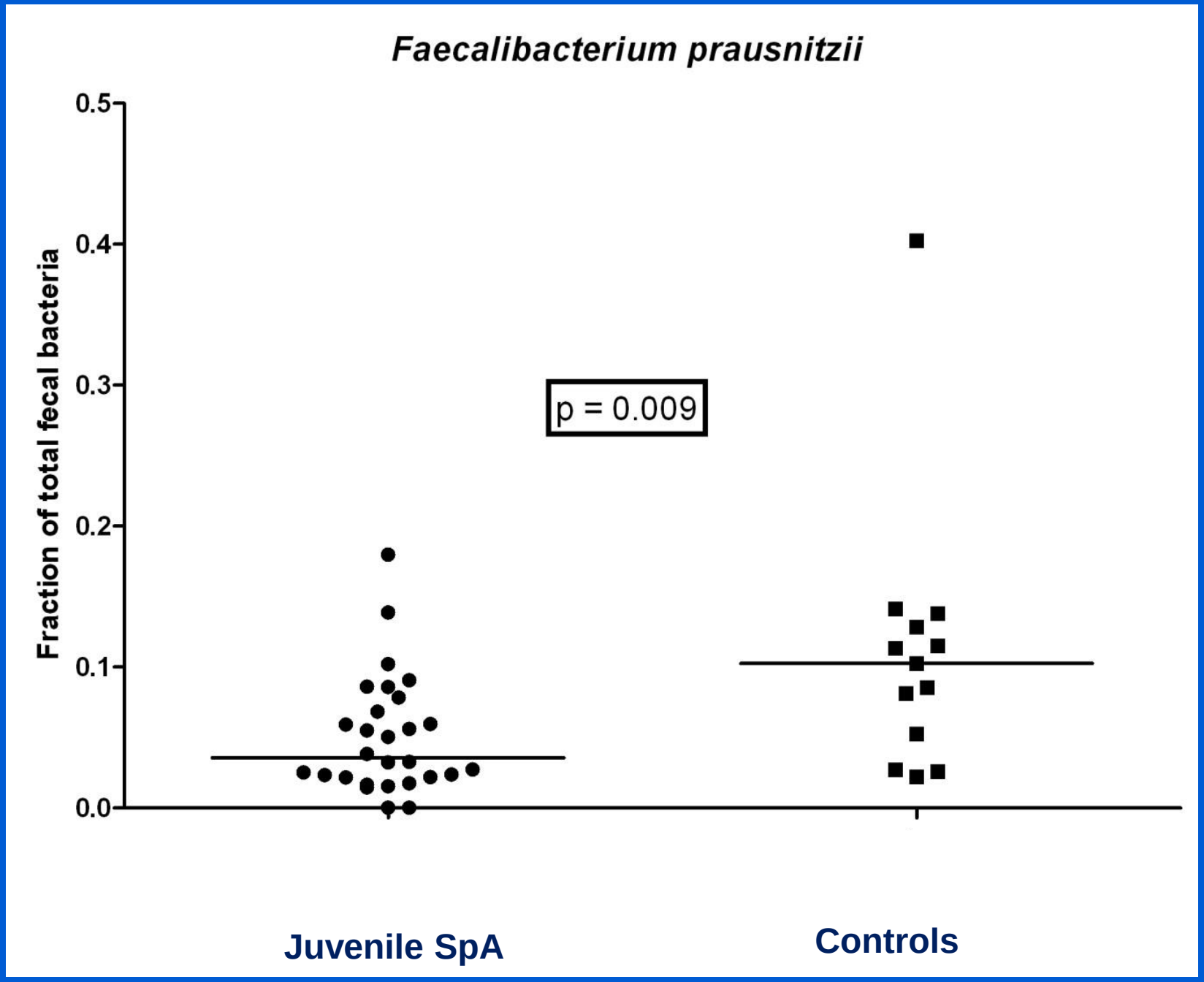
https://huttenhower.sph.harvard.edu/galaxy/root

Microbiota data

- Studied pediatric subjects with a form of juvenile idiopathic arthritis (JIA)
- This form, called spondyloarthritis, has clinical and genetic overlap with inflammatory bowel disease
- Comparator group are healthy children

Taxonomy

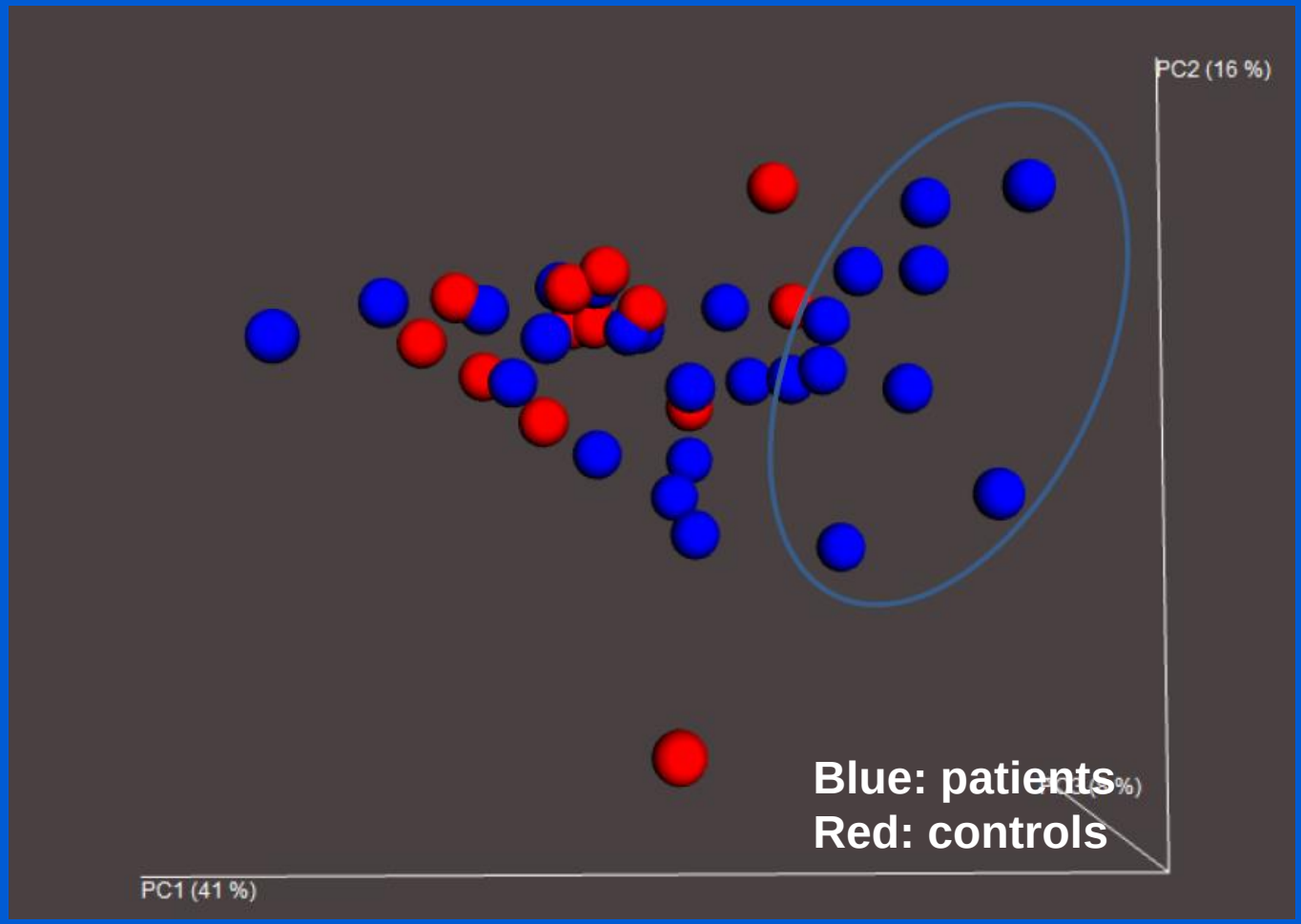

Faecalibacterium
Lachnospiraceae_Other
Prevotella
Ruminococcaceae__Ruminococcus
Oscillospira
Clostridium
Roseburia
Ruminococcaceae_Other
Coprococcus
Rikenellaceae
Clostridiales_other
Clostridiaceae
Blautia
Lachnospiraceae__Ruminococcus
Parabacteroides
Enterococcaceae_Other
Bifidobacterium
Akkermansia
Clostridiaceae_SMB53
Bacteroides
-0.10
-0.05
0.00
0.05
0.10
Controls
Relative difference in abundance
ERA



Fraction of total bacteria

Stoll, *Arth Res Ther* 2014;16:486

PCoA identified a small cluster



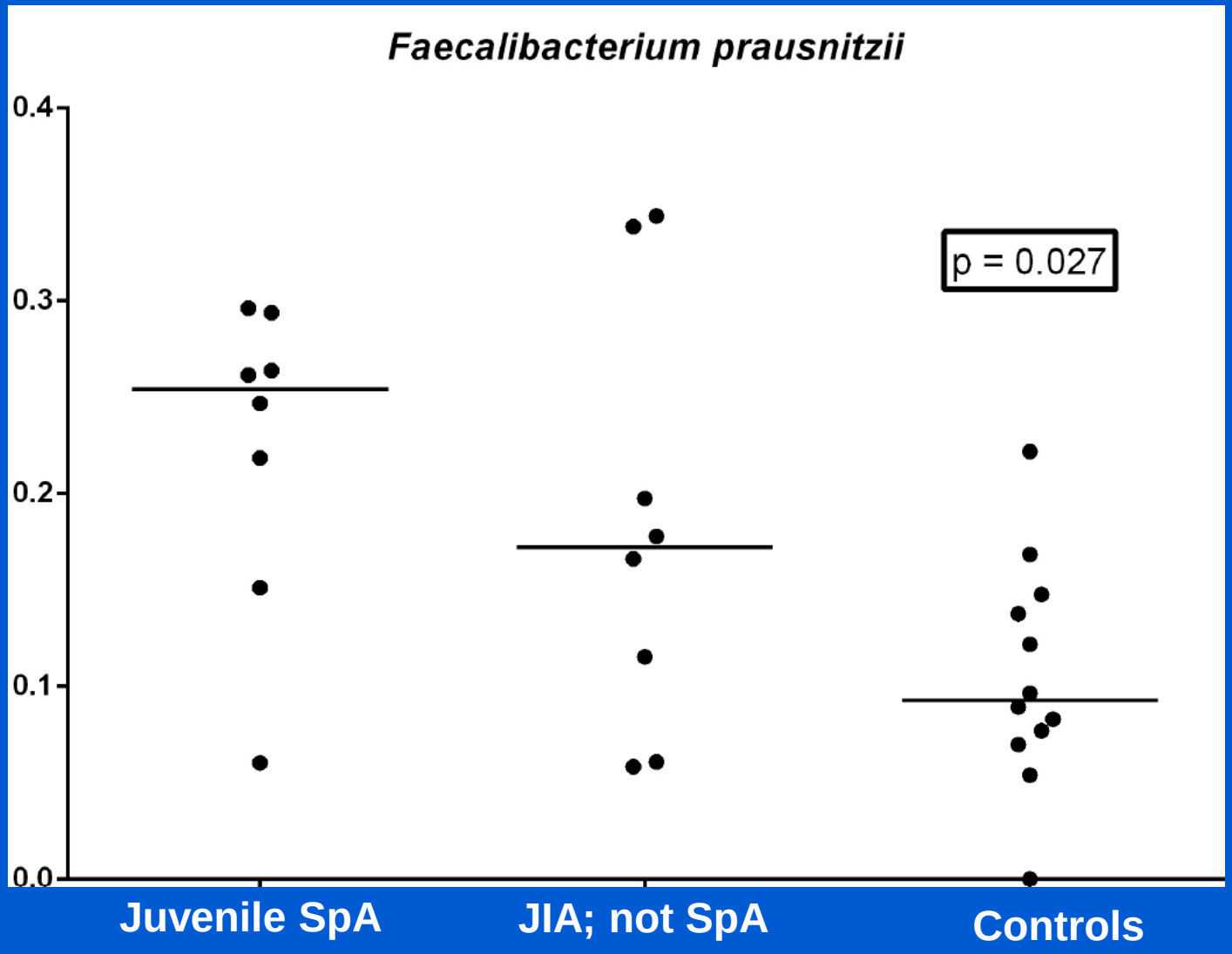
Stoll, *Arth Res Ther* 2014;16:486

Cluster 1 vs 2 of juvenile spondyloarthritis subjects

Species	SpA Cluster 1	SpA Cluster 2	p-value
n	8	19	N/A
F. prausnitzii (% of total bacteria, median)	3.2%	4.4%	0.897
Bacteroides (% of total bacteria, median)	41%	13%	< 0.001

Stoll, *Arth Res Ther* 2014;16:486

Altered *F. prausnitzii* is largely limited to SpA subtype

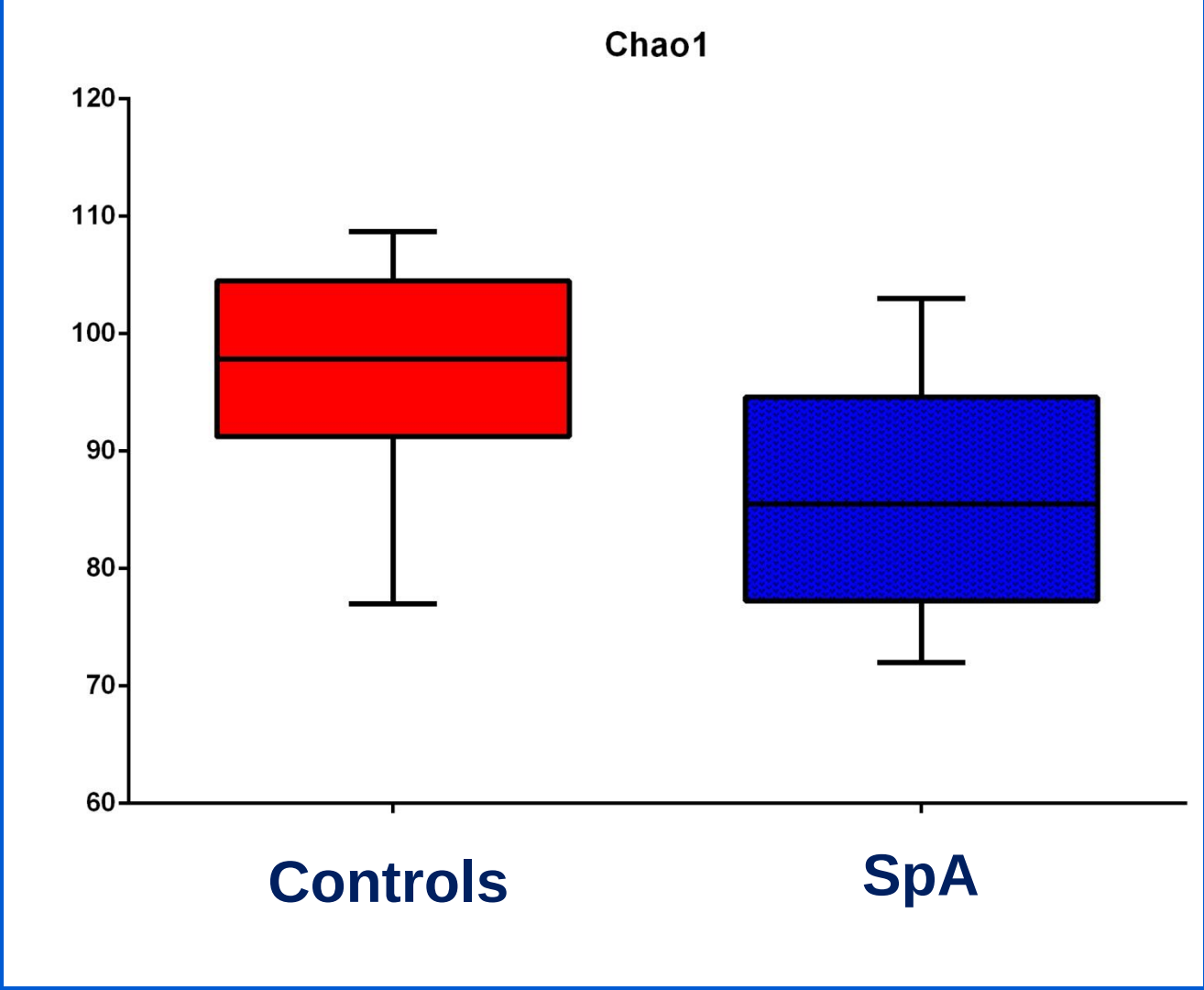


Stoll, *Clin Immunol* 2015;159:134

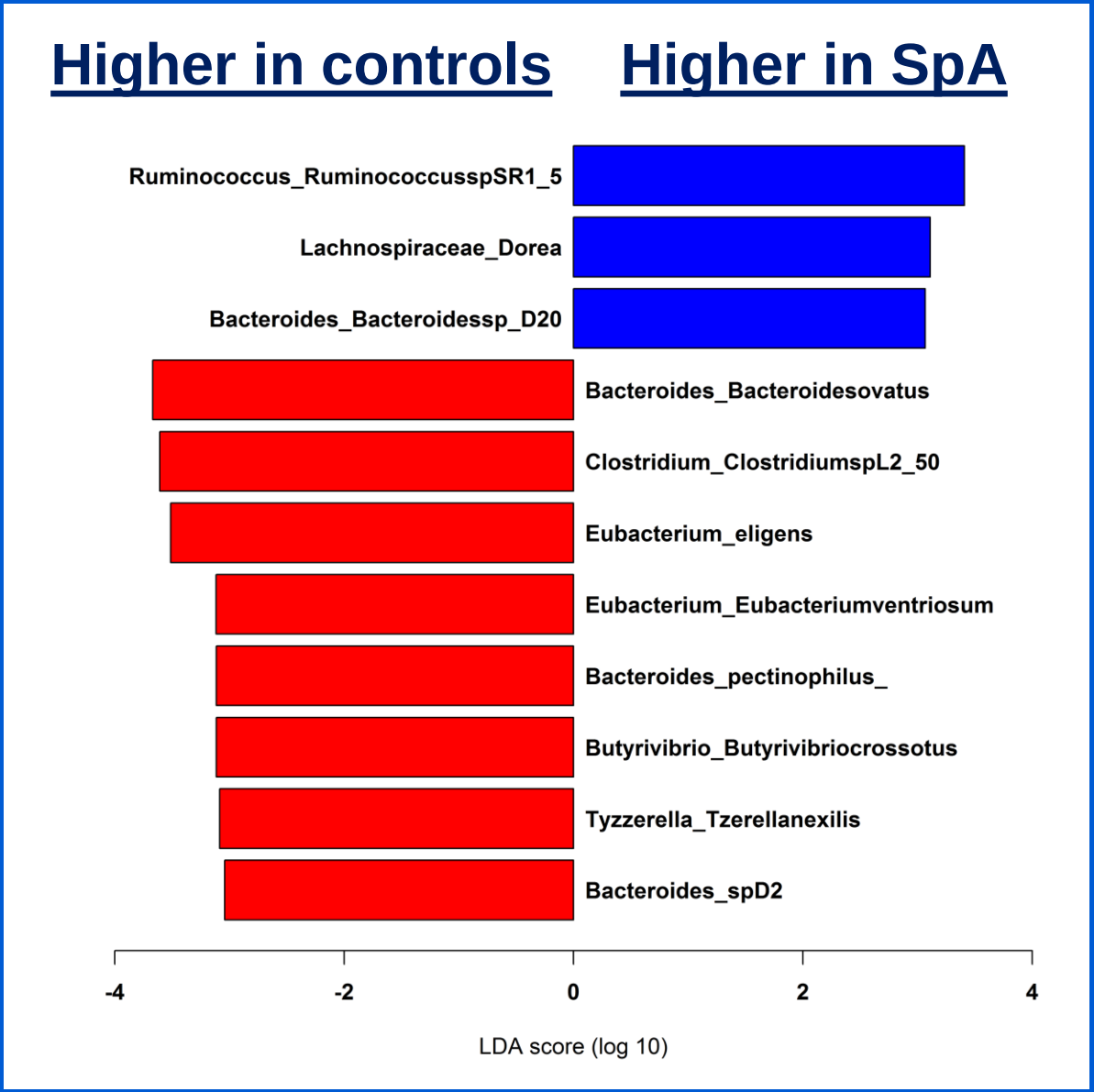
16S sequencing in SpA: summary

- **Differences at taxonomic level identified**
- **16S sequencing does not provide functional information**
  - **Educated guesses are possible: *F. prausnitzii* is a major butyrate producer**
- **We proceeded to assess enteric bacteria at the functional level**
  - **Whole genome sequencing**
  - **Fecal water metabolomics**

Lower alpha diversity in patients



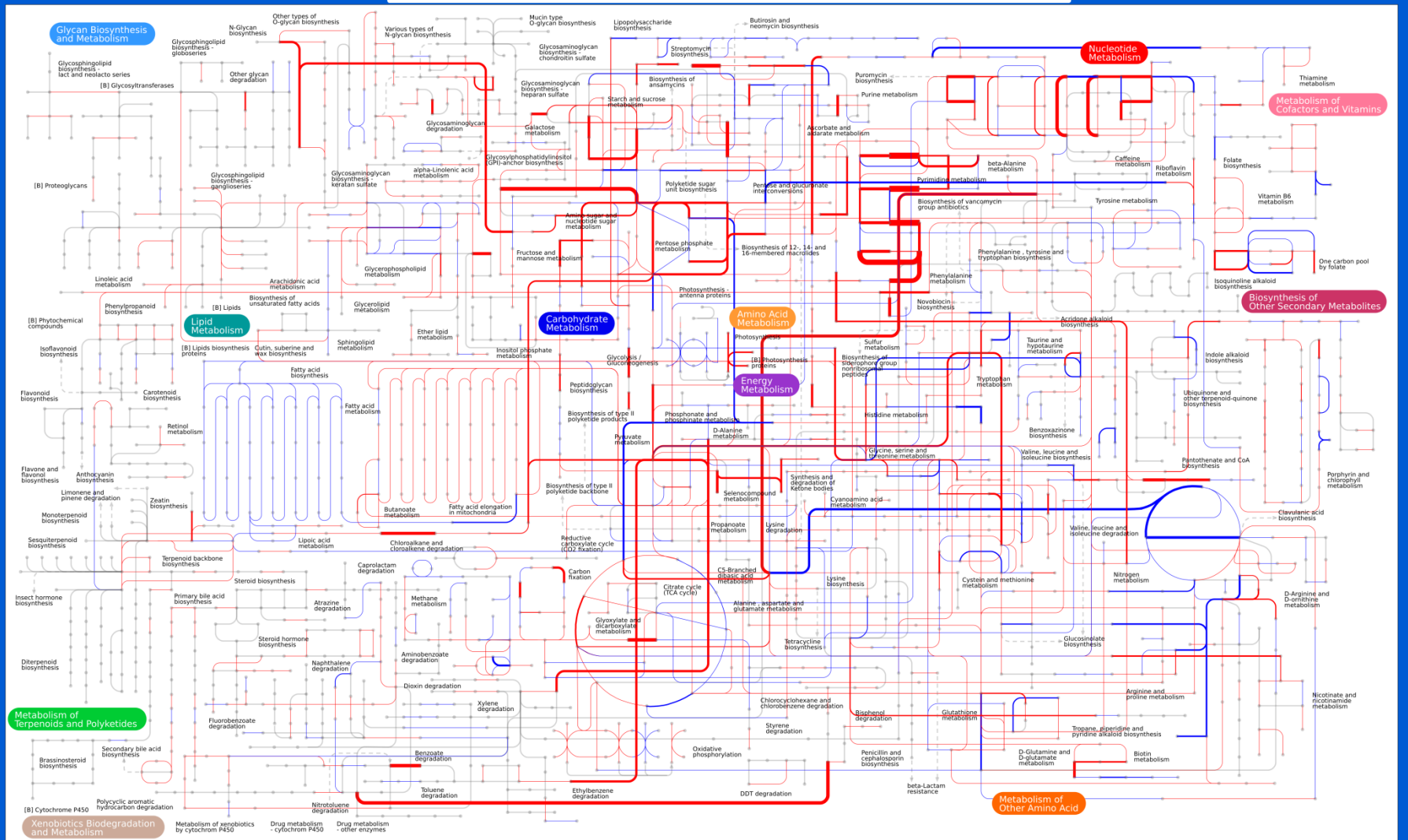
Taxonomic differences



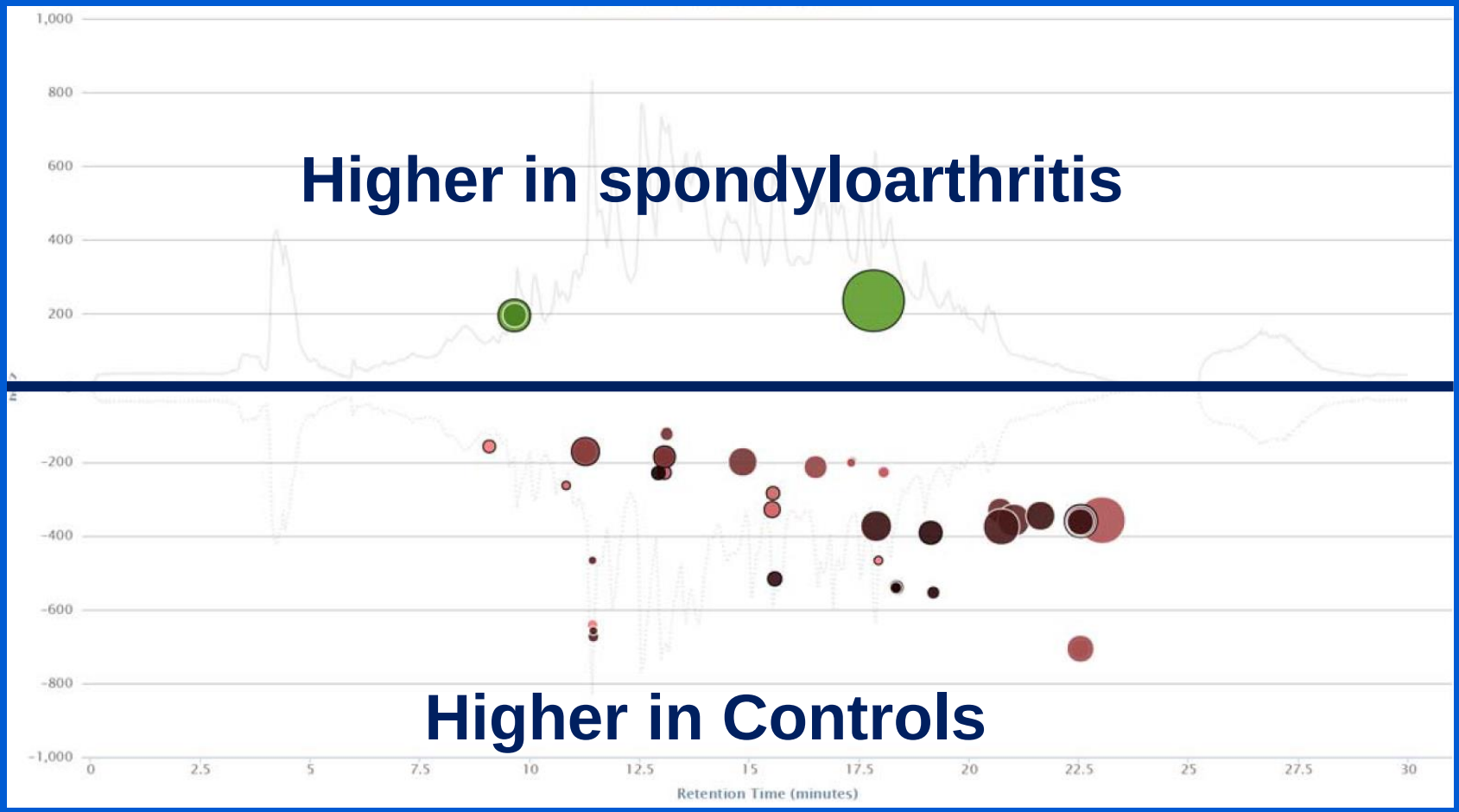
HUMAnN output: iPath2.0

Red: Higher in controls

Blue: higher in SpA



Differentially present ions



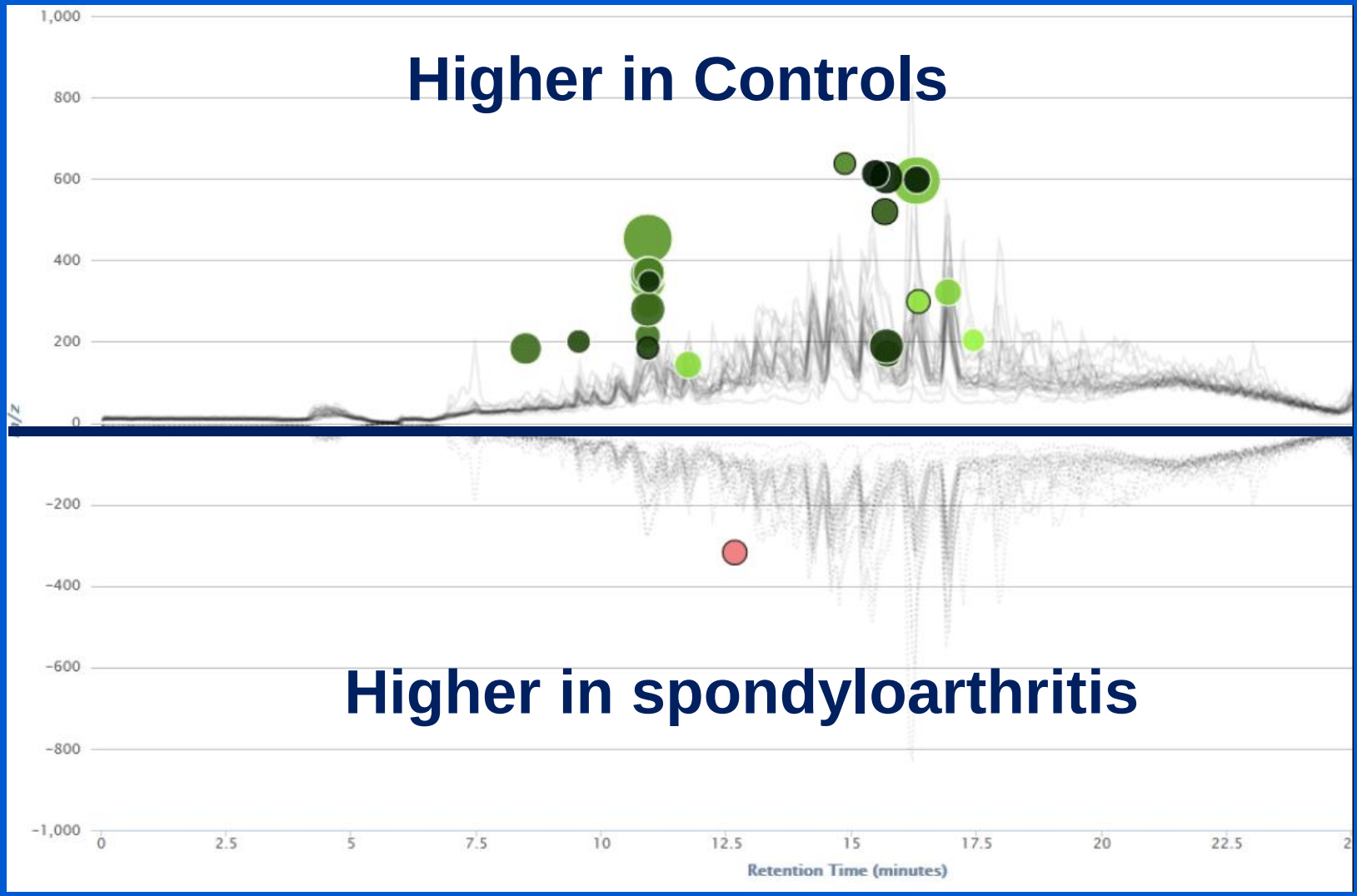
Mass : charge

Retention time

Pathways represented in controls

Pathway	Overlap	p
Butanoate metabolism	2	0.05127
Tryptophan metabolism	2	0.0982
Aspartate and asparagine metabolism	2	0.00587
Bile acid biosynthesis	2	0.01256
Xenobiotics metabolism	2	0.01668
Tyrosine metabolism	2	0.03864

Differentially present ions



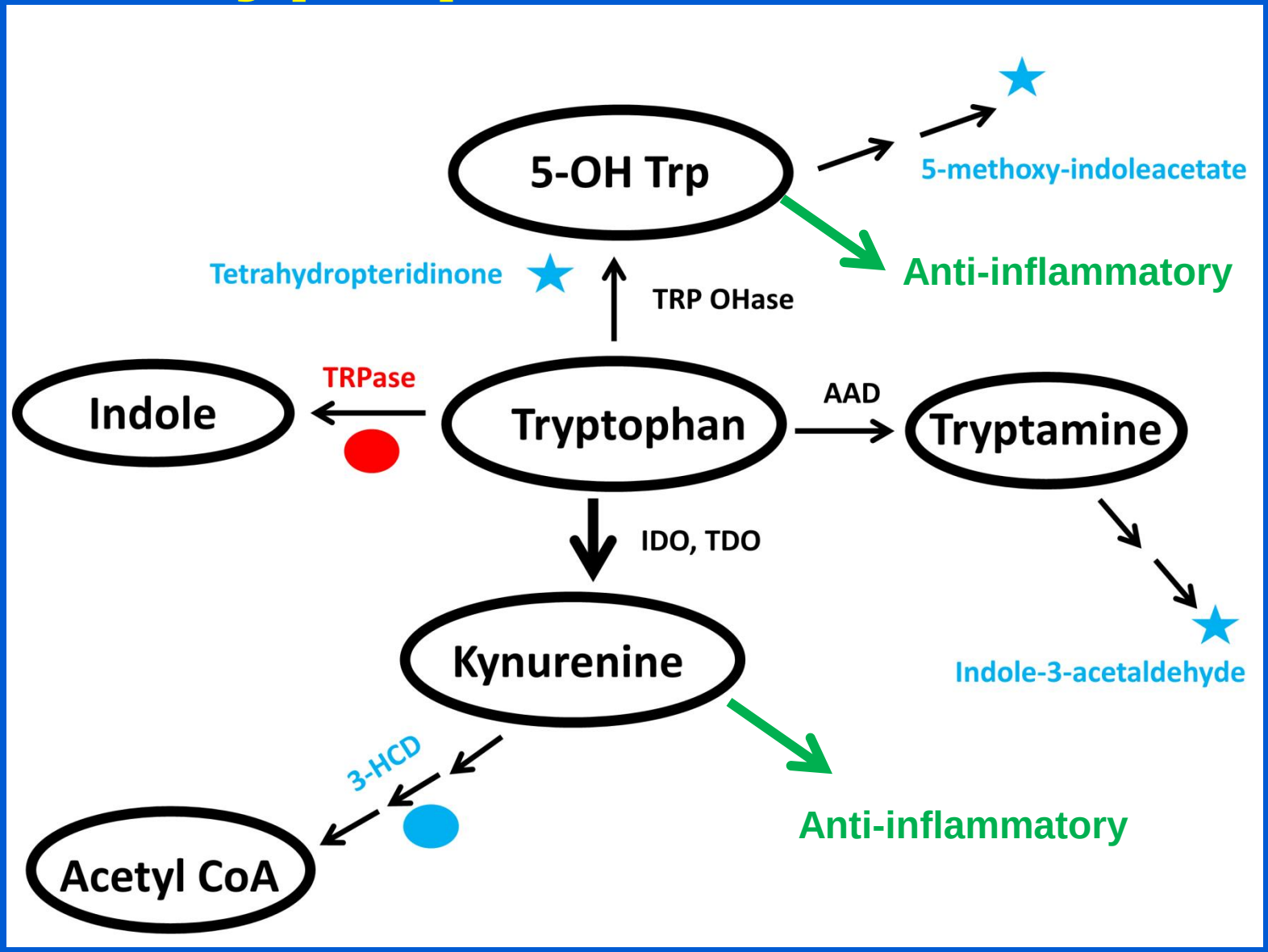
Mass : charge

Retention time

Pathways represented in controls

Pathway	Overlap	p
Biopterin metabolism	2	0.00042
Tryptophan metabolism	3	0.00198
Glycerophospholipid metabolism	2	0.00206
Urea cycle	2	0.00405
Tyrosine metabolism	3	0.01106
Drug metabolism - cytochrome P450	6	0.00171
N-Glycan biosynthesis	2	0.00313
Ubiquinone Biosynthesis	2	0.00507
Hexose phosphorylation	2	0.00777
Linoleate metabolism	2	0.00777
Histidine metabolism	2	0.01597
Drug metabolism - other enzymes	2	0.01867
Galactose metabolism	2	0.02839
Squalene and cholesterol biosynthesis	2	0.02839
Glycerophospholipid metabolism	2	0.04056

Tryptophan metabolism



Metabolomics and metagenomics of SpA: summary

- Patients had lower diversity at the taxonomic, genetic, and metabolic level
- Patients had decreased metabolites from the Tryptophan metabolism pathway
- Patients had increased genes coding for tryptophanase, which results in production of indole
  - Alterations in Trytophan metabolism may be associated with disease

Questions?