

Computational microbial genomics

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Chapter 1

Introduction

1.1 Microbes

Microbes are defined as whatever is not visible at the human eye: bacteria cells' dimensions are in the order of micrometre, while viruses in the order of nanometre. It is obvious how there is no visible part by eye. This is particularly true for viruses: their dimension make them almost impossible to perceive by any other method than genomics.

1.1.1 Prevalence of microbes

We are living in a microbial world: more than 90% of the biomass is composed of them and they are responsible for a great part of the biochemical cycle. Microbes can thrive in a variety of environment and according to some estimates they compose $10^{17} g$ of biomass. To put that in context the overall weight of the human species is three or four order of magnitude less. They also form the human microbiome, with important medical implication.

1.1.2 Difficulties in studying them

Of the predicted 30 million species to exist only thousands can be cultured in isolation in the lab. There is a need to create a way to directly study and characterized samples taken from the environment.

1.2 Genomics

Once the genetic material is isolated and sequenced a huge amount of information needs to be interpreted. The first sequenced gene was one of a bacteriophage. Also the first complete genome was one of a bacteriophage and is used by ILLUMINA as a control. The first bacterial genome was published in 1995 and was that of *Haemophilus influenzae*. It has a dimension of $1.8 Mb$ and sequencing took a year to complete. The first archaea was sequenced in 1996. In 1996 the genome of *S. cerevisiae* has been sequenced and it was noticed that the genome shows a considerable amount of genetic redundancy. The next step was to elucidate the biological functions of these genes.

1.3. LEVERAGING COMPUTATIONAL POWER

1.2.1 Comparative genomics

Studying two different strains of the same organism difference in the genome can be linked to the difference in phenotype.

1.2.2 Metagenomics

Metagenomics is the study of the DNA from all the genomes in an environment. By sampling all of the DNA from a given environment, it is possible to study the presence of bacterial ecosystems, independent of the ability to culture bacterial in the lab. Large evolutionary radiation of bacterial lineages whose members are mostly uncultivated and only known through metagenomics and single cell sequencing have been described as nanobacterial. They have small genomes and lack several biosynthetic pathways and ribosomal proteins.

1.3 Leveraging computational power

Despite the advantages in DNA sequencing technology the sequencing of genomes has not progressed beyond clones on the order of the size of λ because of the lack of sufficient computational approaches that would enable the efficient assembly of a large number of independent, random sequences into a single assembly. When moving from low-throughput to high-throughput biology statistical power is needed: the genome of a bacterium must comprise all the DNA coding molecules present in the cell/ With millions of reads from NGS of an environmental sample, it is possible to get a complete overview of any complex micro biome with thousands of species.

1.3.1 Comparing low-throughput and high-throughput pipeline

In a low throughput pipeline to find the pathogenic agent for a novel outbreak a panel of reasonable putative causative agents is identified. Then one-by-one cultivation protocols to grow the agents from the infected tissue are performed. This is very time consuming. High-throughput instead sequences the full DNA repertoire of the sample and try to identify the pathogen by its genomic signature.

Chapter 2

Escherichia Coli general informations

2.1 *E. coli* genomics

Escherichia Coli is a Gram-negative, facultative anaerobic, rodshaped, coliform bacterium, it pertains to the phylum of proteobacteria and to the family of Enterobacteriaceae. It can be grown easily and inexpensively. It has got genome with a length between 4.5 - 4.7 M bases, including about 4000-5000 genes, and about seven ribosomal RNA operons. Only the 38% of the genes of K-12 (one of the most studied bacterial strains of *E. coli*) were experimentally identified, overall 40-50% of the genes are to date without a known function. The original *E. coli* strain K-12 was obtained from a stool sample of a diphtheria patient in Palo Alto, CA in 1922.

2.1.1 *E. coli* long-term evolution experiment

The *E. coli* long-term evolution experiment led by Richard Lenski is one of the longest evolutionary experiments ever made (search "The Longest-Running Evolution Experiment"). The experiment started on 24th February 1988, and since that moment 12 populations of *E. coli* have been cultivated in the same environment. After each day (corresponding to the time of development of approximately 7 generations), a portion of bacteria from each flask was introduced in a new one, and let proliferating in it. Every 500 generation, it has been saved a sample of the bacteria of each flask, in order to track the evolutionary changes made. Today the experiment is on-going, and researchers reached approximately the 66000th generation. The study suggests a series of conclusions, to cite "long-term adaptation to a fixed environment can be characterized by a rich and dynamic set of population genetic processes, in stark contrast to the evolutionary desert expected near a fitness optimum" (Good et al 2017). In fact, despite of the fixed environment, some bacteria developed the capacity to aerobically grow on citrate, which is unusual in *E. coli* (around generation 31,000) and developed complex mutation patterns.

2.1.2 *E. coli* strains

E. coli could be found as commensal strains, pathogenic strains, or environmental strains. The pathogenic strains could pertain to these categories (which are not exclusive): enteropathogenic

2.1. *E. COLI* GENOMICS

(EPEC), enteroinvasive (EIEC), enterotoxigenic (ETEC), diffusely adherent (DAEC), adherent invasive (AIEC), shiga-toxin producing (STEC), enteroaggregative(EAEC), extraintestinal pathogenic (ExPEC). Resistances to antibiotics make even more difficult the process of categorization of *E. coli*. In 2011 in Germany, an outbreak of Stx-EAEC was responsible of the death of some people. An efficient counter-measure was found by sequencing the genome of those bacteria.

Shigella is *E. coli* with shiga toxin. It had been an issue for taxonomists.

Most of the genes are on plasmids, circular, additional to chromosome, and can be moved easily horizontally. Plasmids between different strains can be moved in enterobacteriaceae, this doesn't happen normally in other families. Some *E. coli* strains are even capable of causing tumors in humans: for example, colibactin-positive *E. coli* can cause colon and rectal cancer, by creating mutations which are responsible of the cancer onset.

several antigens can be used by taxonomists to categorize *E. coli* strains. In particular there are the O, H, K antigens, respectively related to the somatic, the flagella and the capsule. O antigens are 171, Ks are 80 and Hs are 56.

2.1.3 PanPhlAn - strain detection and characterization

Pangenome-based Phylogenomic Analysis (PanPhlAn) is a strain-level metagenomic profiling tool for identifying the gene composition and in-vivo transcriptional activity of individual strains in metagenomic samples. PanPhlAn's ability for strain-tracking and functional analysis of unknown pathogens makes it an efficient tool for culture-free infectious outbreak epidemiology and microbial population studies (PanPhlAn reference). This tool was for example used to study the strain responsible of an outbreak in Germany in 2011. This strain was a shiga-toxigenic Escherichia coli (STEC), and the study was conducted by Loman and colleagues in 2013. This method outlasted the traditional one.

sequencing means generally to sequence everything, it's normally difficult where to find it, although in *E. coli* is quite easy to understand the provenience. from all the world, populating diversity of *E. coli*, every time we sample we find points which are different from the reference genomes. points overlapping are people living together and share bacterias

2.1.4 Genomes of *E. coli*

In the genome of *E. coli* strains, it is possible to distinguish:

- **Core genome:** the set of all genes shared by all members of a bacterial species, it includes 1000 up to 3000 genes.
- **Accessory or dispensable genome:** the set of genes present in some but not all genomes within the same bacterial species. found on a single strain or in a subset of strains.
- **Pangenome:** core genome + accessory genome. set of all the genes foundable in the species strains. It is said to be "*closed*" when pangenome size tends to a maximum as number of genomes increases, instead it is "*open*" when pangenome keeps increasing as you add new genomes

Sequencing more organisms of the same species means to lower the amount of genes in the core genome and augment the number of those in the pan-genome (figure ??). Because of technical problems the probability of getting a gene and not forget it is different from 0, so why probably the sequencing of other genomes would lead technically to a plummet to 0 of the pangenome. With some mathematical formulations we can predict a more probable plateau (Rasko David 2008).

2.1. *E. COLI* GENOMICS

each *E. Coli* genome contains in a balanced way genes of the core genome and of the pan genome, for a total amount of genes correspondant to about 4700 genes (figure ??). Core genomes' genes are responsible of some basic cellular functionalities and utilities to survive environment, while instead elements of the pangenome are quite usually specific to the single strains, they are not always functionally well known.

ratios of the pan-genome and the core-genome are not equal in other organisms behave differently.

Chapter 3

NGS principles (second gen. sequencing) - From Sanger to third gen sequencing

Figure 3.1

- 1959 – First homogenous DNA purified
- 1970 – First discovery of type II restriction enzymes
- 1972 – First RNA gene sequence published (lac operon)
- 1975 – Sanger first publishes his plus/minus method of sequencing (unable to distinguish homopolymers)
- 1977 – Maxam & Gilbert publish their method (could distinguish homopolymers)
- 1977 – Sanger publishes Dideoxy sequencing method

NGS stands for ***Next Generation Sequencing***, and it represents the method of sequencing most used nowadays. Before that, a series of other discoveries were done, elenicated in the figure 3.1.

3.1 History of Sequencing

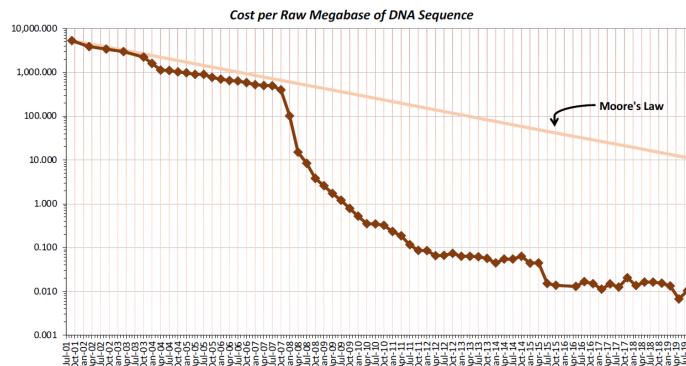
3.1.1 Progresses of sequencing

As the time passes, the cost of sequencing the DNA is diminishing. The rate of decrease actually is higher than the one predicted by the **Moore's Law**, "*Democratization of sequencing*" is seen nowadays, because of the costs lowering. After the Human genome project, several other animals and plants' genomes were sequenced.

The methods of sequencing actually can be grouped in three **groups**, that are:

3.1. HISTORY OF SEQUENCING

Figure 3.2



- **Chemical degradation** of DNA: it includes the method of Maxam-Gilbert
- **Sequencing by synthesis (“SBS”)** which is the most common approach and the first to be developed. It uses DNA polymerases in primer extension reactions Illumina, Pacific Biosciences, Ion Torren and 454
- **Ligation-based**: sequencing using short probes that hybridize to the template, the technologies pertaining to this class are SOLiD, Complete Genomics
- **Other**: Nanopores

3.1.2 The Chain Terminators

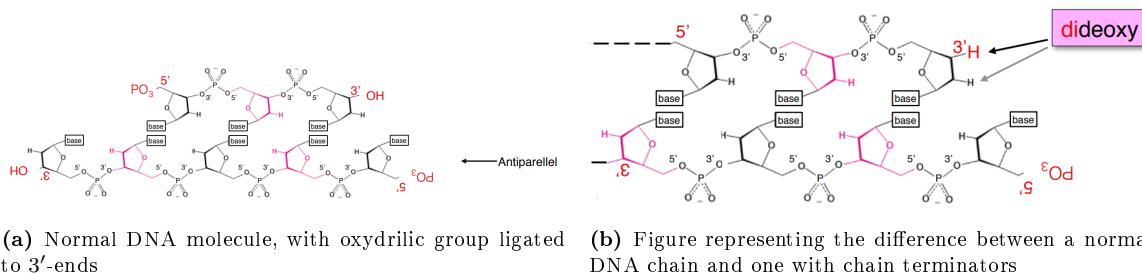


Figure 3.3: Normal DNA synthesys vs Chain terminators

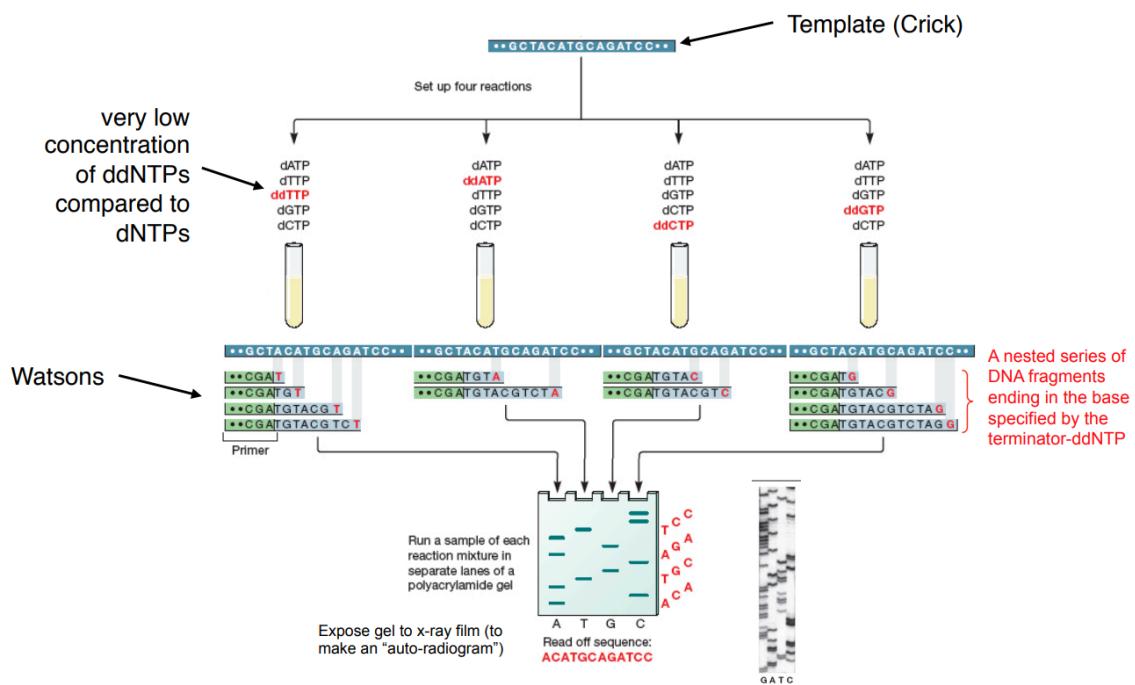
Normally, the addition of new nucleotides to a generated molecule of DNA happens with the 3'-end of the nucleotide chain (figure 3.3a). Chain terminators are dideoxy nucleotides, ddNTPs, that cannot be further extended. These nucleotides aren't able to add a new nucleotide on the 3'-end, as they have not the needed oxydrilic group (figure 3.3b).

3.1.3 Sanger method: the first one

The first method ever used to sequence DNA was designed by Frederick Sanger. The Sanger manual sequencing system consists in an *in vitro* process, which is described in figure 3.4, also named as

3.1. HISTORY OF SEQUENCING

Figure 3.4: The Sanger's method process



"primer extension" method. It is performed over a single-filament DNA sample, and it uses the chain terminators nucleotides, a type for each nucleobase: ddATP, ddGTP, ddTTP, ddCTP.

The reaction is done inside four different reactions tubes, each containing the sample DNA to be reproduced, a DNA polymerase, the normal nucleotides and one of the four possible chain terminators. The chain terminators are marked with sulfur-35, a radioactive atom. In each tube, the corresponding dideoxy-nucleotide was used with a concentration 10 times lower than the other "normal" nucleotides.

From the polymerization reactions, several molecules of DNA were produced, with different length: each replicative cycle is in fact terminated after the addition of a chain terminator nucleotide.

To reconstruct the initial DNA sequence, a long PAGE gel was prepared, with high concentration of urea ($6 - 7M$) to avoid the coiling of the DNA single-filaments. To run the gel, high voltages were required and it had to be highly resolute, as DNA's fragments are different only for a nucleotide. It was needed to do an auto-radiography of the gel in order to see the bands, in order to evidence the fluorescent signals.

To read the sequence you have to start from the shortest fragments, at the end of the gel, and carefully go up along the gel, looking for the first presence of a band in one of the four runs.

Past procedures: In the past, the Klenow's fragment (part of the 1st DNA polymerase) was used to perform the Sanger method, and the DNA to be sequenced was inserted inside the genome of an M13 phage.

3.2. DEVELOPMENT OF SEQUENCING MACHINES

3.1.3.1 Automatic sequencing

It does not use the radioactive signals, instead it uses fluorescent proteins. Several versions were developed after modifications of the Sanger method, in this order:

1. fluorescent primers marked with a single fluorochrome.
2. four aliquotes of the same primer were used marked with four different fluorochromes, able to emit different fluorescences.
3. four different fluorochromes were used to mark the single ddNTPs

Thanks to the use of 4 different fluorochromes, it was possible to use a single electrophoretic lane to carry the sequencing reaction. For this type of sequencing also, a cyclic replicative reaction was performed, with this procedure, made possible by using a thermal cycler:

1. **Denaturation at 95°C** of the DNA to be sequences
2. **Annealing at 50 – 70°C** of the primer specific to one of the two filaments
3. **Extension at 72°C** by using a *Taq*-polymerase. The use of the *Taq*-polymerase makes it possible to avoid the formation of coiled structures in the DNA molecule to be sequenced.

Traditionally, also in this type of sequencing it is used a long PAGE gel, as the Sanger's one, but with a great difference: all the ddNTPs are inserted in the same electrophoretic run, and after it, it is not needed an auto-radiography. Fluorescence, instead, can be triggered simply by irradiating the DNA molecules synthesized, which produce different fluorescences with different wavelengths.

More usefully, this sequencing method is performed by using a capillary, filled with a synthetic polymer, with the same function of polyacrylamide.

At the end of the analysis, it is produced an electropherogram, with a color depicting the probability of each nitrogen base in each position. The production of the electropherogram is made better thanks to algorithms to boost signal/noise ratio, to correct the dye-effects, and other effects that generate systemic errors.

Regarding the Sanger machines in use, the upgrades viewable in figure ???. Based on the same technology, new machines were developed and it was obtained a 1000 fold improvement.

When talking about the **human project**, it is important to specify that most of the job was done by using the Sanger automatic sequencing method.

3.2 Development of Sequencing Machines

The way you can get to the point could be different based on technology and the wanted output, comprised quality.

SOLID gave only 35-75 sequenced bases, and it is not used anymore; Sanger sequencing, the capillary, can give up to 1000 read length, with a low output; on MINION it was possible to sequence an entire genome of *E. coli*. A plethora of sequence machines are available today. None of the machines are able to sequence DNA from a sample of blood, some things have to be done. Nowadays machines producing bigger outputs of short reads are preferred.

3.2. DEVELOPMENT OF SEQUENCING MACHINES

Figure 3.5

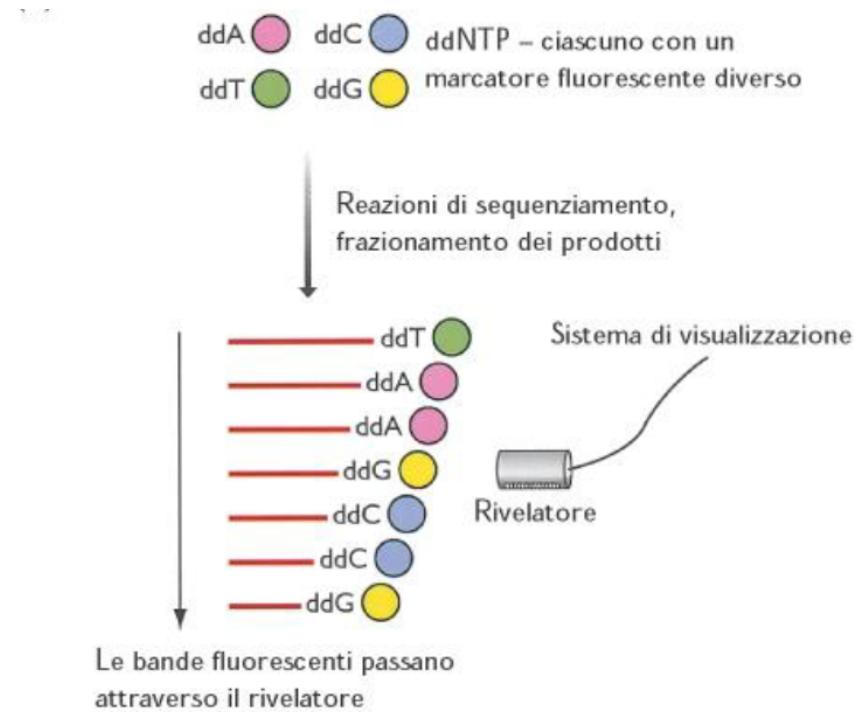
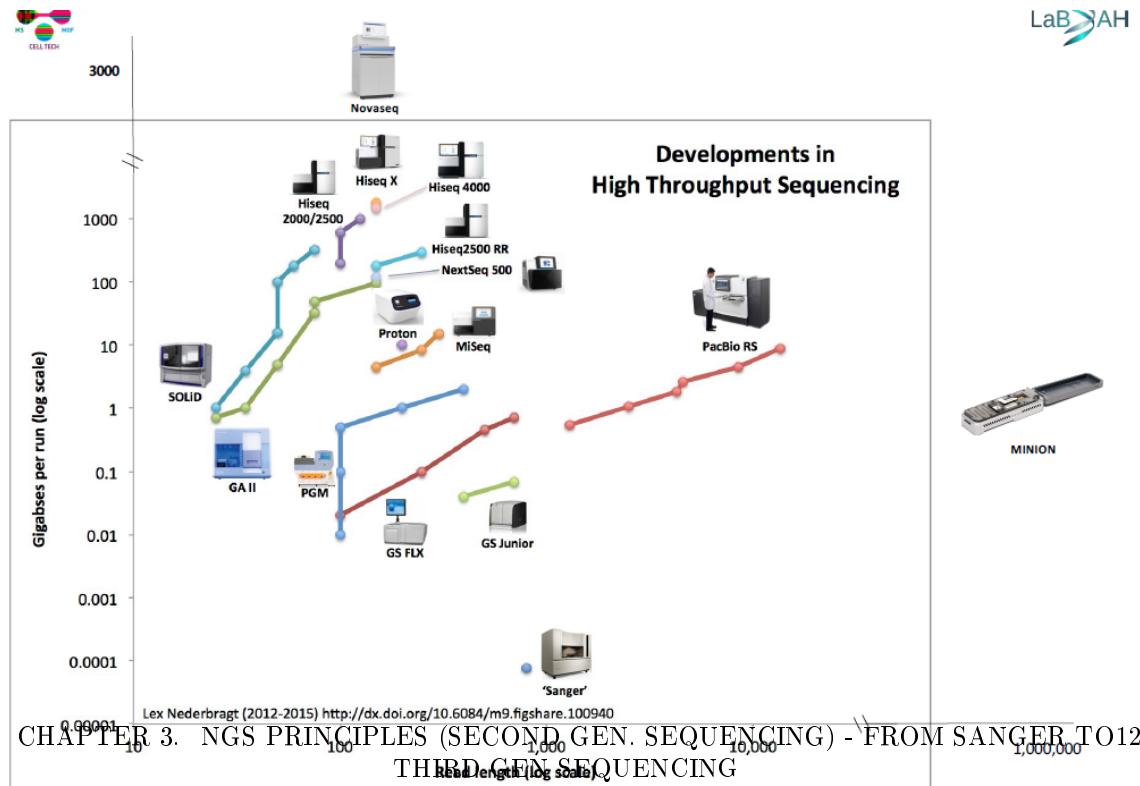
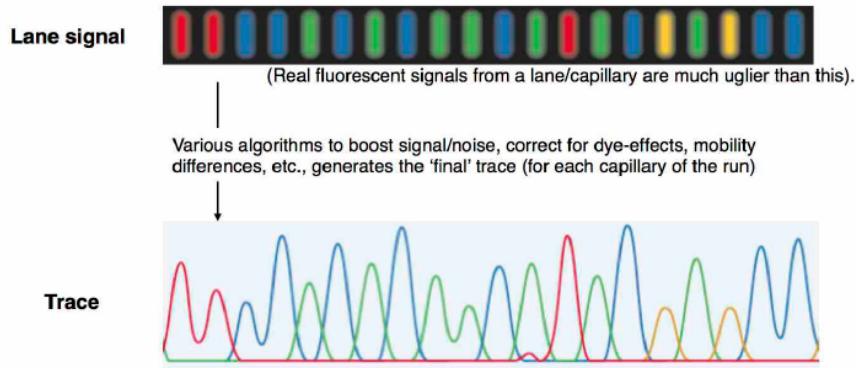


Figure 3.8: It can be noticed how recent developments had the scope of increasing the output data



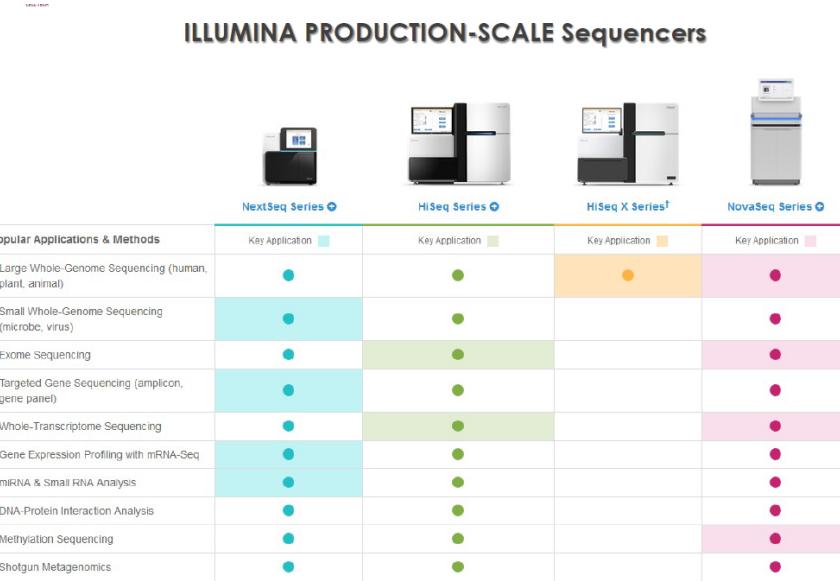
3.3. NEXT GENERATION SEQUENCING NGS

Figure 3.6



At the top of the market nowadays there are ILLUMINA machines, that use sequencing by synthesis method, NovaSeq is the biggest one. They need to amplify the signal through clusters formation.

Figure 3.9



3.3 Next Generation Sequencing NGS

The NGS protocol requires 3 steps, that are:

1. **Sample preparation:** series of fragments added

3.3. NEXT GENERATION SEQUENCING NGS

Figure 3.7: The implementation of capillary sequencing machines gave the possibility to make more runs than with the others. A 1000 fold productivity increase was allowed

		
Radioactive polyacrylamide slab gel Low throughput, labor intensive	AB slab gel sequencers (370, 373, 377) Fluorescent sequencing 1990-1999 6 runs/day 96 reads/run 500 bp/read 288,000 bp/day	AB capillary sequencers (3700, 3730) 1998-now 24 runs/day 96 reads/run 550 – 1,000 bp/read 1-2 million bp/day

2. **Clonal amplification:** which is needed to replicate fragments attached to the solid surfaces, since machines are not sensible to single molecules
3. **Sequencing:** ILLUMINA sequencing is one of the techniques used to obtain sequence data nowadays

Tools pertaining to the 3rd generation are those that permit to read a molecule without replicating it.

3.3.1 Fragments/Library preparation

Most of the sequences sequenced are fragmented in short read sequences, since most of the machines today used aren't able to sequence reads longer than some hundreds of nucleotides.

The fragments obtained have to be prepared for the sequencing process, through a process called **tagmentation**. The obtained fragments are shown in the figure. Those fragments are provided with one or two indexes, called also barcodes, two sequencing primer binding sites and regions complementary to the oligonucleotides present in the chamber (see in Clonal amplification chapter). The fragments' length has to be checked, depending on the scope of the process. Indexes are needed to run sequencing process on multiple samples, they are needed to distinguish those; when they are two, they permit to distinguish also the 2 types of sequencing that are performed, forward and reverse.

P5 and P7 are the oligos needed to attach fragments to ILLUMINA sequencing machines.

3.3.2 Clonal amplification and ILLUMINA sequencing procedure

Clonal amplification are necessary to amplify the signal from each single fragment. ILLUMINA machines make use of clusters to sequence DNA. Clusters are a group of DNA strands positioned closely together and generated from a single DNA filament. Generally, Each cluster represents thousands of copies of the same DNA strand in a 1–2 micron spot (figure 3.11).

3.3. NEXT GENERATION SEQUENCING NGS

Figure 3.10: Figure representing the a good prepared fragment, it has two indexes, two sequencing primer binding sites and regions complementary to the oligonucleotides present in the chamber

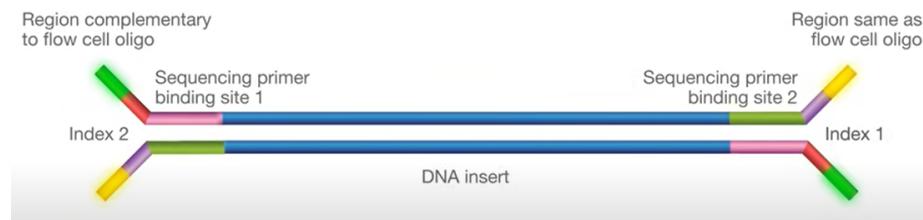
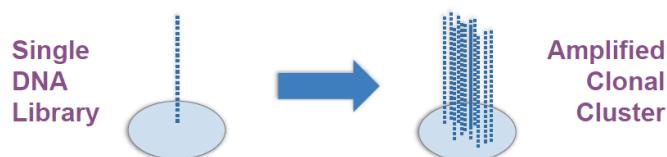


Figure 3.11



On patterned flow cells, the clusters' formation location could be known or not, in the first case it is said that there is a "Rigid registration" (figure 3.12).

ILLUMINA sequencers normally use slides of glass, named flow cells, and the fragments to be sequenced are able to flow over the channels. The temperature inside the cells can be changed to produce ligations or separations. The surface of the cells is functionalized with a series of oligos complementary to library adapters.

Two kinds of flow cells, the *patterned* flow cell permits to create clusters in specific positions, inside nanowalls, contrarily to *Random Flow Cell* which instead have randomly positioned clusters.

Once the fragments are made flow over the chambers, they can bind only to p5 or p7 (ILLUMINA oligos), the two oligos functionalizing the plate. Once the fragments are attached to the surface, using temperature and solvents flows you can control the sequencing process. To see the entire procedure: (procedure on Youtube: video about ILLUMINA sequencing). In the video, it is shown the two index sequencing process.

3.3. NEXT GENERATION SEQUENCING NGS

Figure 3.12

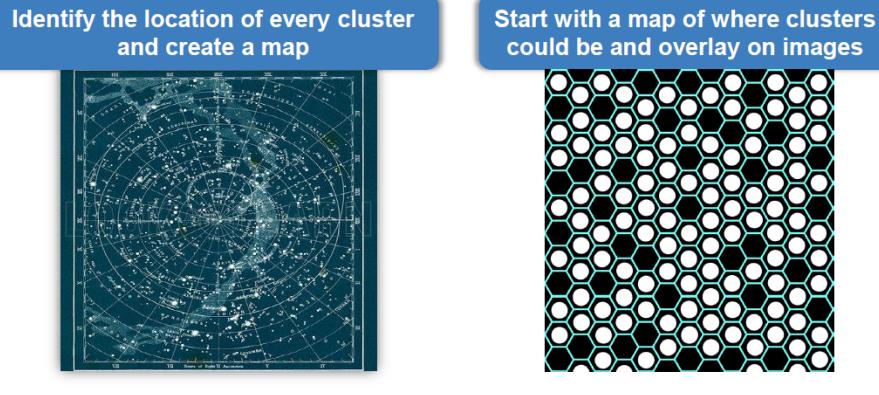


Figure 3.13

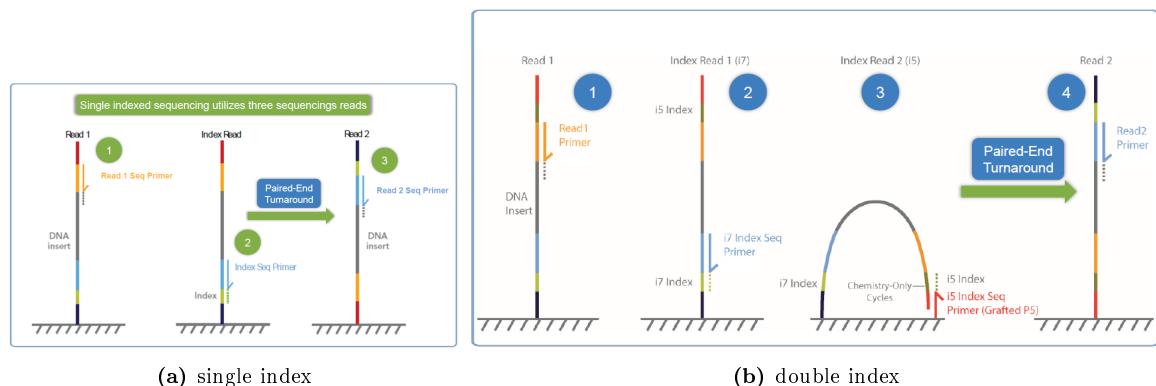
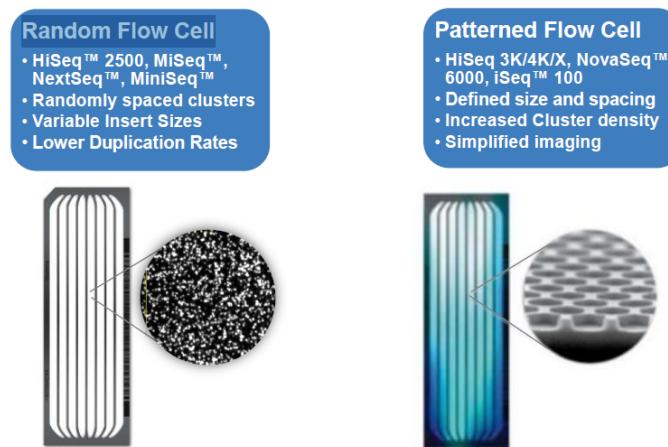
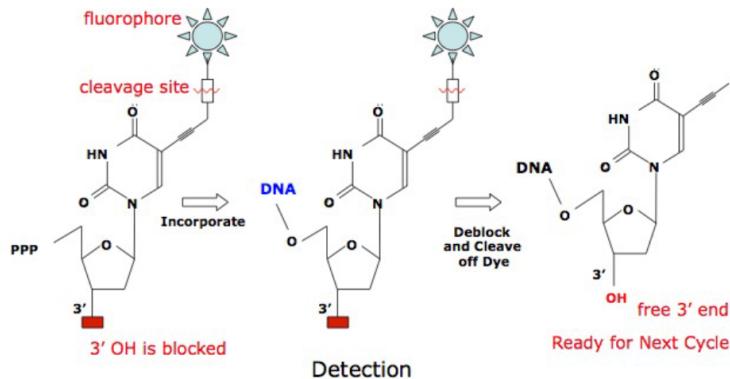


Figure 3.14: Single/double index for ILLUMINA sequencing

3.3. NEXT GENERATION SEQUENCING NGS

With 1 and 2 indexes the sequencing process actually differs, as shown in figure 3.14

Figure 3.15



To perform the sequencing process, ILLUMINA machines utilize *reversible terminators* (figure 3.15). They permit a real time analysis of the sequencing by synthesis reaction, and because of this they are different from the Sanger method. Fluorophores are reversible, they can be cleaved to eliminate the light signal.

Figure 3.16

4-Channel Chemistry				2-Channel Chemistry				1-Channel Chemistry						
	A	G	T	C		A	G	T	C		A	G	T	C
Image 1	●				●				●		●			
Image 2		●				●					●			
Image 3			●				●					●		
Image 4				●				●					●	
Result	A	G	T	C		A	G	T	C		A	G	T	C

----- Intermediate chemistry step -----

To perform their activity, ILLUMINA sequencers could be of 3 different types: 4-Channel, 2-Channel or 1-Channel, depending on the number of fluorescent molecules used. In the case of the 4-Channel technology, 4 images are taken in each cycle, and each cluster appears in only one of four images 3.16. 2-Channel technique is used by some sequencing machines, like NextSeq 550, MiniSeq, NovaSeq 6000

4-Colors base calling is needed to make the true signal the purest, and after, The base with the highest intensity becomes the called base for that cluster. In the case no base is clearly related to a position, *N* is the result.

The reading process could be done in two ways: through single reads, on a single extreme of the fragments, or paired-end, on both the extremes. The second one in particular gives structural and 2 sequence informations.

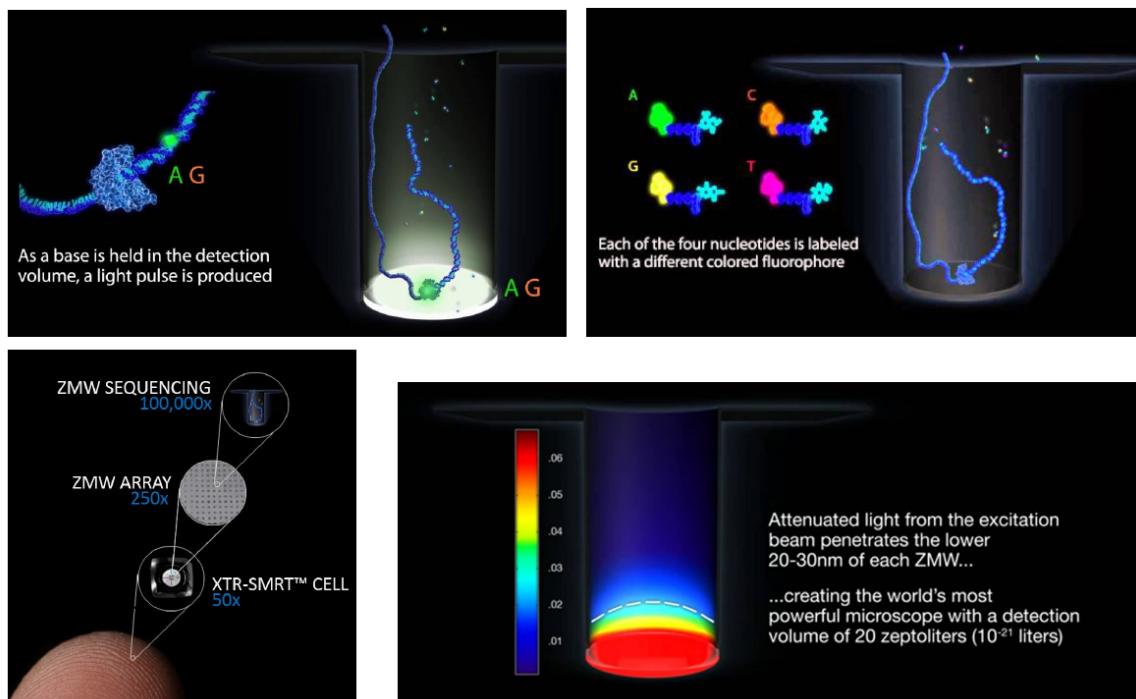
3.3. NEXT GENERATION SEQUENCING NGS

3.3.3 Pacific Bioscience (PacBio)

the long DNA filament to be sequenced is attached to a polymerase, over the surface of a SMRT (Single Molecule Real Time) cell. This cell is really small, and at each nucleation process a light signal is emitted. The produced light is not able to get out of the walls, and its duration is extremely restricted. Sequencing, also in this case, is made by sequencing, and the main advantage consists in the possibility of sequencing really big DNA molecules.

In the video PacBio procedure it is briefly shown how the process works and what are the strategies used to reduce errors.

Figure 3.17



3.3.4 Nanopore sequencing

Intramembrane proteins are used, sequence detected through the passage of DNA nucleotides, which produce different voltage changes. During the first periods, this type of instruments gave great amount of errors, nowadays the technique is improving. Nanopore Sequencing

Chapter 4

Sequencing data

After all, genetics/genomics studies a code of a digital information (4bases/2bits). We should try to be as hypothesis driven as possible and use the already available and processed data to guide new data analysis. Be aware that, in genomics, data generation is the starting point of the study (the ratio of wet experiments vs computational effort is 1:10)!

Given the biological problem at hand, we need to choose the optimal sequencing machine. To achieve this, we need to consider:

- Throughput;
- Cost;
- Read lengths,
- Data output (reads per run);
- Coverage;
- Sequencing errors (indel, substitution, CG delation, AT bias). Although the error rate is decreasing with new technologies;
- Library preparation compatibility;
- Speed (run time)

Suppose that we want to sequence a genome of a bacterium: which is the best machine that we can use?

Illumina NovaSeq : if one wants to sequence a lot of DNA molecules at the same time, genomes, metagenomes. It can't go over the 300 bp readlengths runned, but it has the highest throughput so far (3TB of output). It is capable of multiplexing, so we have a unique barcode for any input sample.

Illumina iSeq : If you need to sequence shorter genomes. From iSeq to NextSeq (increasing the reads lengths).

NanoPore (minion) : pocket-sized wet-lab free sequencer for DNA, RNA and (possibly) proteins, but the read lengths is smaller than Illumina's. The machine is cheap; the running flow is more expensive (going down by time). It's a real-time sequencer.

4.1. INTRO TO SEQUENCING RAW DATA

PacBio : carries a high amount of error. A solution to this is using more than one technology for the same project. For example, if the goal is to put together a genome of a bacterium, the challenge of using PacBio is errors in the sequence, while Illumina is unable to reconstruct the sample because there are ambiguities. By using both technologies, PacBio will construct the genome and Illumina will correct the sequence.

Anyway in the case of bacteria, you can use only PacBio because you have to sequence more and more, and since the PacBio error is random (not systematic), you will able to overlap the reads and solve the errors. PacBio has problem with homopolymers (same base or same set of bases repeated multiple times), which is a systematic error, more difficult to overcome just by sequencing more. If one wants to sequence a complex sample - with more than one genome - we cannot do the trick of overlapping, because we don't know a priori which reads are coming from which organism. Another trick to work on this problem (complex samples), adopted by miniON and also PacBio, is to sequence the same molecule multiple times. By doing this you will have a molecule sequenced multiple times, and then we can go on with the other reads, and in conclusion do the consensus on that (sequencing error is usually declared by the machine).

4.1 Intro to sequencing raw data

All sequencing platforms can produce FASTQ output files or they can at least convert their format to FASTQ. FASTQ contains the sequencing reads and the quality of each base and needs to be stored in a compressed format.

The first output is an internal output, not directly accessible by the user. You will see only the translation of the raw signal (FASTQ format). The internal raw signal can be completely different (ionTorrent measures pH, Illumina takes photos of light signals, nanoPore looks at a signal coming from a little hole, . . .). However, all machines, internally, interpret this physical signal into a file which is in FASTQ format (the standard format).

Sequencing might lead to short (100nt) strings of few digital values (A T G C), puzzles of 3,000 M letters (solved then by assembling and mapping) or thousands of puzzles of 5 M up to 5,000 M letters, meaning 3 TB of letters that need to be made sense of. Furthermore, many pieces do not fit due to sequencing error/SNP/structural variant or low complexity region/microsatellite/repeat.

More on the FASTQ format will be covered in section 4.1.2.

4.1.1 Base caller

A base caller is an instrument (algorithm) inside the machine needed to understand how to work with the sequence, in particular, it translates the analogical signal of the reading into numbers/nucleotides. The most popular algorithm is **Phred**.

Phred is an algorithm based on ideas about what might go wrong in a sequencing reaction and in electrophoresis. It was tested on a huge dataset of 'gold standard' sequences (finished human and *C. elegans* sequences generated by highly-redundant sequencing). Its results were compared with the ABI base caller and Phred was considerably more accurate (40-50% fewer error).

NB: When things are getting too messy, the algorithm needs to understand that it is impossible to recover an high quality sequencing and so the base caller just needs to be able to give up

4.1. INTRO TO SEQUENCING RAW DATA

for some reads (the ones without a high quality). The confidence that the base caller has to call a certain nucleotide ATCG is annotated in the FASTQ file, so one can later decide what to do with the bases that are not supported or clear.

4.1.1.1 Errors solved by Illumina's base caller

Phasing noise ϕ : when a certain base is not seen frequently, the first time in which you will see this specific base (or pair of bases) there will be a peak (a spike) in the graph, increasing the signal of the nearby bases. There are going to be some errors in estimating the real nucleotide that is occurring in the site. This problem can be solved by waiting more time between a reading and another, but the sequencing will be less efficient (slower) and the throughput will be lower. So the machine needs to find a trade-off between the efficiency and the clear reading.

Signal decay δ : after a while one has read the same base, or the same repeated couple of bases, the signal will go down and at some point will be indistinct (so at some point you will need to cut the read since it will be not trustable after a while).

Mixed cluster μ : when you have a cluster mixed in two sequences, the machine will read the signal resulting by this two strings simultaneously.

Boundary effects ω : in this case the machine needs to interpret an image (a digital object.. and a blue point will appear if you magnify it a little bit). To estimate the intensity of the blue charter here you should probably look at the internal part here and average out all the pixels. Putting the edge of the internal part is tricky, since it is a gradient and not clear. So this error occurs when you can't figure out what is the signal and what is the around background.

Cross-talk χ

Fluophore accumulation τ

4.1.1.2 Density on the flow cell

There is an optimal clustering in the flow cell and the presence of readings that go one through the other represent a problem. In the case of the dark yellow spots, the one that are too closer are going to be considered as a single peak. It is even more a problem if the spots are of different colours, and the signal will be a result of different bases.

In the case of under clustered the machine doesn't read simultaneously as many reads as it should do (and it is not a problem of the sequencing technology, it is a problem for the researcher who did not optimize the method). By increasing the flow' density you will have an optimal clustering. If you increase the density too much you will have and over clustered sequencing.

4.1.1.3 An ecology of base callers

With base callers the matter is finding a satisfying trade-off between accuracy and computational efficiency.

The quality associated to a single nucleotide is internally estimated by the machine. We need to check later if the quality of the read is right. This quality is the estimation of the probability that that nucleotide in that position is wrong.

4.1. INTRO TO SEQUENCING RAW DATA

There's a difference between PHRED score reported by base caller vs. PHRED score when mapped to a reference sequence. There are some base callers more calibrated than the others (like the Ibis). In general base callers need to be calibrated with a standard to make sure that the estimations of the quality are accurate enough.

4.1.2 FASTQ format

1. @SEQ_ID
2. GATTGGGGTTCAAAGCAGTATCGATCAAATAGTAAATCCATTGTTCAACTCACAGTT
x N 3.
3. +
4. !”*((***+))%%%++)(%%%%.1***-+*)**)55CCF»»»CCCCCCC65

Chapter 5

Mapping

Chapter 6

Assembly

Chapter 7

16S-rRNA sequencing

7.1 Introduction to metagenomics

7.1.1 Definition of metagenomics

The term **metagenomics** refers to the "*study of uncultured microorganisms from the environment, which can include humans or other living hosts*" whith "*focus on taxonomic and functional characteristics of the total collection of microorganisms within a community*". The main way to analyze the entire microbial population of an environment is through **high-throughput sequencing** of nucleic acids isolated from the sample; we can further distinguish two approaches, namely **16S rRNA gene sequencing** and **shotgun metagenomics**.

7.1.2 Why studying the metagenome

Microbes are basically everywhere, in and outside of our bodies, in oceans, glaciers, hot springs and rocks. Given how widespread and abundant microbes are, studying the metagenome provides us plenty of information (both on human and non-human microbiome and environment). For instance, it has been shown that the microbiome correlates to several diseases, therefore it can be used as a non-invasive **biomarker** (colorectal cancer, immunotherapy efficacy, autoimmune diseases...); the list of activities microbes are involved in is evergrowing.

7.1.3 Differences with older microbiome studies

The microbiome was discovered many years ago but there were no tools to analyze it properly; the only way was to culture and isolate each bacterium, which is an unfeasible approach to study the entire community, since only some bacteria can be grown in lab and it still would take an unreasonably long amount of time. The advent of high-throughput technologies is what made possible to study the microbiome of a sample, reducing significantly times, costs and increasing substantially the fraction of the microbiome that can be known.

7.1.4 Example: skin microbiome

Some studies were performed on skin microbiome (Segata et al, Nature Methods 2012, Truong et al, Nature Methods, 2015); only about 60% of the contigs (of various size) were mapped to known microbes while 40% belonged to unknown species. When separating these sequences based on GC

7.2. 16S RRNA SEQUENCING

content and abundance, many clusters formed, some with higher abundance while others with lower abundance, probably due to the low GC content that makes more difficult for the machine to sequence them, therefore causing them to be underestimated. Studying this 40% of unknown sequences is one of the main tasks of metagenomics.

7.2 16S rRNA sequencing

16S rRNA sequencing is one of the first techniques developed to study the microbiome, since it does not require a huge amount of sequences nor excessive costs; for these reason the technique became popular.

7.2.1 Simplified 16S rRNA analysis workflow

The general workflow for a 16S rRNA analysis is the following:

- **DNA extraction** from the entire community present in the sample; some bacteria will be over-represented while other will be under-represented.
- **Selective PCR amplification of 16S rRNA gene** (due to the characteristics explained below)
- **High-throughput sequencing**
- **Sequence mapping** against genomes in databases; this allows to define which bacteria (and which variants of those) are present in the sample and to find new and unknown bacteria.

7.2.2 16S rRNA gene

The ribosome is one of the most conserved, if not the most conserved, structure in all living organisms, making it one of the best phylogenetic markers. In prokaryotes, the ribosome is composed of several elements, both proteic and RNA based. Of the RNA based ones, 3 of them are **ribosomal RNAs** (rRNAs), namely 5S, 16S, 23S. Since these components are fundamental for any bacterium, all bacteria present the genes codifying for these rRNAs; most of the sequences are highly conserved but some regions have some variability which, since this variability is species-specific, can be used as a *barcode* to find and classify species (it also allows to distinguish between Archea and Bacteria). The most conserved of the rRNAs is 23S but the one used for microbiome analysis is 16S (which corresponds to the human 18S). The 16S rRNA gene is a few thousands nucleotides long, most of which are highly conserved; the bulk of the differences among species is in the hypervariable regions (named V1 to V9), which are terminal loops of the structure (basically regions far away from the catalytic site, therefore more free to mutate). Despite the high degree of conservation, some variability can be found outside the hypervariable regions too (eg. 530 loop structure). The annotation of which portions of the 16S rRNA gene are conserved has been performed using *E. coli* as a reference; for a few hundred organisms the gene has been compared to the reference one to define the degree of conservation of each stretch of nucleotides. Some totally conserved regions (meaning pretty much identical in all species) are present but they are not very big.

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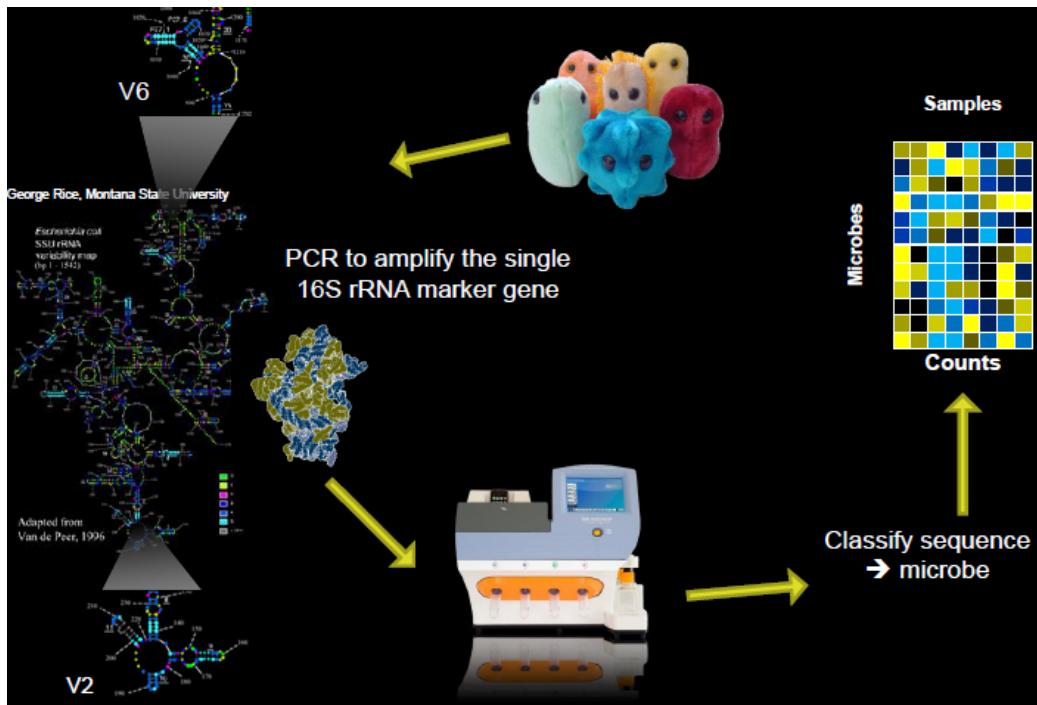


Figure 7.1: General 16S gene analysis workflow

7.2.3 Primer and high-throughput machine choice

One could sequence the entirety of the 16S rRNA gene, for example using NanoPore seq, but this would introduce many errors that could lead to mapping the sequence to the wrong organism; for this reason it is preferred to amplify only certain specific regions of the gene. To study the microbiome in a high-throughput way you need primers which can bind to all species, but since the sequences conserved in all species are too short, you use primers that bind highly conserved regions; for this reason, regardless of which primers you choose there will be bias in your results (some species will not be identifiable using those primers). This bias can be somewhat minimized using *in silico* primer validation, which means testing your primers against databases of 16S tRNA genes (silva and green genes), to test and decide the best pair of primers for your experiment.

Still, two experiments conducted with different primers will always have some differences. Moreover the binding regions must flank some variable region, in order to include it in the amplicon; finally you need pair end amplification (both primer back and forward) in order to have the complete amplicon (to make comparison easier). Given these characteristics there are multiple possible priming sites, based on the sequence and on chemical properties of the primers. Moreover, primers can be used as forward or reverse to obtain different combinations and sequences.

As an example of the importance of the choice of primers, in some skin microbiome analyses, researchers could not find two bacteria always present on human skin due to the choice of primers. Moreover *S. aureus* seemed over-represented due to the non-amplification of other important species. Despite the biases, this technique is still extremely useful even today.

There are different protocols to target conserved regions also based on the machine used. In general:

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Figure 7.2: Structure of the 16S rRNA in *E. coli* and *T. thermophilus*

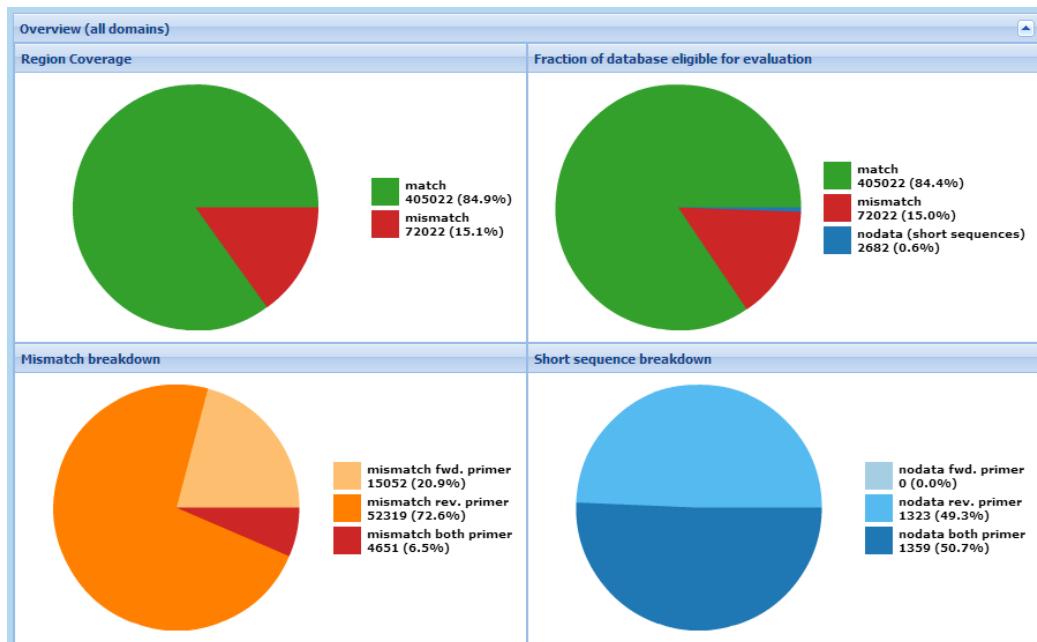


Figure 7.3: Example of *in silico* primer validation using silva; you can notice the different efficiency of the primers relative to different parameters.

7.2. 16S RRNA SEQUENCING

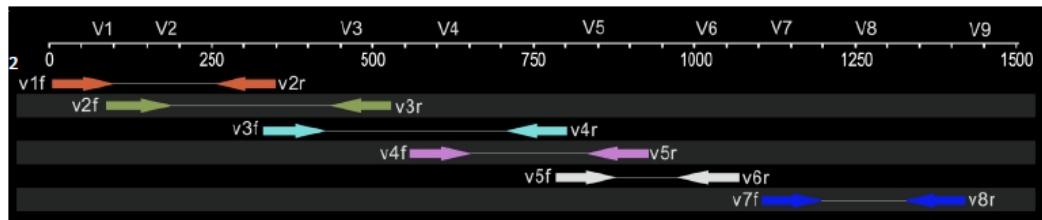


Figure 7.4: Examples of common primer placements relative to hypervariable regions

- Sanger machines are not very good for this application since they have low throughput and they are more suited for longer sequencing tasks (full genomes for instance)
- Roche 454 machines have historically been well suited, since it was possible to sequence three hypervariable regions together using 400 nucleotides reads, providing a good cost/throughput trade-off.
- Illumina HiSeq is not the optimal choice since it has shorter reads and unnecessarily high throughput; Illumina MiSeq and IonTorrent can be a decent compromise.

7.2.4 In depth 16S rRNA analysis workflow

A more in depth 16S rRNA analysis workflow is the following:

- **DNA extraction** from each of your samples
- **Selective PCR amplification of 16S rRNA gene**, introducing a barcode in the sequences using tagged primers.
- **High-throughput sequencing** of all the samples in a single run (to reduce costs); the result is a set of amplicons belonging to different samples and with a barcode attached.
- **Demultiplexing**, which means removing the barcodes and assigning each sequence to the corresponding sample. Sequencing noise must be taken into account, therefore low quality reads must be removed.
- **Multiple sequence alignment** against reference sequences. Some reads will probably not map to any reference sequence.
- **Group related sequences into OTUs** (operational taxonomic units), which means grouping sequences that share some common variants; since there are some SNPs in the microbial genome, the similarity threshold between sequences cannot be too restrictive. OTUs can be used to define the relative abundance of each species in the sample, but in order to do so it is necessary to normalize for the copy number of the 16S gene sequence; this is very difficult since an accurate estimate can be made only if long read sequencing has been performed on the organism, which is almost never the case since for microbes that basically corresponds to full genome mapping (needless to say it is therefore non applicable on unknown microbes).
- **Build phylogenetic tree** using one representative for each OTU.
- **Annotate** the OTUs using 16S gene databases.

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- **Downstream analysis** is performed, such as clustering to visualize similarities among samples.

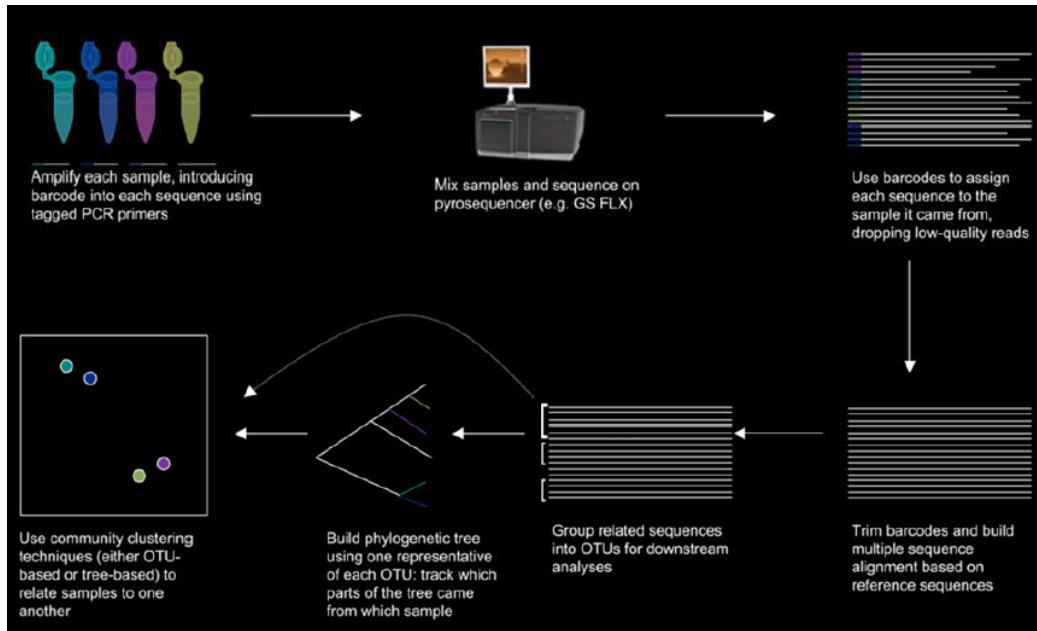


Figure 7.5: Expanded 16S gene analysis workflow

7.2.5 OTU clustering

Defining OTUs requires using multiple sequence alignment; since this approach is a generalization of the mapping algorithm (meaning you have to compare every sequence with every sequence) it is quite complex in terms of speed, but still feasible. Generally, greedy algorithms, which add the lowest possible amount of gaps, are used to perform multiple sequence alignment. After the alignment, sequences are split into OTUs (operational taxonomic units), which are basically groups of 16S sequences very similar to each other. Generally a sequence is defined as the representative of the OTU, meaning that it has a certain threshold of identity with all other sequences in the OTU (usually 97% when considering species) and that minimizes the differences of all other sequences of the OTU with itself. Some OTUs can be assigned univocally to a species, some others may be associated to more species, some others cannot be mapped to known species. The fact that a species may map to multiple OTUs is often a negative factor (confusion in the analysis) but it may sometimes allow to find subspecies.

After sequence alignment, OTU clustering (= splitting the sequences into OTUs), can be done through several supervised or unsupervised learning methods. Each method has pros and cons, therefore there is not an always optimal method. The most common unsupervised clustering methods are:

- **Single linkage clustering** (nearest neighbour): assign the sequence to a cluster if that OTU already contains **at least a sequence** similar enough (97%). However two distant sequences

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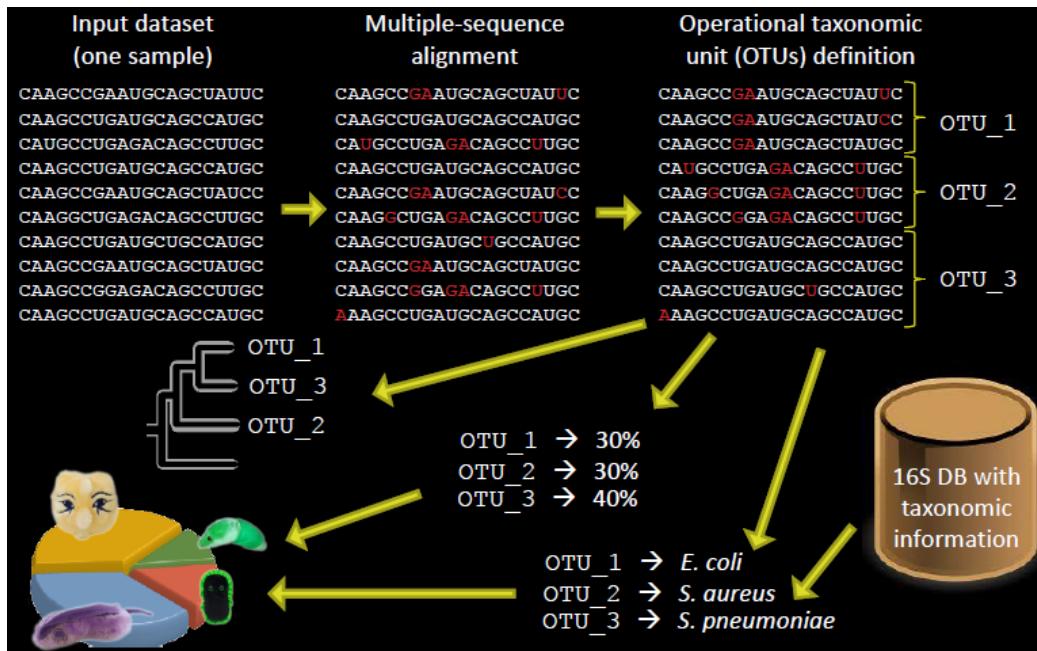


Figure 7.6: Zoom in on 16S gene analysis workflow

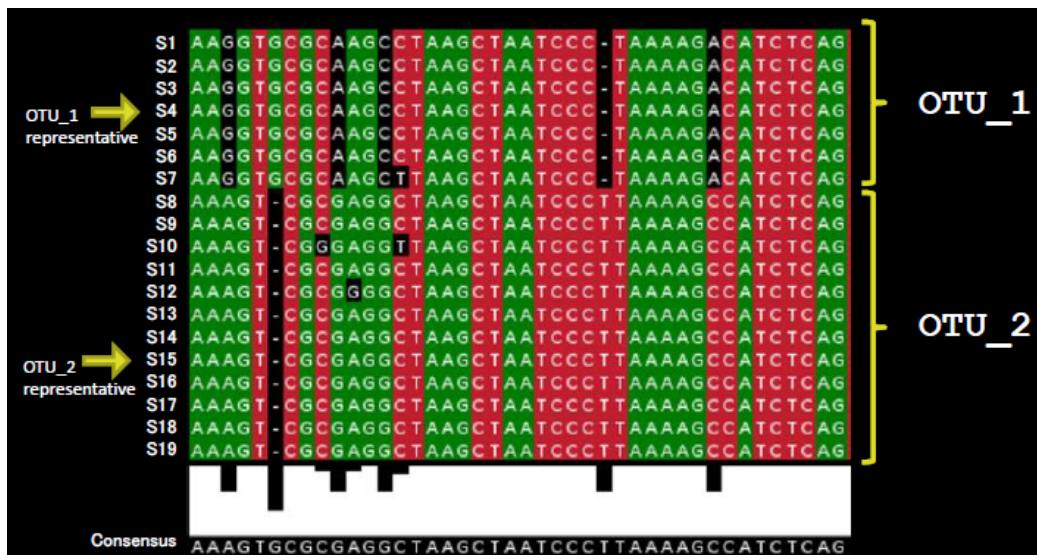


Figure 7.7: Example of multiple sequence alignment for OTUs

in the OTU network could share a similarity which is way lower than 97% (because of a series of connections above 97%); this could result in **underclustering** (defining too few clusters).

- **Complete linkage clustering** (furthest neighbour): assign the sequence to a cluster only

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if **all the sequences** of the OTU are similar enough (97%). However two sequences may be similar enough (97%), yet belong to different OTUs, because the overall cluster width, or **divergence**, is at most 3%; this approach could then generate different solutions, based on the order the points are added in. Moreover, if the clustering conditions are too stringent, sequencing errors and SNPs in the microbial genome may result in **overclustering** (defining too many clusters).

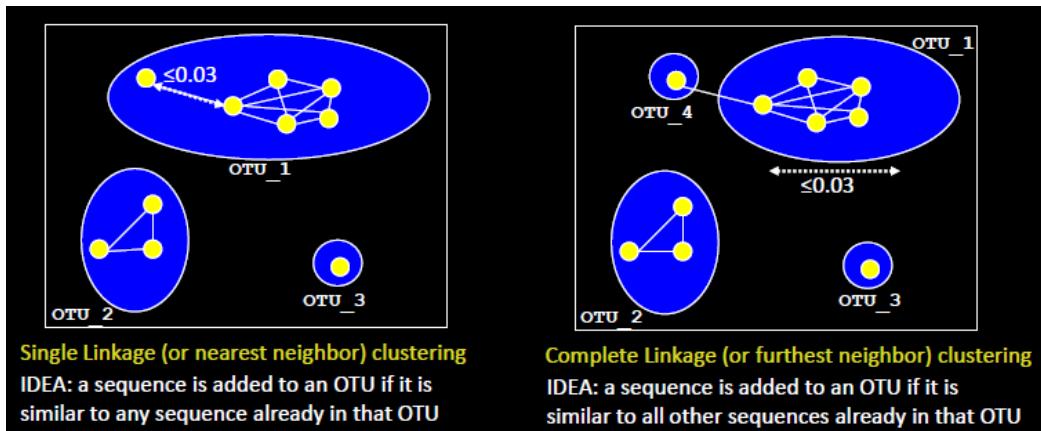


Figure 7.8: Visualization of single linkage analysis and complete linkage analysis

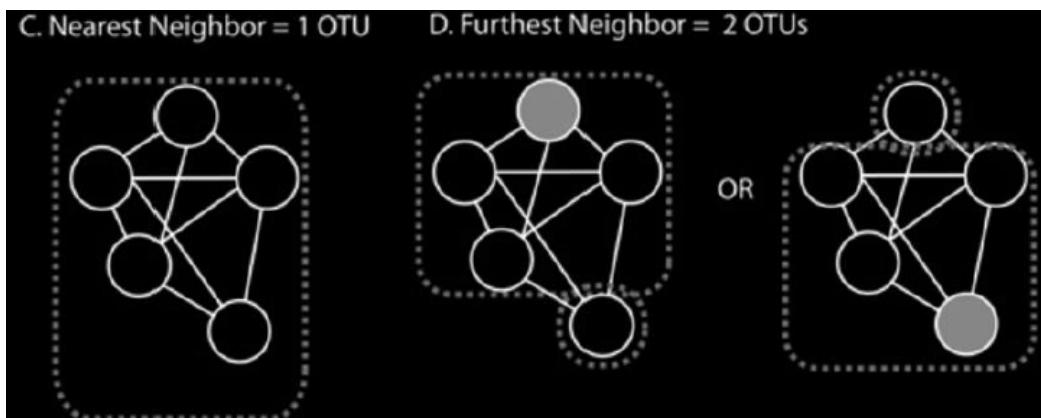


Figure 7.9: Example of overclustering and result multiplicity due to complete linkage analysis

7.2.6 OTU taxonomic annotation

NOTE: this topic continues in the next lecture; when done merge together Assigning a taxonomic annotation to an OTU cannot be done simply using BLAST to get the best matching sequence; this is because there is too much noise in the sequences and because it is difficult to classify new strains. A better way is using some other algorithm that assigns the terms of the taxonomic notation (since it is more than just one label) and provides some degree of confidence

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in the prediction. For instance the algorithm may be able to correctly assign the first taxonomical terms, up until *Enterobacteriaceae*, but then it provides a prediction of the OTU belonging to *E. coli* with some confidence interval, say 85%, and some alternative like *S. dysenteriae*, say with 15% confidence.

Chapter 8

Shotgun metagenomics

Chapter 9

Staphylococcus aureus