



*University of Naples "Federico II"*

# Marine Microbial Diversity

# Methods in Marine Microbial Diversity



***General (first order)  
questions in studying  
microbial diversity***

*Who's there*

*What are they doing*

*Who's doing what?*

*How are they doing it?*

*To what extent?*



## *Higher order questions in studying microbial diversity*

*How is diversity influenced by environmental conditions?*

*How is functional diversity influenced by environmental conditions?*

*How is diversity and functioning influenced by species interactions?*

*How are metabolic rates influenced by environmental conditions and species interactions?*

# *Approaches to Microbial Diversity*

*Each time we approach the study of microbial diversity we are following a similar workflow generally consisting of:*

- 1- Definition of the study question and study design*
- 2- Sample collection*
- 3- Sample preservation*
- 4- Sample processing and data acquisition*
- 5- Data analysis*
- 6- Interpretation of results*

# Approaches to Microbial Diversity

Each time we approach the study of microbial diversity we are following a similar workflow generally consisting of:

1- Definition of the study question and study design

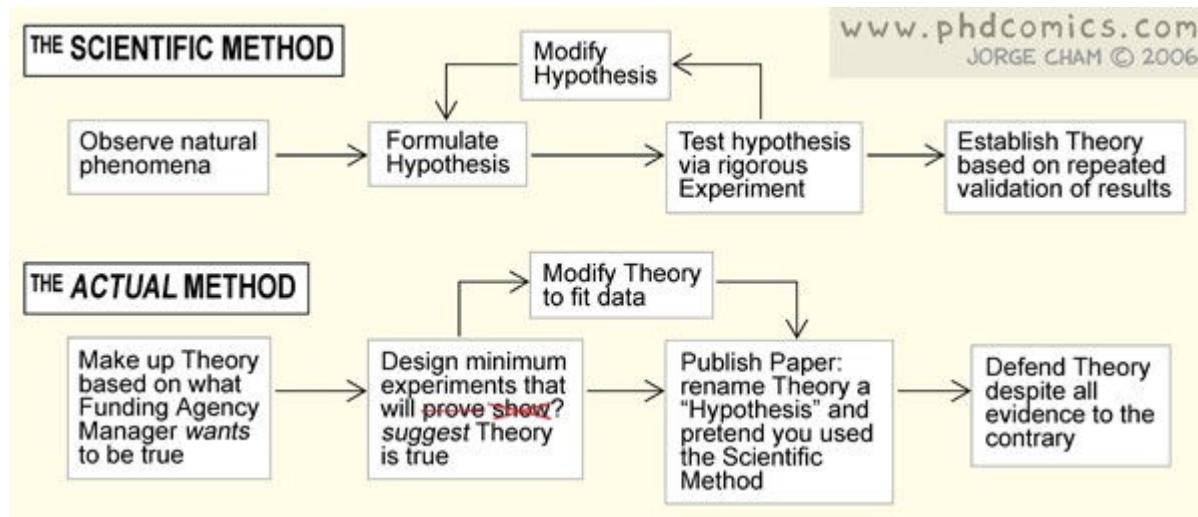
**2- Sample collection**

3- Sample preservation

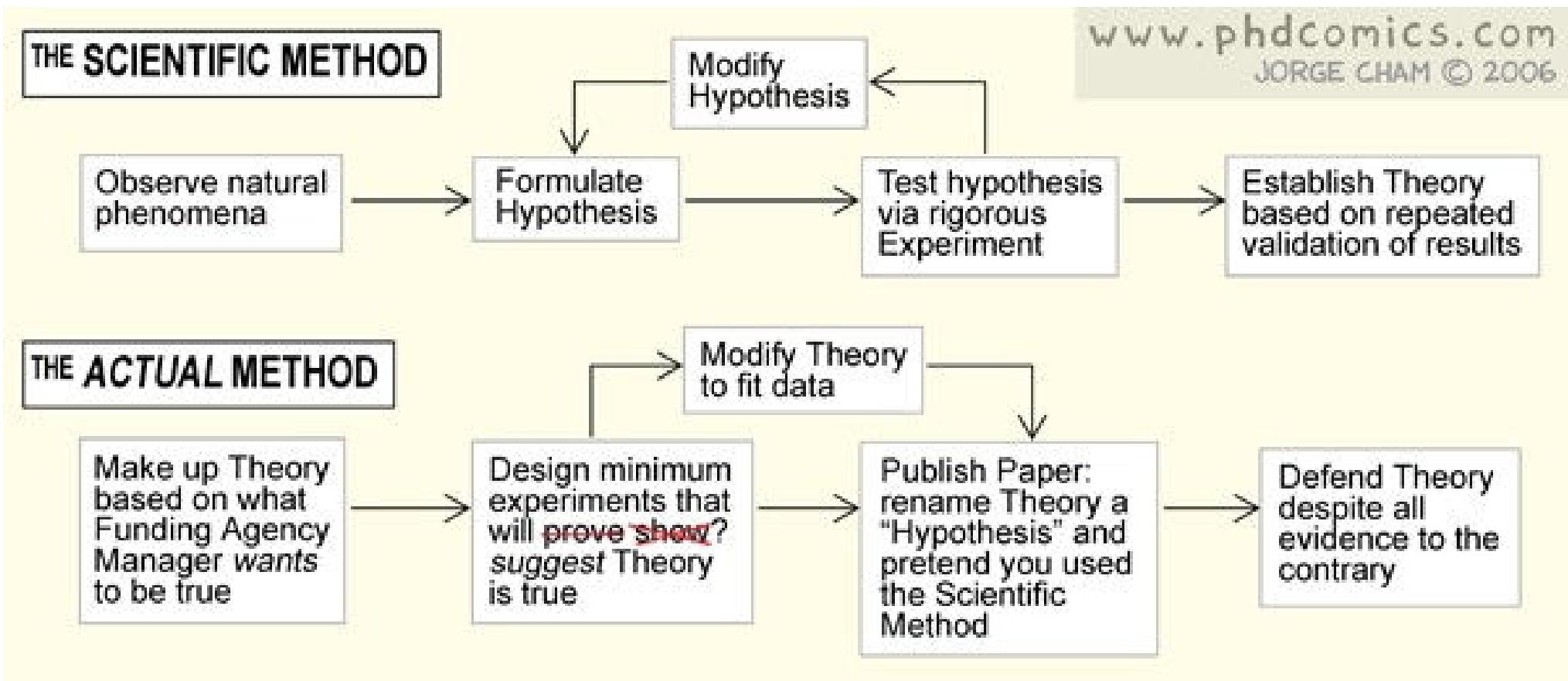
**4- Sample processing and data acquisition**

5- Data analysis

6- Interpretation of results



# Approaches to Microbial Diversity



# *Richard Feynman on Scientific Method (1964)*



<https://www.youtube.com/watch?v=0KmimDq4cSU>

# *Problem in studying microbial diversity*

**Spatial heterogeneity** – most methods need several hundreds milliliters of waters or few grams of sediments

**Inability to culture** – Yet most of the microbial diversity is uncultured, with big implications for what we can understand

**Taxonomic ambiguities of microbes** – Difficult definition of “species”, high genomic plasticity, horizontal gene transfer

**Technical bias of chosen methods** – Each techniques has its own bias, that need to be considered while making inferences

# *sampling for Marine Microbial Diversity*

*Sampling the marine environment can be done in several different ways depending on the scientific question, the environment and the technique to be used*

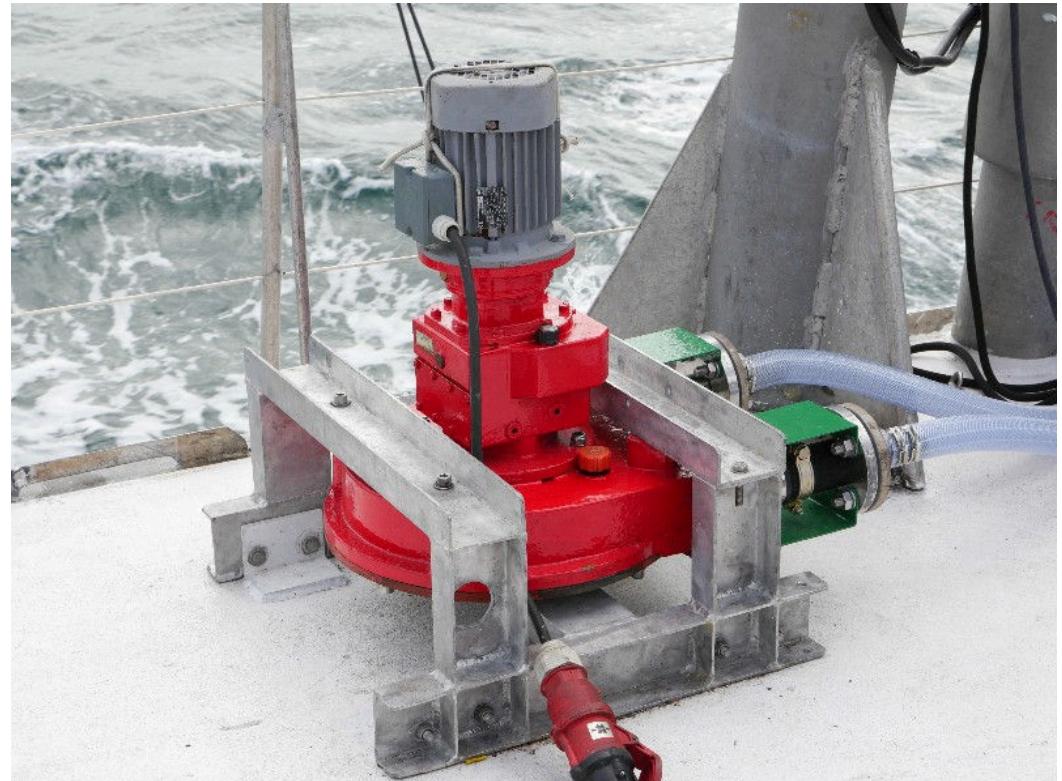
*Generally speaking samples can be collected of seawater, specific fluids, sediments, biofilms or flora/fauna*

*The difficulties and technological hurdles associated with collecting these sample increase with increasing water depth*

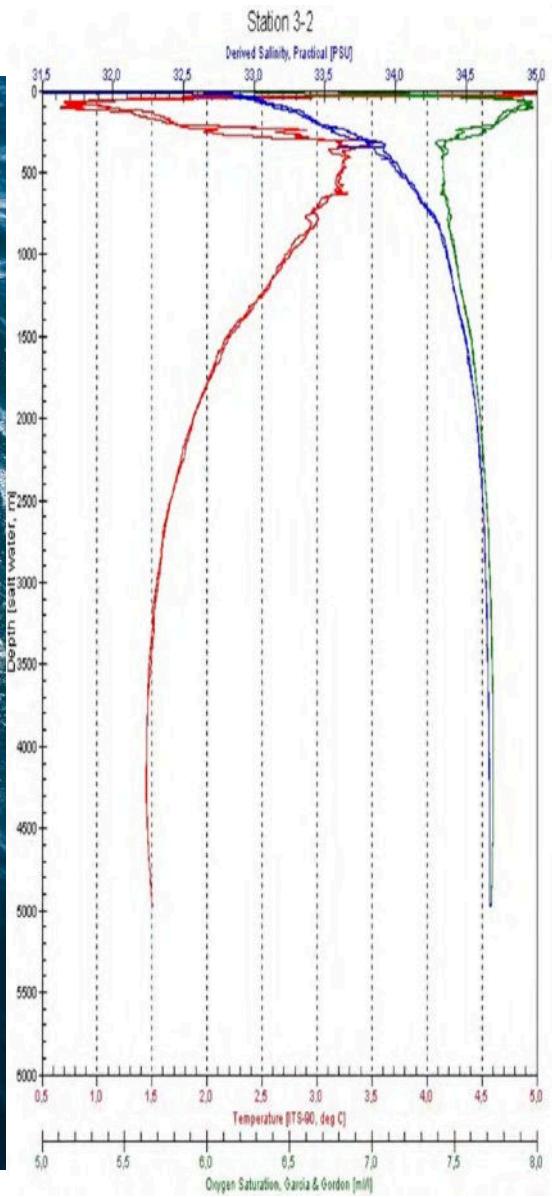
*The need for specific sample preservation protocols might also complicate the sampling procedure*

# sampling Seawater

Seawater can be sampled in several different ways. The principal techniques are the use of hand-operated sterile bottles or containers, collection of known volumes using pumps and using the Niskin bottles, often mounted on a CTD-Rosette sampler



# sampling Seawater

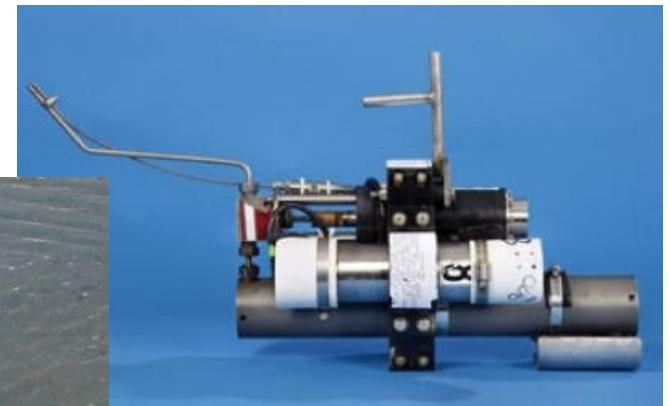


# *sampling Seawater*

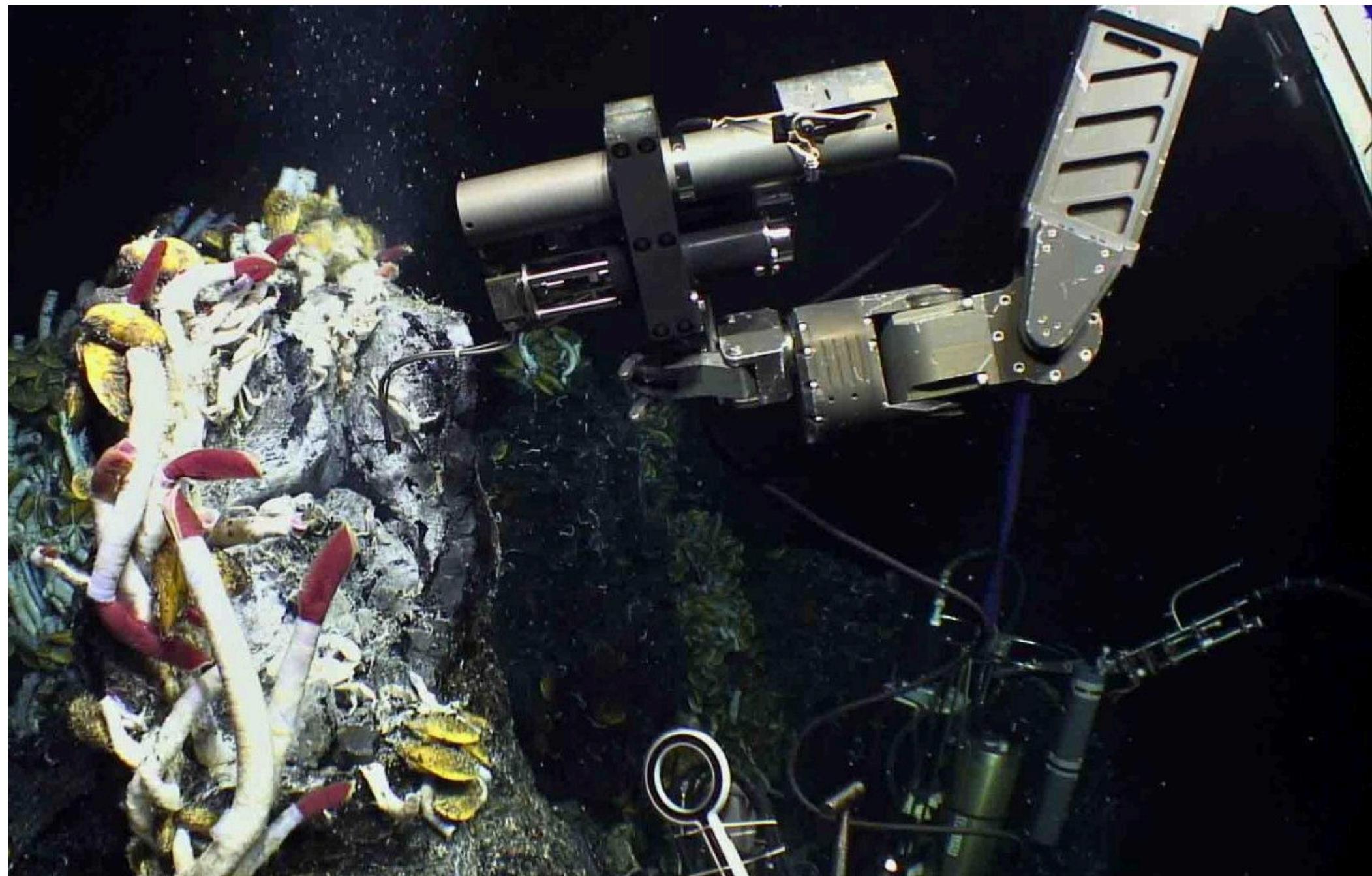


# sampling Fluids

Fluids, like sediment pore fluids, or fluids seeping from a cold seeps or hydrothermal vent can be sampled using different approaches, that are specific to each case. Common approaches include centrifugation of sediments (pore fluids), core suction (pore fluids), pumps (fluids) and syringes (pore fluids and fluids)

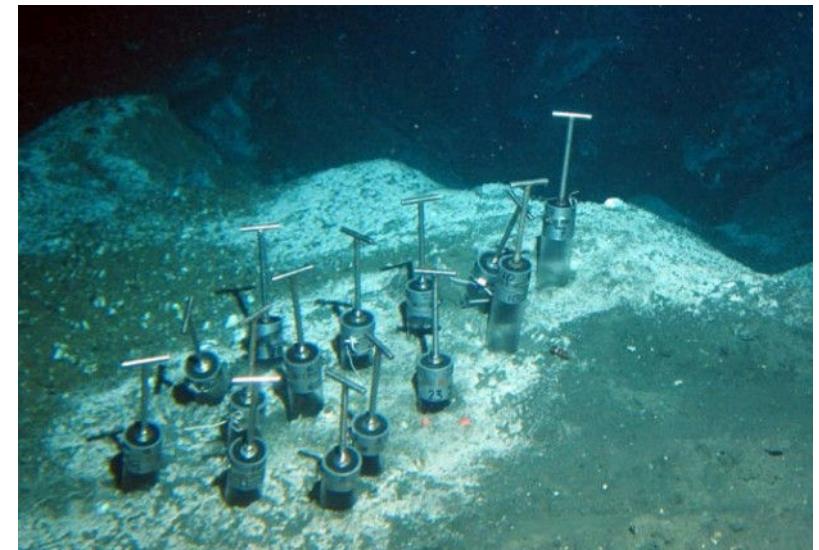


# sampling *Fluids*



# sampling *Biofilms*

Biofilms can be sampled through a syringe, a push core, or directly swabbing the surface

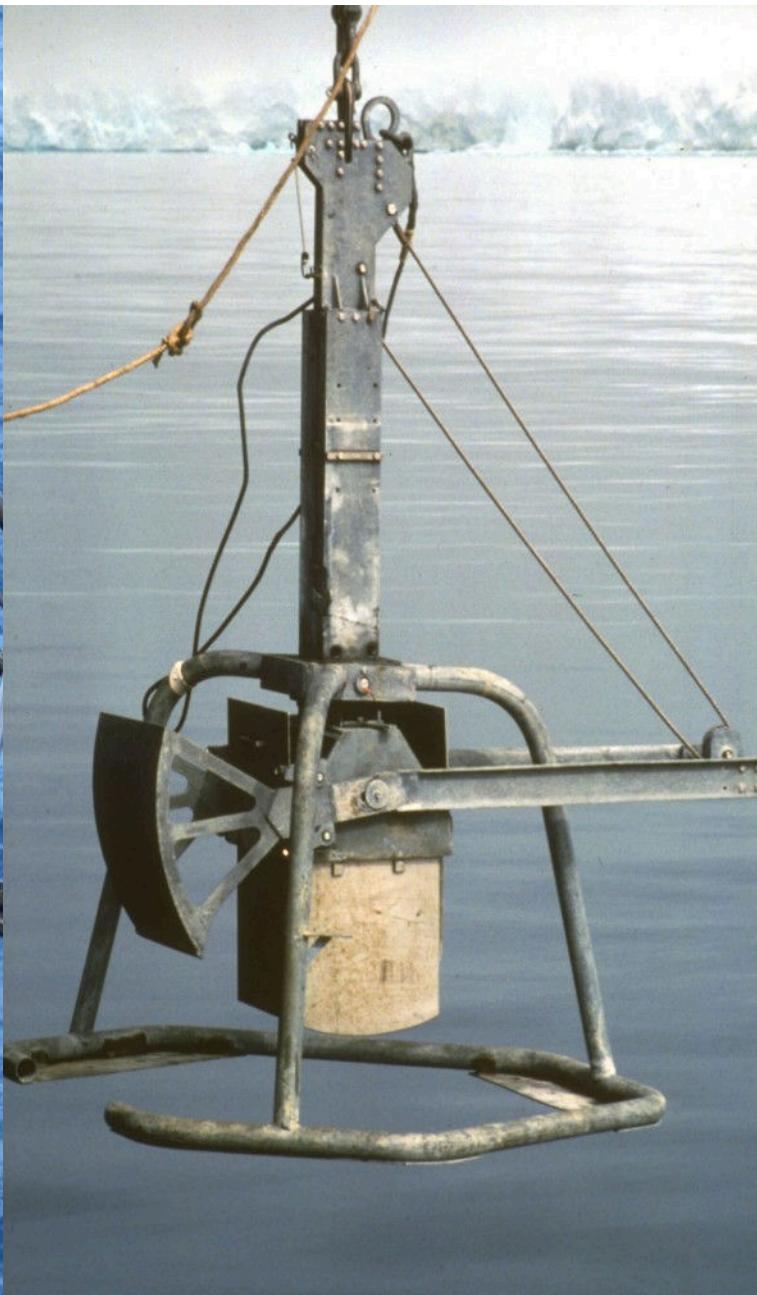


# sampling Sediments

Sediments can be sampled in a variety of ways. The simplest way is to use a sterile container (like a falcon tube), however the most used method is a push core. Beyond that the use of multicorers, boxcorers, gravity corers and drilling is also used, depending on the purpose and location

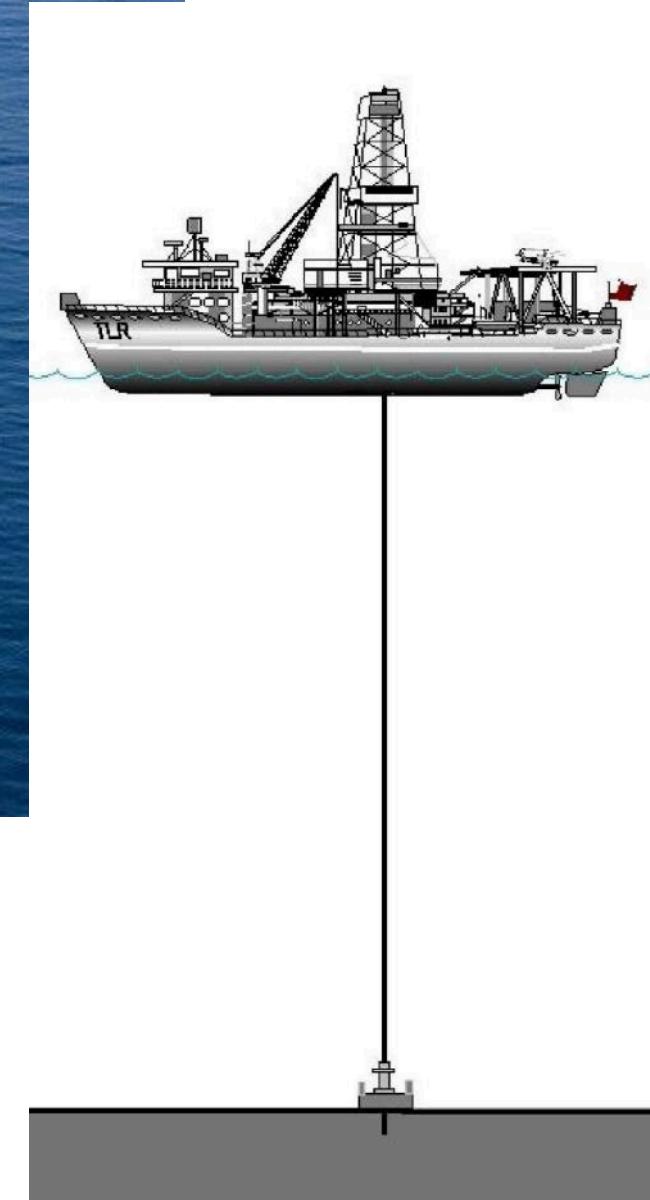
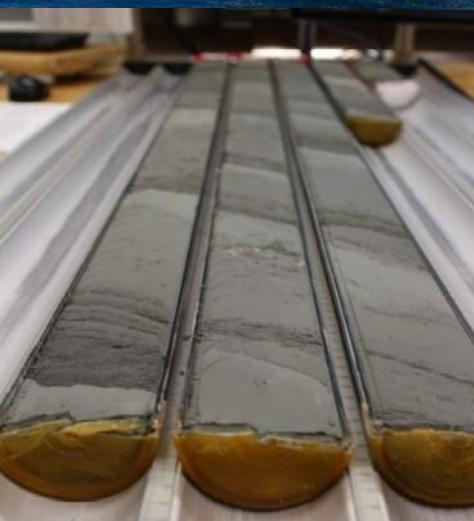


# *sampling Sediments*



# sampling Sediments

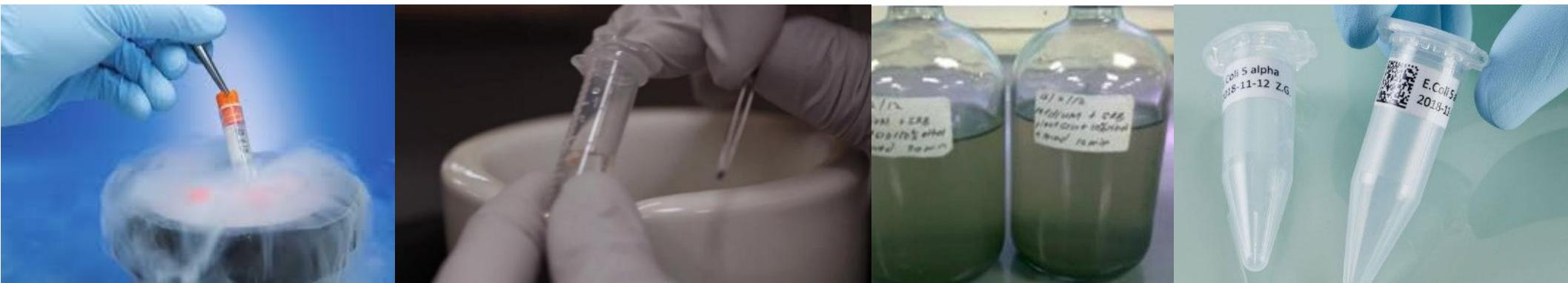
研究航海



# sample *Preservation*

Preservation is highly dependent upon the type of downstream analysis that needs to be carried out. There are some common preservation strategies that include the modulation of temperature or the addition of specific preservatives. These are often used in combinations

- Refrigeration at +4°C (culturing, enrichments, live specimens)
- Freezing at -20°C (viral counts, chemistry, DNA)
- Freezing at -80°C or liq-N<sub>2</sub> (DNA, RNA, proteins)
- Preserving solutions like formaldehyde, gluteraldehyde, RNA later, PBS (counts, DNA, RNA)



# sample Analysis

The technique used depends on the scientific question and the analytical principles used. We can divide techniques on different ways:

**Culture-dependent** and **culture-independent** techniques, refers to a grouping of techniques based on the use of culturing as a basic step in the investigation. Since the majority of the microbes is currently uncultured, the decision of using culture-dependent vs independent techniques is very important

**Qualitative, semi-quantitative** and **quantitative**, refers to the type of information obtained from the analysis. Often semi-quantitative and quantitative techniques are all referred as quantitative, although not correct. Bias in the chose technique usually impair our ability to obtain true quantitative data

**Chemical, biochemical, molecular, isotopic**, or a combination refers to the analytical principles used by each technique

# **Culture-dependent vs culture-independent**

Given our current inability to grow a large portion of marine microbes, the choice between culture dependent and independent technique is very important

Culture-independent techniques allow you to probe the natural diversity of microbial communities **avoiding the bottleneck of culturing.** Despite this the inferences about functional diversity are entirely dependent upon information obtained from pure microbial cultures

Culture-dependent approaches, while highly selective for a small subset of microorganisms, have the advantage of resulting in a **new model system** that can be used for probing functional diversity using physiology, genetics and biochemistry

Some approach are an hybrid between the two techniques, for example combining enrichments with molecular tools

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Some approach are an hybrid between the two techniques, for example combining enrichments with molecular tools

**A successful approach to the study of microbial diversity requires both approaches combined**



## *General questions in studying microbial diversity*

Who's there

What are they doing

Who's doing what?

How are they doing it?

How much?



## *General questions in studying microbial diversity*

Who's there



What are they doing



Who's doing what?



How are they doing it?



How much?

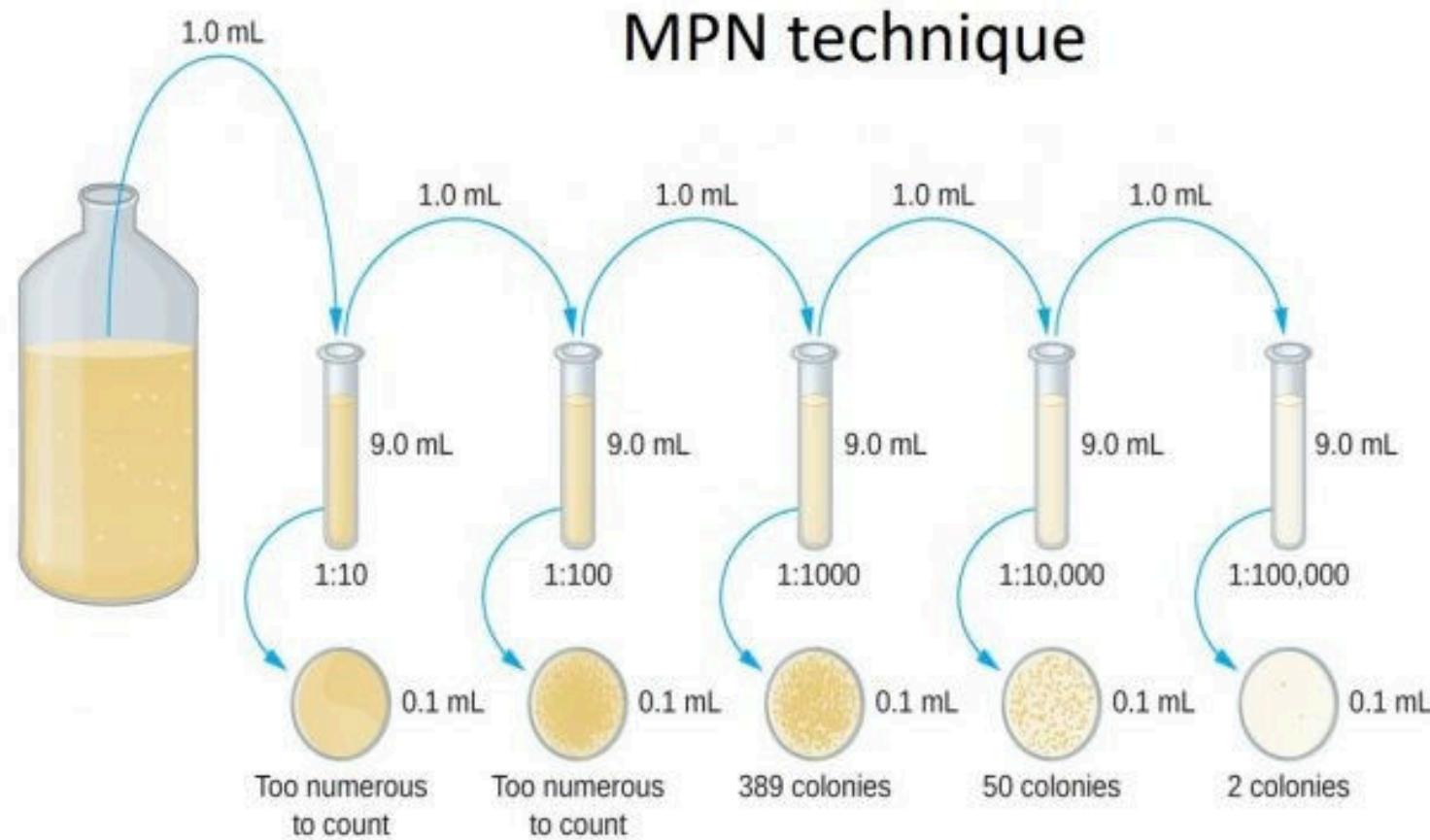


# *Culture-dependent approaches*



# Most Probable Numbers

Most Probable Numbers (MPN) techniques relies on our ability to selectively culture specific trophic groups. A serial dilution of a sample is made on a selective media (e.g. thiosulfate oxidation), and the viable colonies for each dilution are counted on a plate. The number of original cells for that specific metabolism are then back calculated





# Most Probable Numbers

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**PROS:** Gives you a quantitative assessment of the number of active cells performing a given metabolism

**CONS:** Useful only for well defined metabolisms and taxonomic groups; bias toward cultured groups; the amount of cells that can perform a given metabolism is usually underestimated



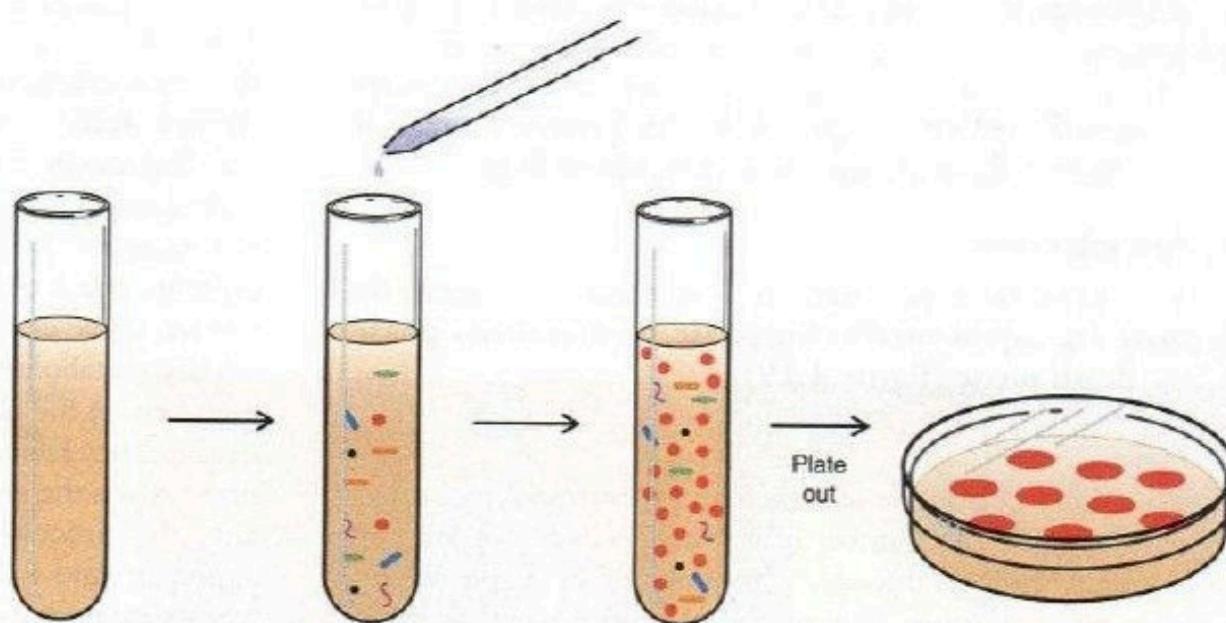
# Enrichments and Isolation



Defined media for a specific metabolism of interest is inoculated with the original sample, following the enrichment for microbial groups able to grow, pure cultures are then isolated and characterized

## Enrichment cultures

Isolating an organism from natural sources



Medium contains select nutrient sources chosen because few bacteria, other than the organism of interest, can use them.

Sample that contains a wide variety of organisms, including the organism of interest, is added to the medium.

Organism of interest can multiply, whereas most others cannot.

Enriched sample is plated onto appropriate agar medium. A pure culture is obtained by selecting a single colony of the organism of interest.



# Enrichments and Isolation



Defined media for a specific metabolism of interest is inoculated with the original sample, following the enrichment for microbial groups able to grow, pure cultures are then isolated and characterized

While the *initial enrichment and isolation* can be based on different techniques (different types of incubators, cell sorting, co-cultures), pure cultures are the **ONLY officially approved approach** that can lead to the description of a **new microbial species**

New species names are officially valid only if they appear on the journal of the International Committee on the Systematic of Prokaryotes (ICSP) in the **International Journal of Systematic and Evolutionary Microbiology (IJSEM)**

Approved name appear as **Genus specie sp. nov.** in their first appearance, and **Genus species** from then on. Not officially approved species appear instead as "**Candidatus Genus species**" or "**Ca. Genus species**"



# Enrichments and Isolation



Officially described species are deposited in at least two culture collections, from where they are available to the community for analysis

Pure cultures give a model organism for **manipulative experiments, physiological, biochemical** and **genetic studies**, allowing for new microbial functions to be characterized

Being “Uncultured” is **an operational definition**, not an intrinsic attribute of the organism. The ultimate goal is to culture the uncultured

Recently the community has started referring to uncultured microbes as “microbial dark matter”, a term that while great for science communication purpose should be avoided within the community (more at <http://merenlab.org/2017/06/22/microbial-dark-matter>)

Even a single isolate from a previously uncultured group can dramatically change our view of the ecological role of the group

# example *Thermovibrio ammonificans*

International Journal of Systematic and Evolutionary Microbiology (2004), 54, 175–181

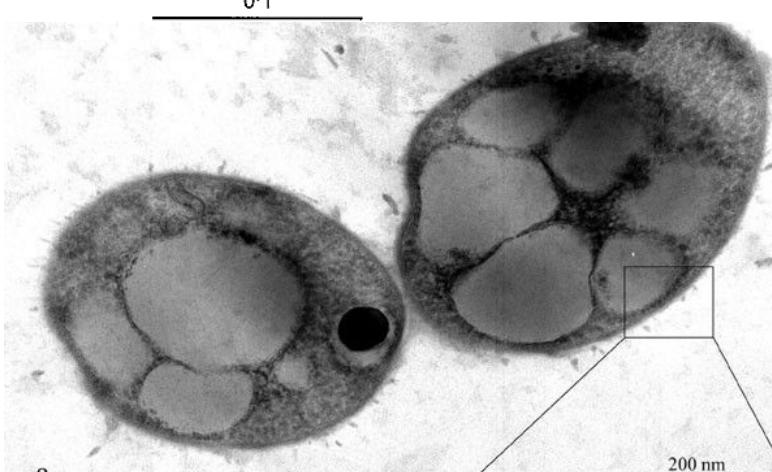
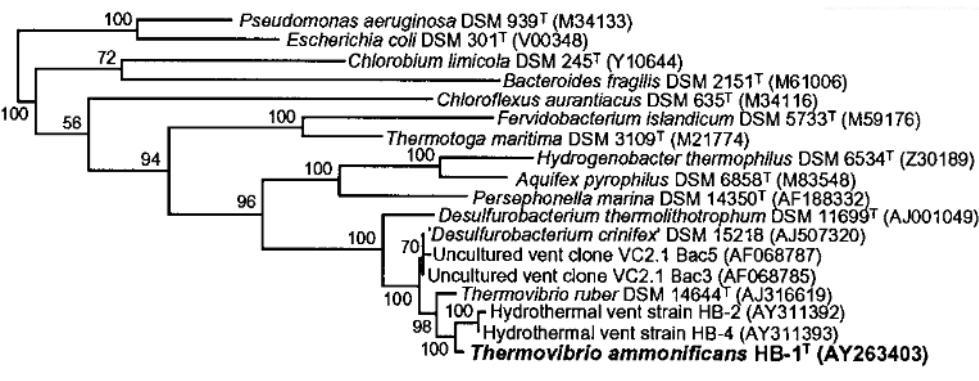
DOI 10.1099/ijs.0.02781-0



research papers

*Thermovibrio ammonificans* sp. nov.,  
a thermophilic, chemolithotrophic,  
nitrate-ammonifying bacterium from  
deep-sea hydrothermal vents

Costantino Vetriani,<sup>1,2</sup> Mark D. Speck,<sup>2</sup> Susan V. Ellor,<sup>2</sup> Richard A. Lutz<sup>2</sup>  
and Valentin Starovoytov<sup>3</sup>



Cys43

***Thermovibrio ammonificans***  
Thermococcus kodakarensis  
Nautilla profundicola  
Pyrococcus furiosus  
Pyrococcus horikoshii  
Shewanella loihica  
Aequifex aeolicus  
Hydrogenobacter thermophilus  
Thermocrinis ruber

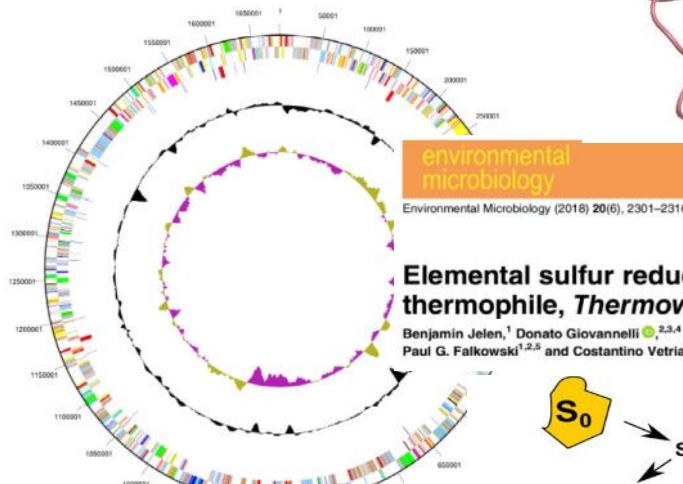
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3: DVVVIGGSAGGLTAAISAKRFPDKSVLVVIKKED-VSMIPC GIPY---IFGTLRSVEDDV--PTERFLKPLG  
2: D I V V I G G V S G F V A A V N A K R F Y P D K S V L V I K K E - V S M I P C G I P Y -- I F D T Y - G I D D D L M H - - L E K K L K K F N  
4: KVVIIGGGAAAGMSAASRVKRLRPEWDVKVFATEWVSHAPCGI PY--VVEGI-APKEKLMHYPPEVFIKKRG  
7: KVVIIGGGAAAGMSAASRVKRLKPEWDVKVFATEWVSHAPCGI PY--VVEGL-STPDKLMYYPPPEVFIKKRG  
2: K I L I I G G V A G G A S A A R A R R L I S T A E I I M F E R G E Y V S F A N C G L P Y - - H I S G E I A Q R S A L V L Q T P E S F K A R F N  
1: K V V I V G N G P A A A S A V E A F R K V D R D E S I I L S D E E F P T Y A P N C M E N - - V I R D D - I S K E A L F Y K G G E K F Y E K Y R  
1: K V V I V G T G M A G S A L V D E L L K T D P S L E L H L F G E E K - - S P P Y N R I Y L T D V L S G K - K L P S Q L L F K S Y Q R F - E E E G  
1: K V V I I G G A G M G S A L V D E L L S L E P S L E I H L F G E E R - - T P P Y N R I Y L T D L L A G A - K L P S Q L L K S L Q K F - E E E G

Sciences (2012) 7:82-90

DOI:10.1126/science.1213300

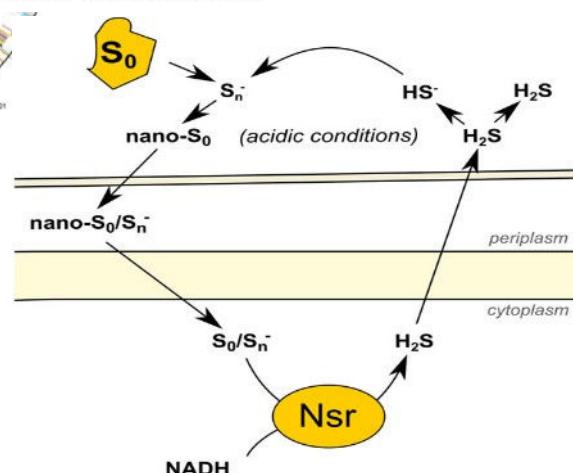
Complete genome sequence of *Thermovibrio ammon*  
HB-1<sup>T</sup>, a thermophilic, chemolithoautotrophic bacter  
isolated from a deep-sea hydrothermal vent

Donato Giovannelli<sup>1,2,3</sup>, Jessica Ricci<sup>1,2</sup>, Illeana Pérez-Rodríguez<sup>1,2</sup>, Michael Hüg  
O'Brien<sup>1</sup>, Ramaydalis Keddis<sup>1,2</sup>, Ashley Grosche<sup>1,2</sup>, Lynne Goodwin<sup>6</sup>, David Br  
W. Davenport<sup>6</sup>, Chris Detter<sup>6</sup>, James Han<sup>5</sup>, Shunsheng Han<sup>6</sup>, Natalia Ivanova<sup>5</sup>,  
Land<sup>7</sup>, Natalia Mikhailova<sup>5</sup>, Matt Nolan<sup>5</sup>, Sam Pitlick<sup>5</sup>, Roxanne Tapia<sup>6</sup>, Tanja I  
Costantino Vetriani<sup>1,2</sup>

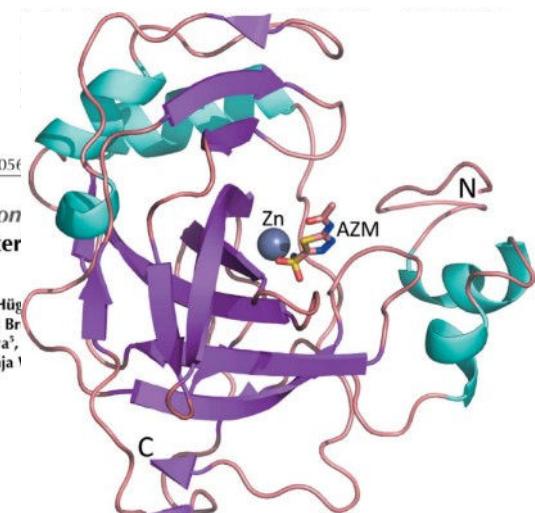


Elemental sulfur reduction in the deep-sea vent  
thermophile, *Thermovibrio ammonificans*

Benjamin Jelen,<sup>1</sup> Donato Giovannelli<sup>1,2,3</sup>,  
Paul G. Falkowski<sup>1,2,5</sup> and Costantino Vetriani<sup>1,2,\*</sup>



The structure of a tetrameric  $\alpha$ -carbonic anhydrase  
from *Thermovibrio ammonificans* reveals a core  
formed around intermolecular disulfides that  
contribute to its thermostability



doi:10.1111/j.1462-2920.14280

# *Culture-independent approaches*

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*There are a number of culture independent approaches to study microbial diversity, and can be distinguished based either the approach (microscopy, molecular, biochemical) or based on the type of information they provide*

*Earlier culture-independent approaches were based on different microscopy techniques, from optic and phase contrast to transmission and scanning electron microscopy*

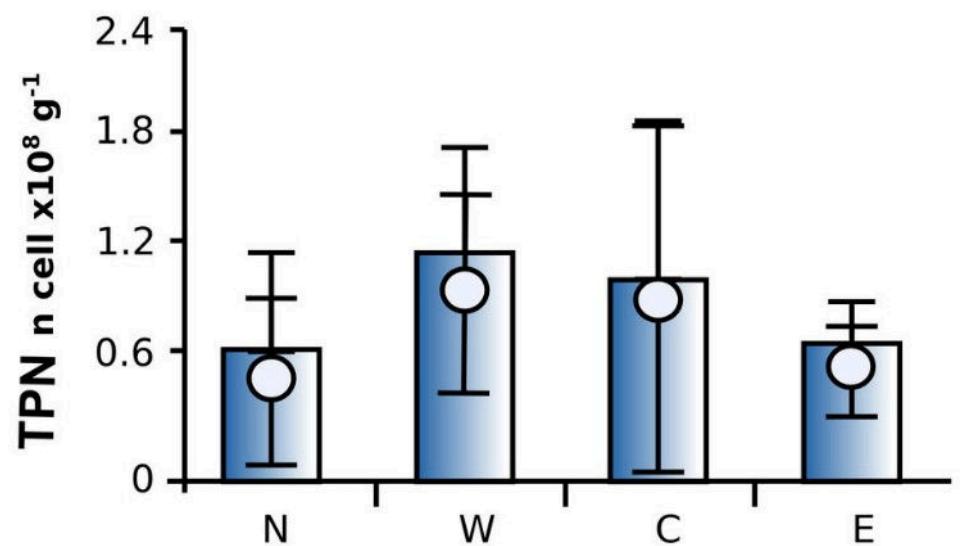
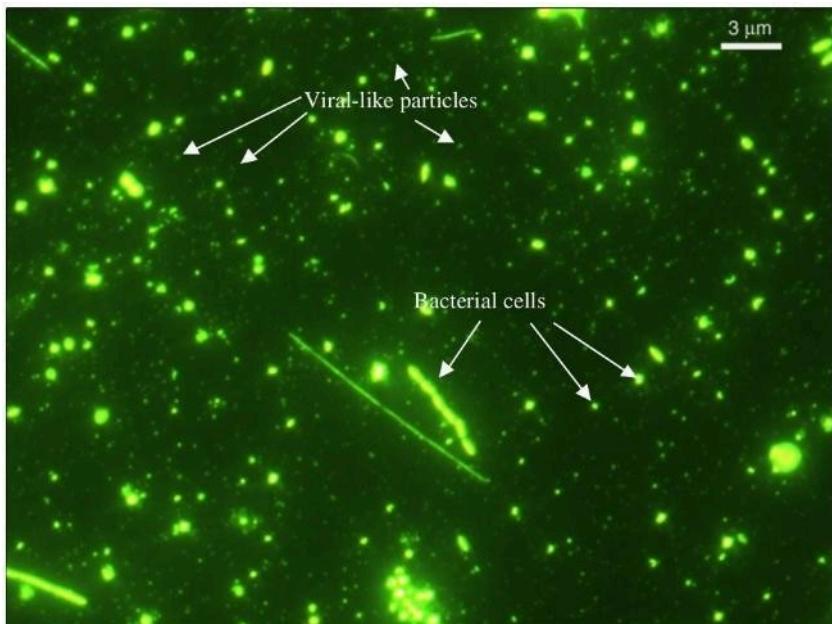
*The big leap in culture-independent techniques started in the seventies with the evolution of molecular biology techniques. These allowed to probe the microbial world in new ways (mainly DNA at the time), bringing upon us a big revolution*

*The establishment of a third domain of life (the Archaea) is a direct result of the application of molecular biology techniques to the study of microbial diversity*

# Epifluorescence Microscopy

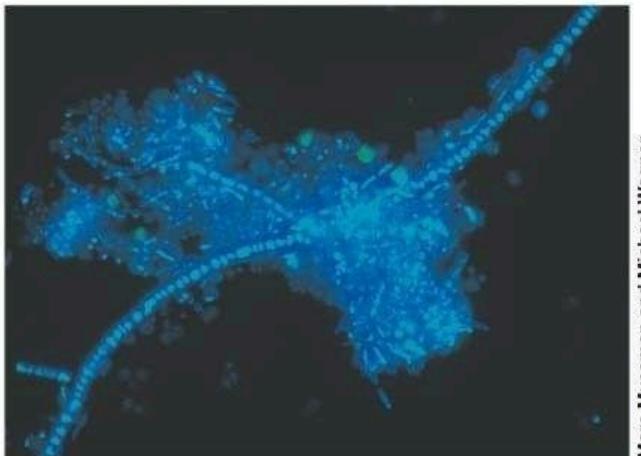
**Counting microbes** in natural samples can be achieved by using DNA staining chemicals that fluoresce under UV light. The staining is aspecific to any DNA (double or single strands depending on the dye)

These approaches have lead to the “Great Plate Count Anomaly” paradox, and are used to obtain quantitative data on population abundance. Data on microbial abundance can be also obtained with flow cytometry techniques, depending on the sample matrix

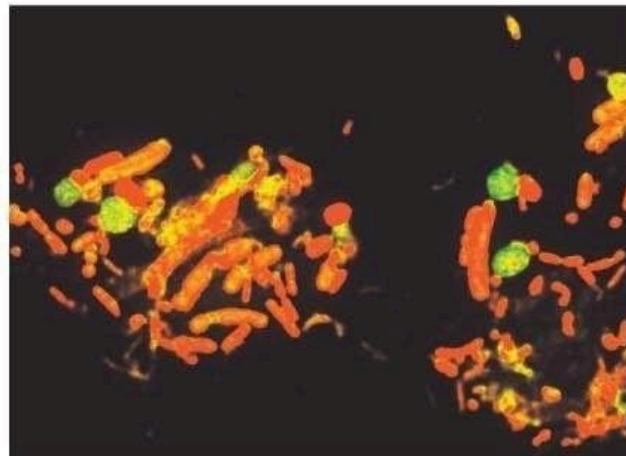


# Epifluorescence Microscopy

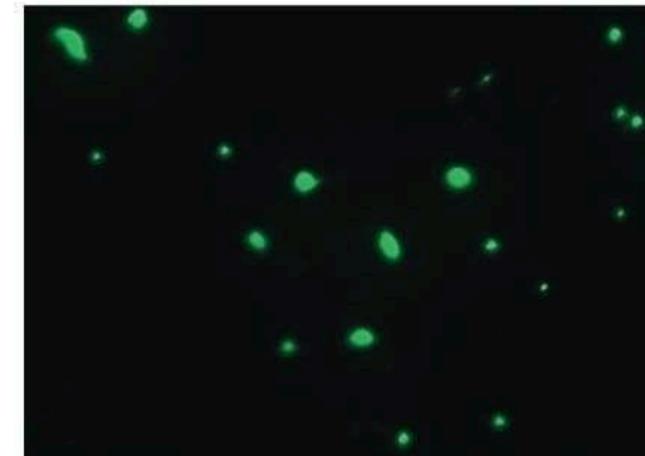
DAPI stain



Acridine orange stain



SYBR Green stain



(a)

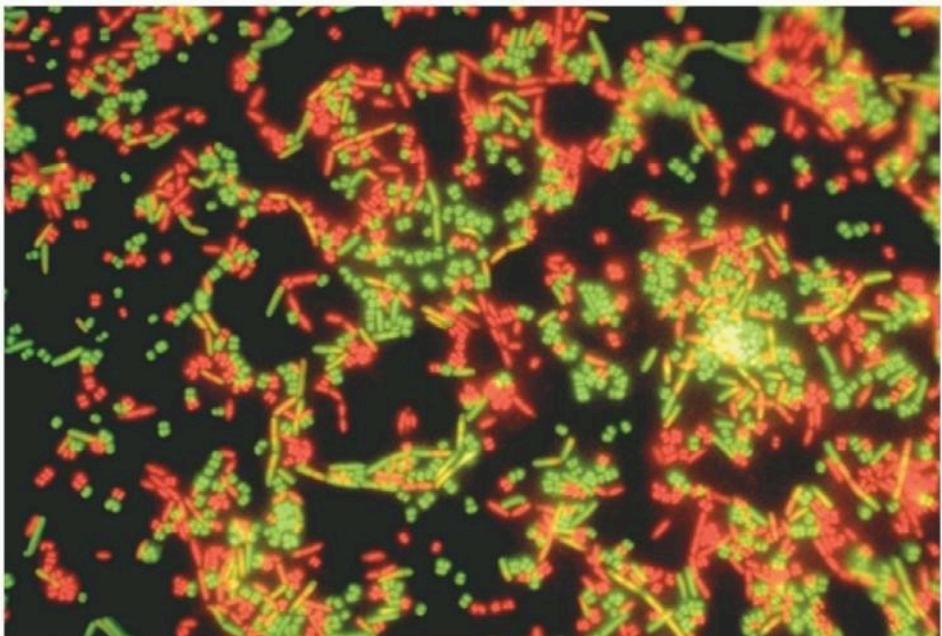
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(b)

(c)

Marc Mussman and Michael Wagner

Willm Martins-Habenna



Molecular Probes, Inc., Eugene, OR

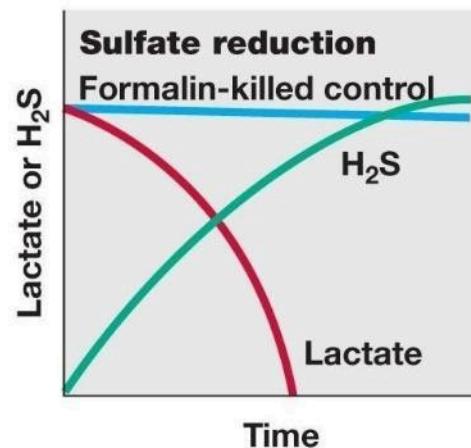
*Viability staining:* Live (green) and red (dead) cells are revealed. The technique is based on the integrity of the cytoplasmic membrane.



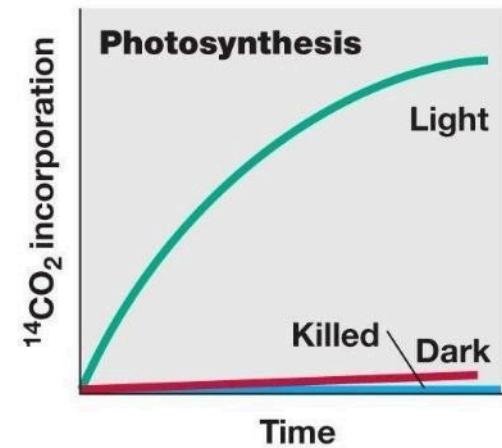
# Activity measurements

Specific metabolic activities can be measured by incubating natural samples with selected substrates. The resulting rates are often not representative of *in situ* rates, since the community is stimulated by substrate addition

Chemical substrates

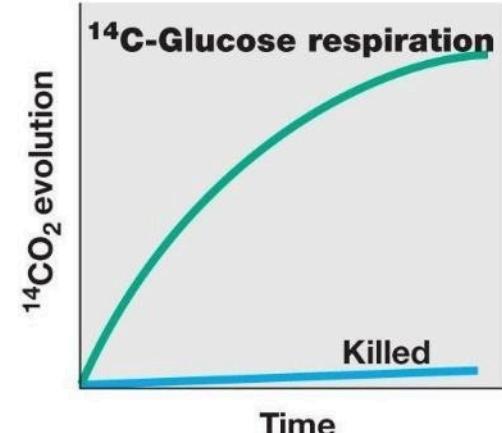
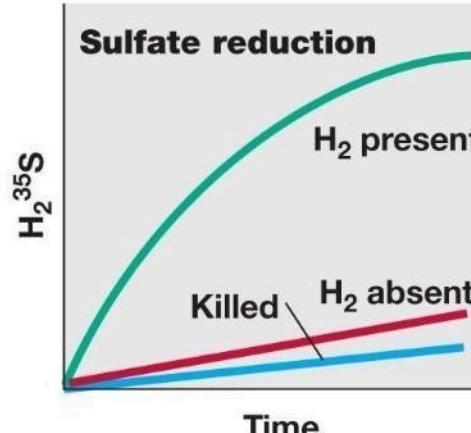


(a)



(b)

Stable or radioactive isotopes



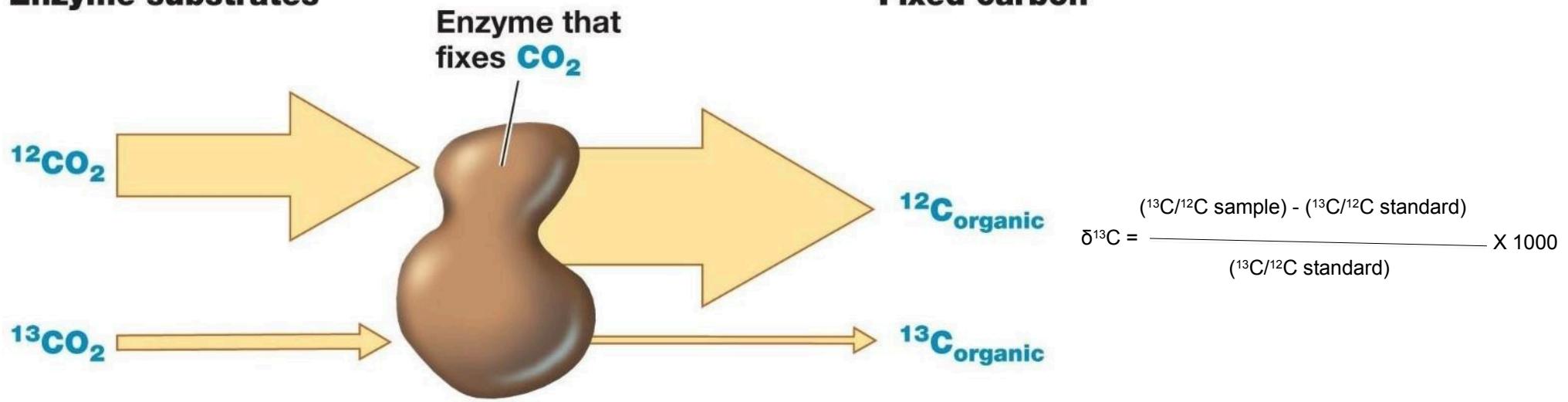


# Stable isotopes

Enzymes can be more or less selective light isotopes during their activity, often leaving behind a specific isotopic signature as a result

Stable isotopes can thus be used not only for tracking rates, but also for linking identity (who) to function (what). They are often combined with other techniques such as FISH and sequencing

## Enzyme substrates

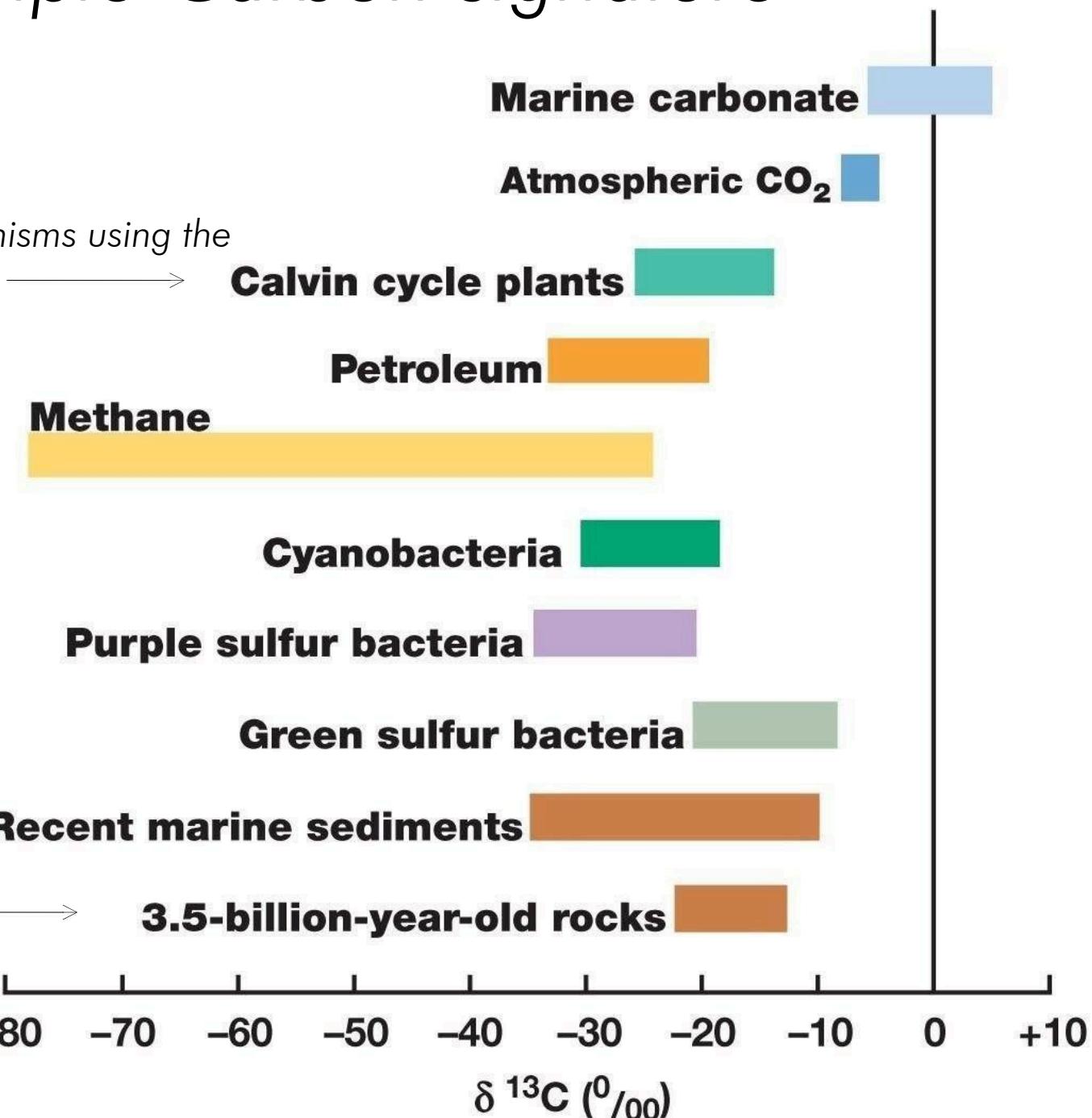


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Biological C isotopic fractionation in enzymes that fix  $\text{CO}_2$  is calculated as  $^{13}\text{C}$  depletion relative to a standard of geologic origin

# example Carbon signature

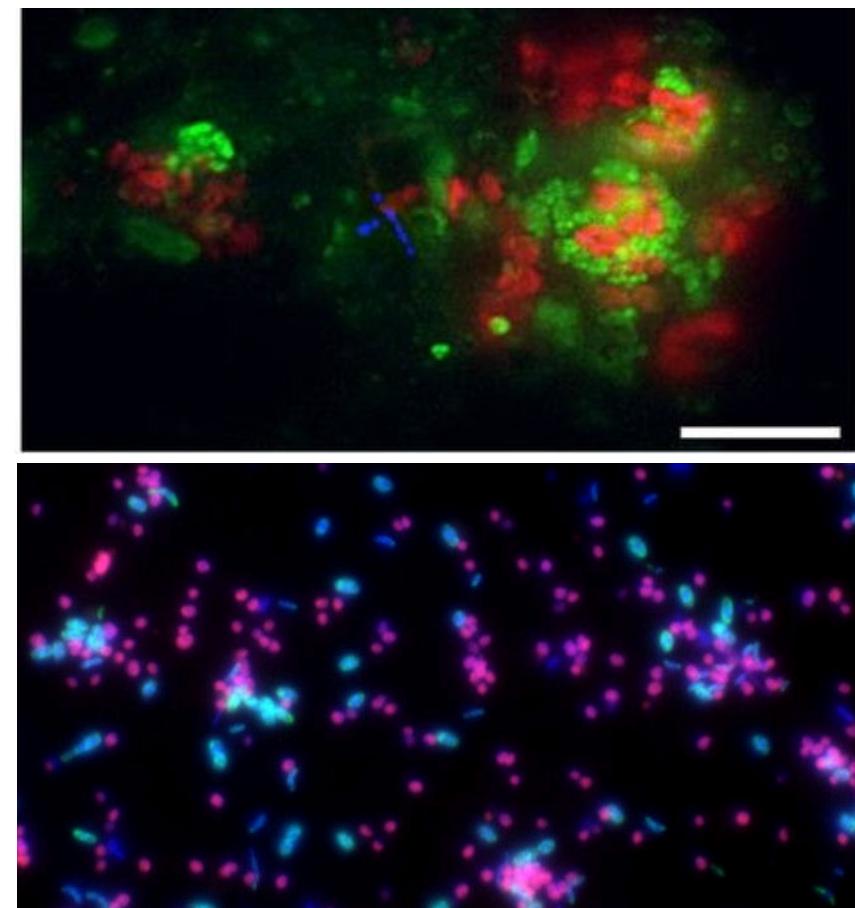
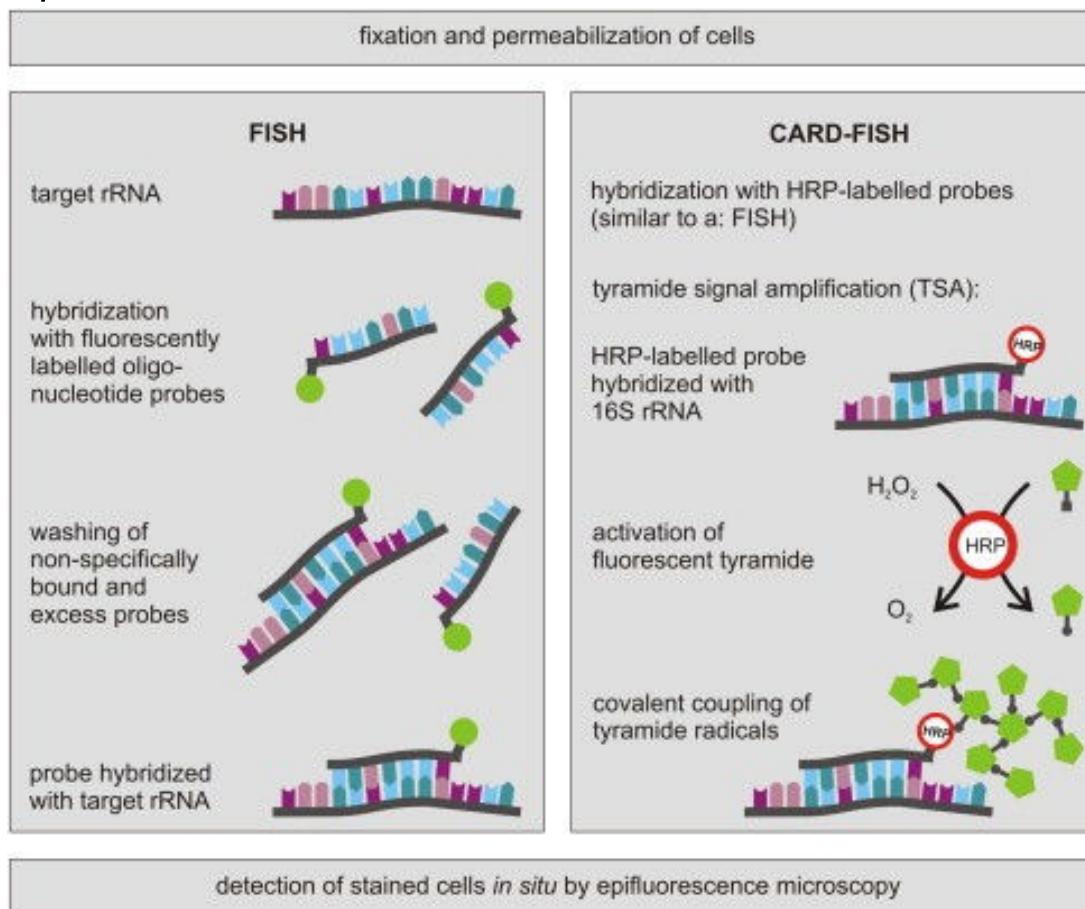
Carbon fixed by autotrophic organisms using the CBB cycle is depleted in  $^{13}\text{C}$



Organic Carbon in ancient rocks  
also shows evidence of  
isotopic fractionation

# Fluorescent in situ hybridization (FISH)

FISH (like CARD-FISH and related techniques) rely on the use of fluorescent probes that bind selectively to specific DNA or RNA sequences, allowing to selectively color and count specific groups of microbes. The specificity of the probe is selected during the design phase. Target sequences need to be known in order to design the probes.



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The diagram illustrates the FISH process and its combination with other techniques. It shows a flowchart of the FISH process: fixation and permeabilization of cells, followed by hybridization with HRP-labelled probes (similar to a FISH), then tyramide signal amplification (TSA). The TSA step involves HRP-labelled probe hybridized with target rRNA, activation of fluorescent tyramide, and covalent coupling of tyramide radicals to the probe. Finally, stained cells are detected in situ by epifluorescence microscopy. A photograph of a micrograph showing multiple colored spots (blue, green, red) is shown on the right.

fixation and permeabilization of cells

FISH

CARD-FISH

target rRNA

hybridization with HRP-labelled probes (similar to a FISH)

tyramide signal amplification (TSA):

HRP-labelled probe hybridized with target rRNA

washing of non-specifically bound excess probes

activation of fluorescent tyramide

covalent coupling of tyramide radicals

HRP

o<sub>2</sub>

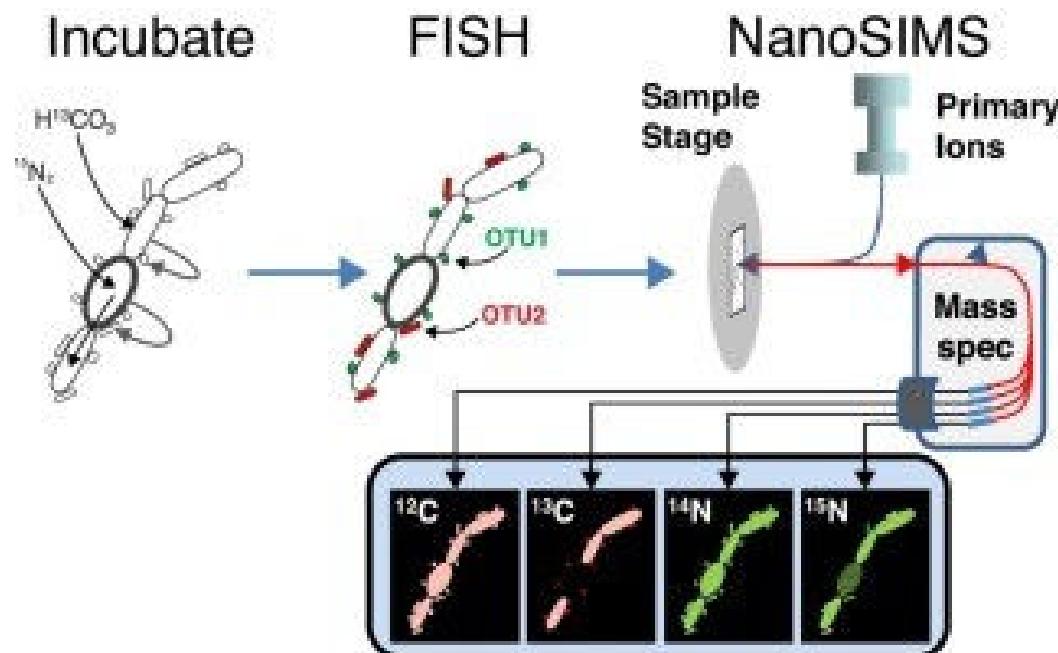
detection of stained cells *in situ* by epifluorescence microscopy



# nano-SIMS

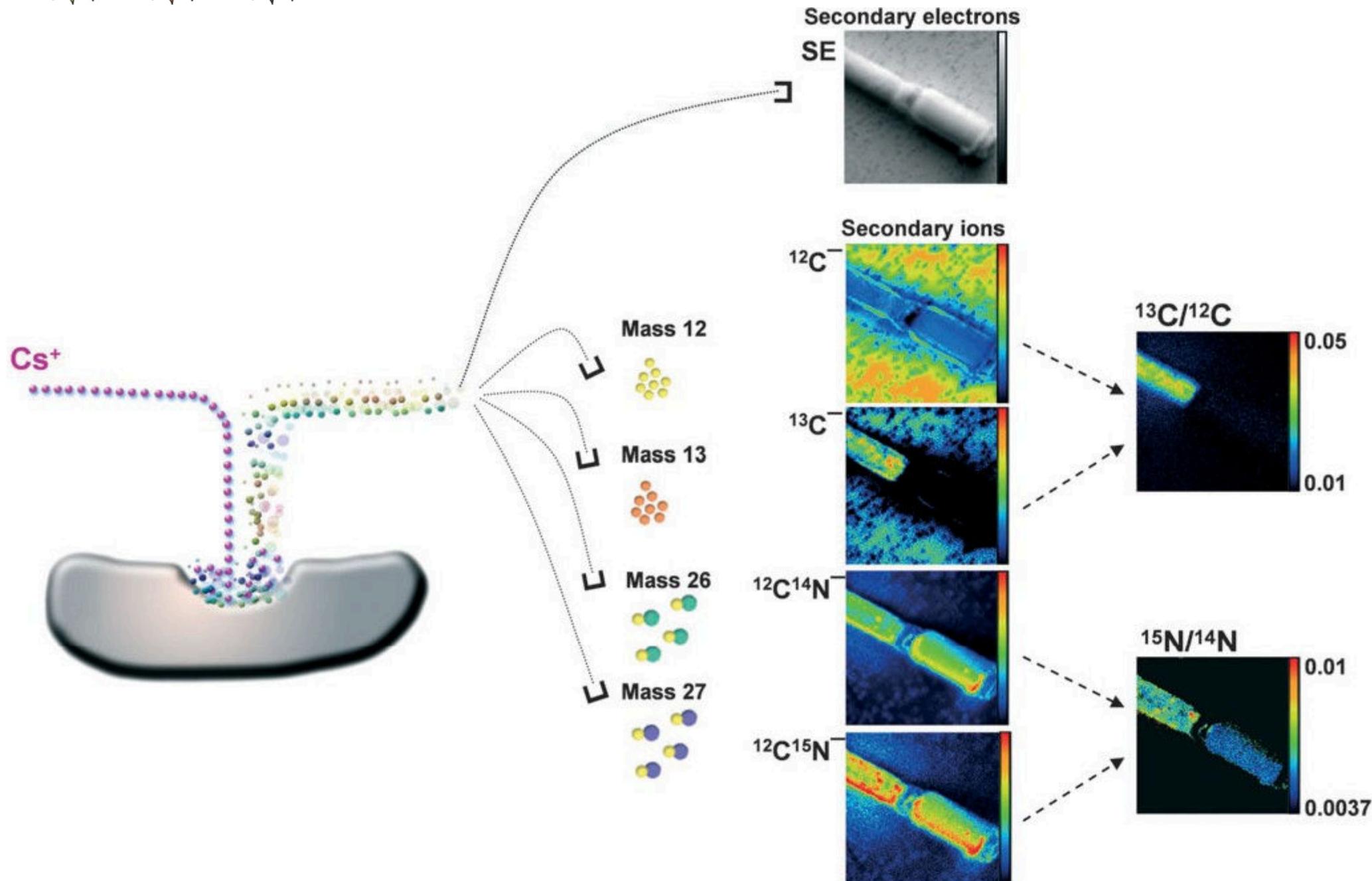
Nano-SIMS (*nanoscale Secondary Ion Emission Mass Spectrometry*) is a relatively recent techniques that combines stable isotope incubations with FISH and spatially resolved mass spectrometry

A natural sample is incubated using selected substrates enriched in a stable isotope (for example  $^{13}\text{C}$  CO<sub>2</sub>). The cells are then extracted and imaged using a combination of epifluorescence microscopy and probed for their incorporation of the label





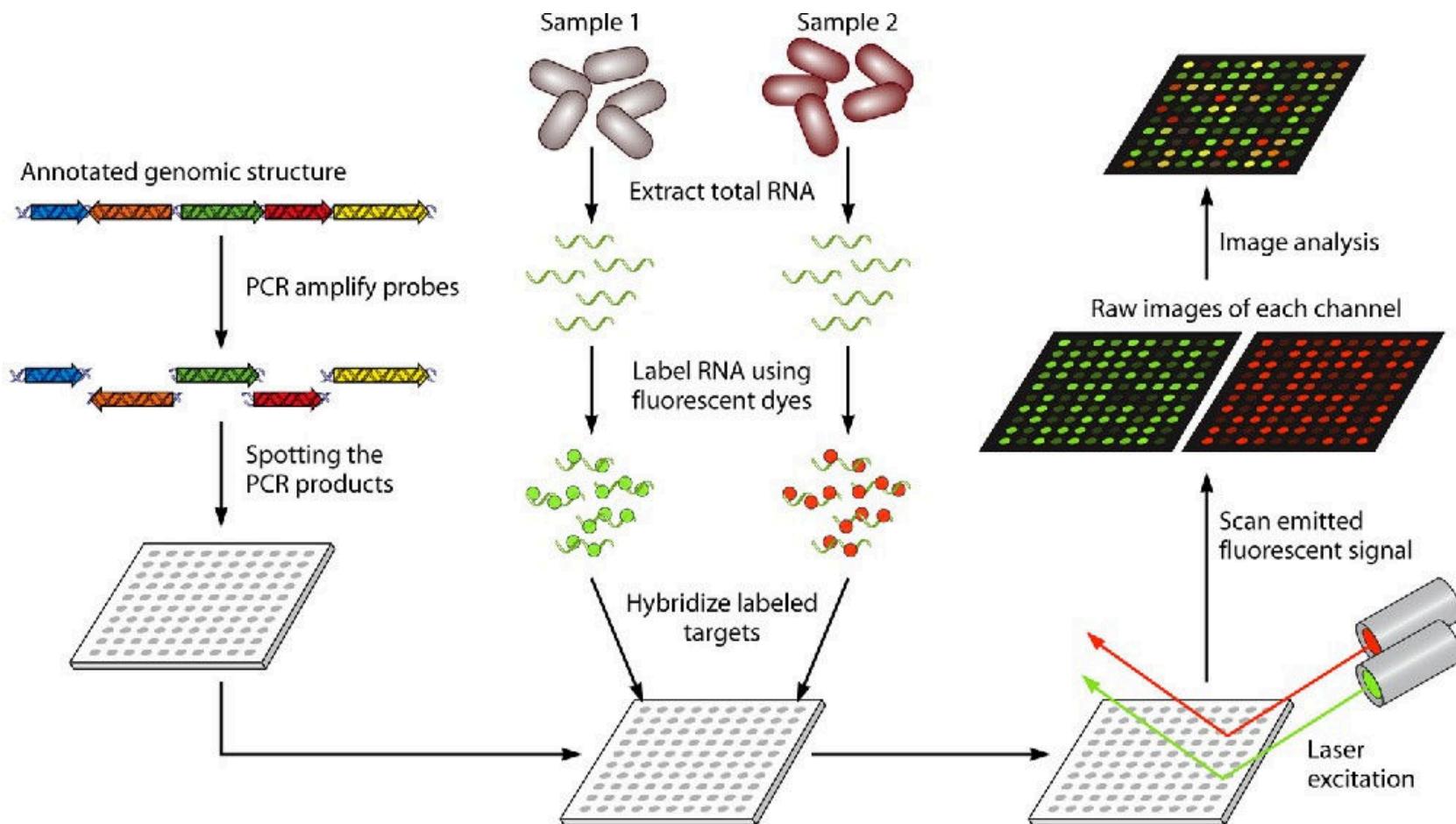
# nano-SIMS





# Microarray

Microarrays (such as the Geochip) are also based on our ability to design specific probes to visualize the presence of specific genes (DNA) or transcripts (RNA). They are limited by our ability to design probes since the sequence must be known



# *Culture-independent approaches: a detour on sequencing*

# Sequencing based approaches

DNA sequencing has revolutionized the study of microbial diversity in many ways. Current technologies and lowering prices allow us to probe the microbial world at an unprecedented rate

Sequencing can be divided based on the throughput in **single sequence** or **massive parallel sequencing** and base of the type of sequencing in **sequencing by synthesis** or more recently **sequencing by reading**

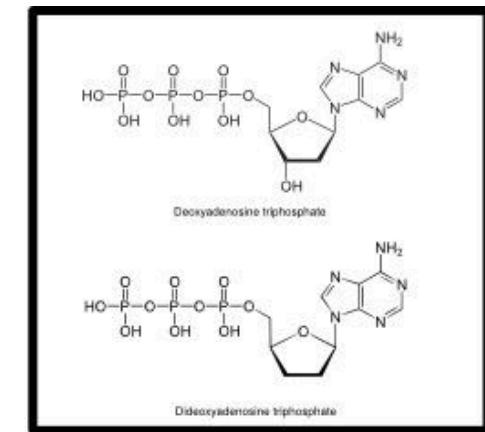
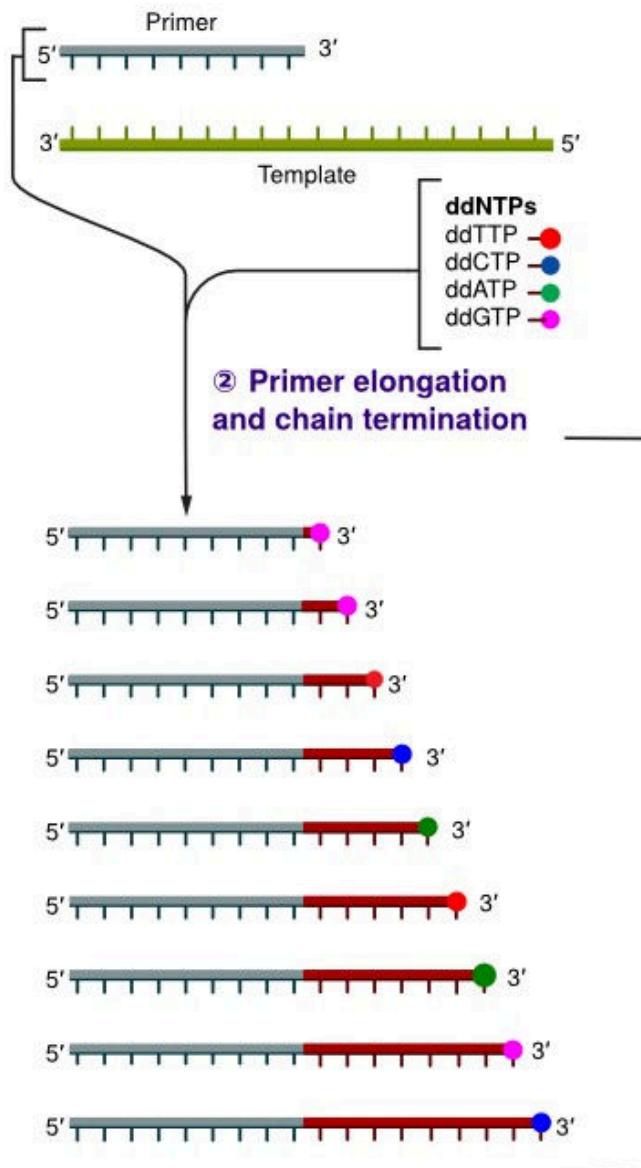
The choice of the type of sequencing to use is highly dependent on the study questions, and all the sequencing approaches have biases (like all the techniques used)

Current sequencing techniques result in millions of DNA reads to be generated, making **computer skills** a **MUST** for any biologist

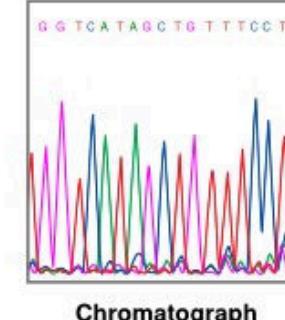
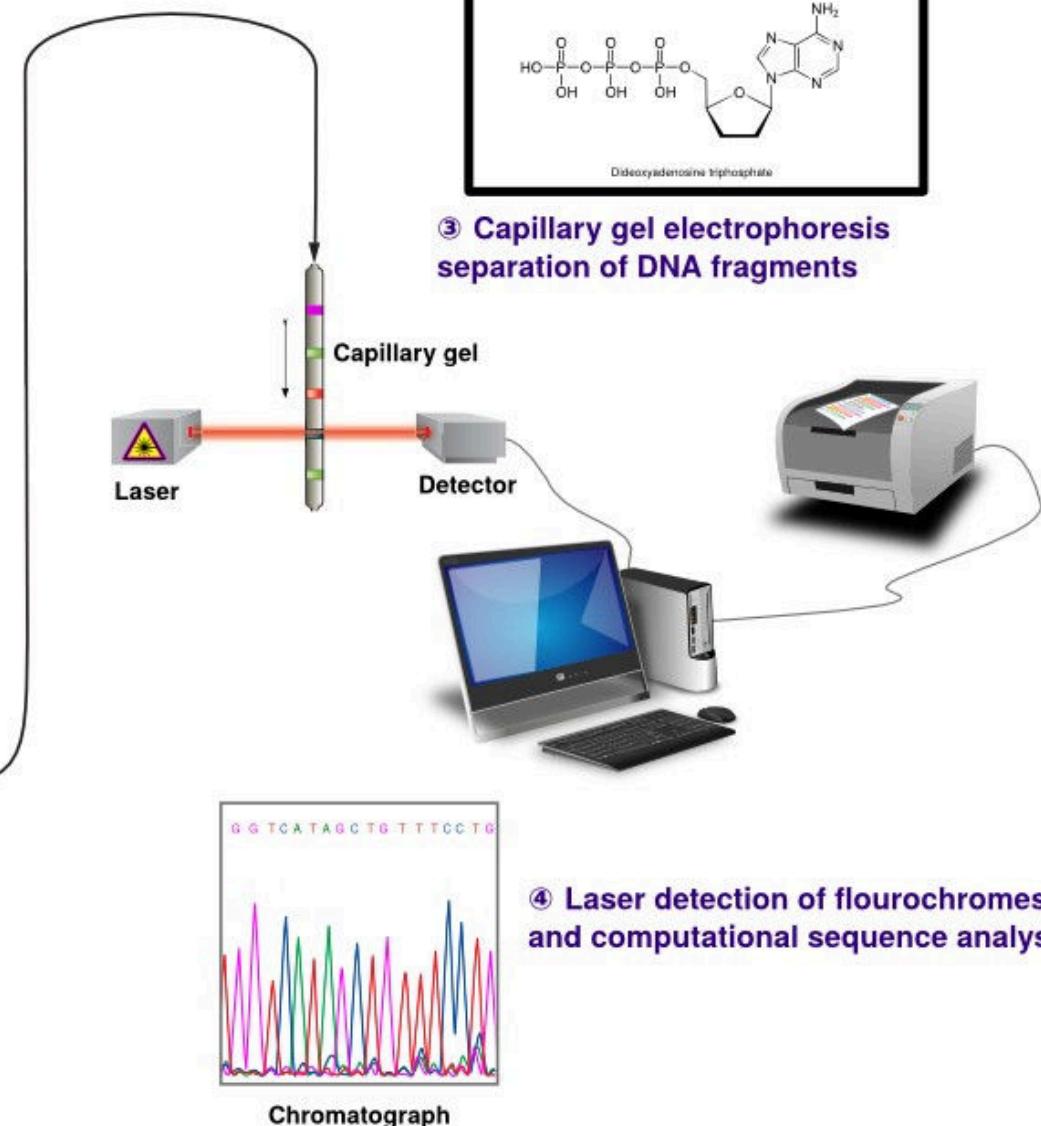
# ABI Solid Dig Dye Terminator (Sanger)

## ① Reaction mixture

- Primer and DNA template ► DNA polymerase
- ddNTPs with flurochromes ► dNTPs (dATP, dCTP, dGTP, and dTTP)



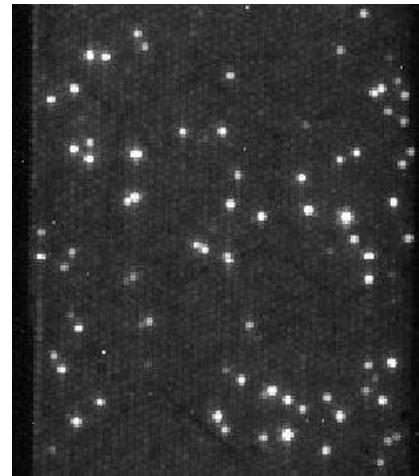
## ③ Capillary gel electrophoresis separation of DNA fragments



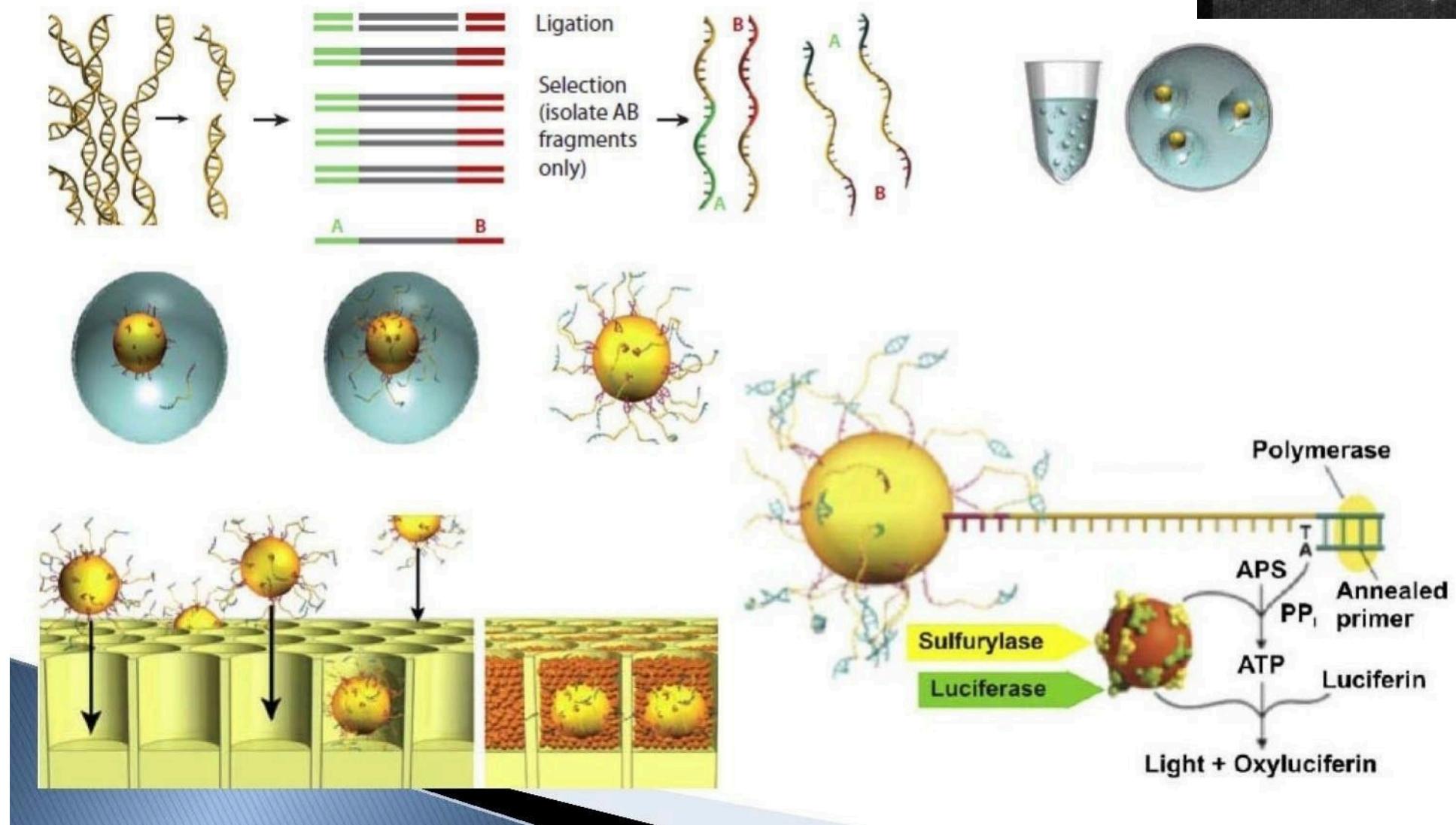
## ④ Laser detection of flurochromes and computational sequence analysis

"Sanger-sequencing" by Estevezj - Own work.

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# Pyrosequencing



# IonTorrent Sequencing

## Construct Library

Fragment / Amplicon



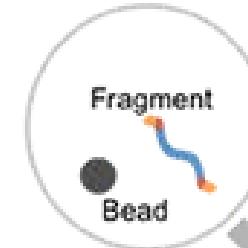
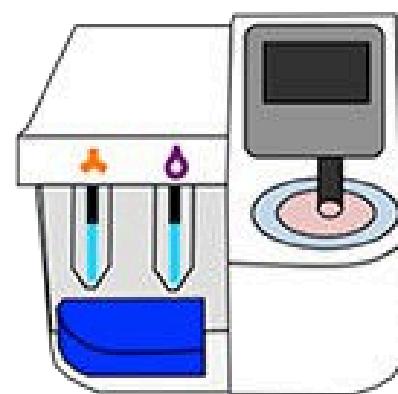
Adaptor Ligation



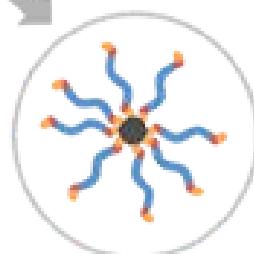
Amplification



## Template Preparation

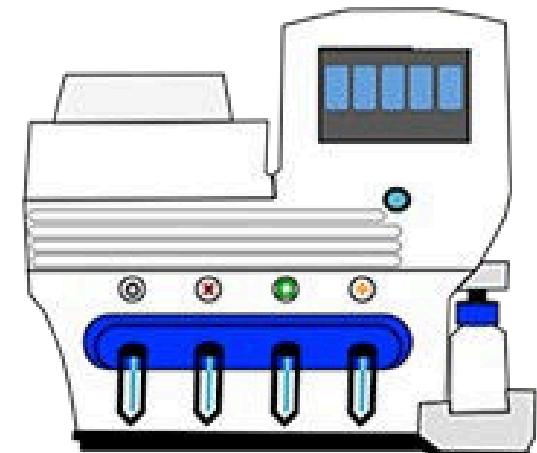
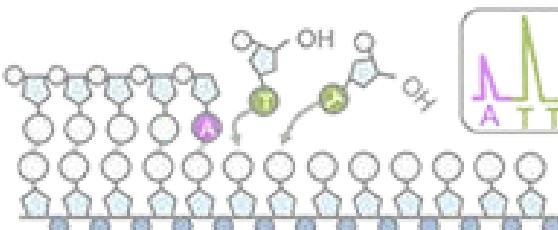
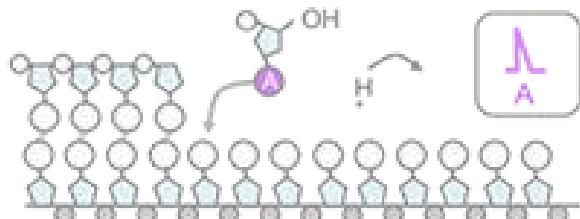


Clonal Amplification

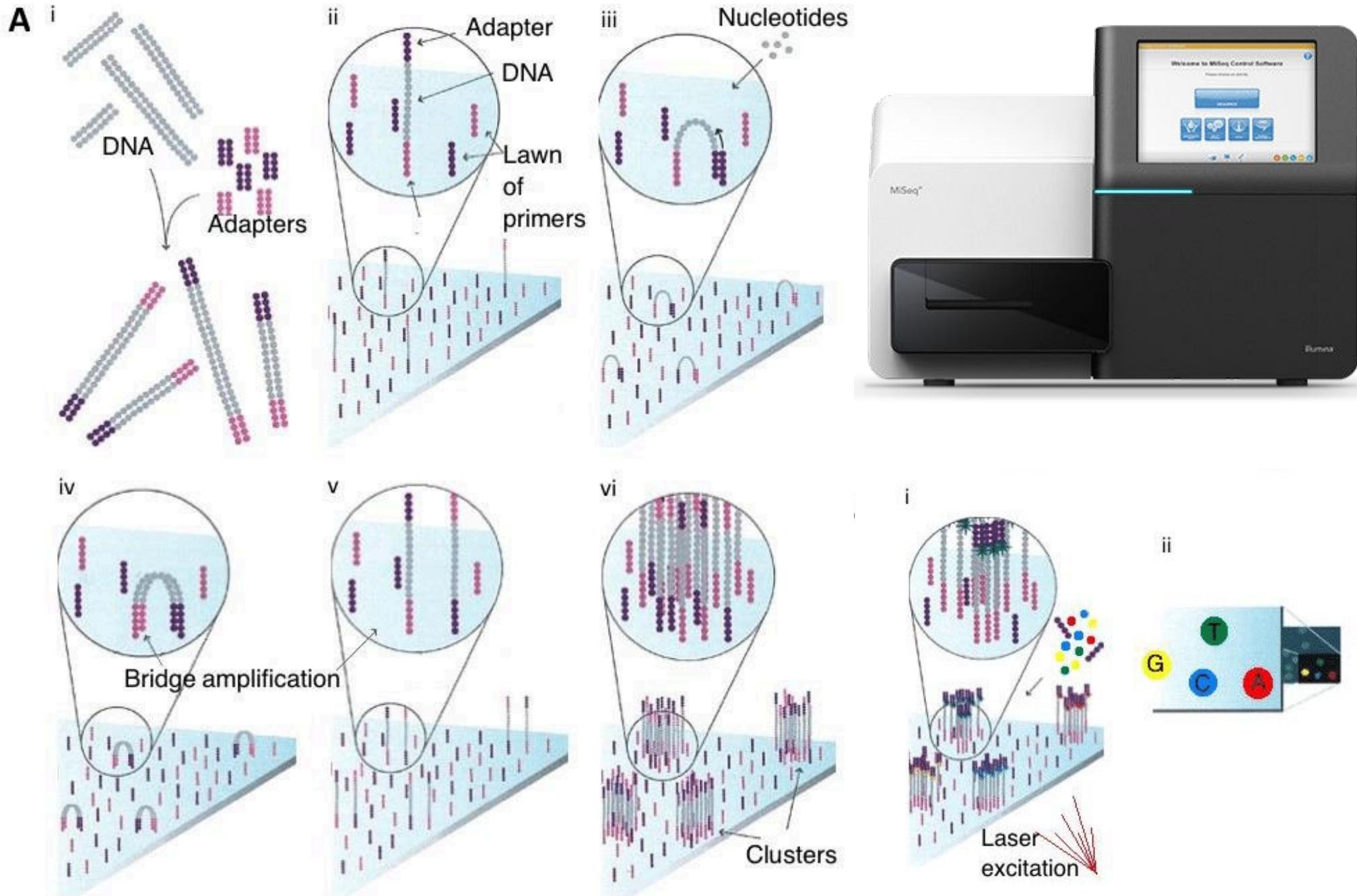


- Clonal amplification
- In-line template amplification
- Ion Sphere recovery
- Template-positive Ion Sphere enrichment

## Sequence



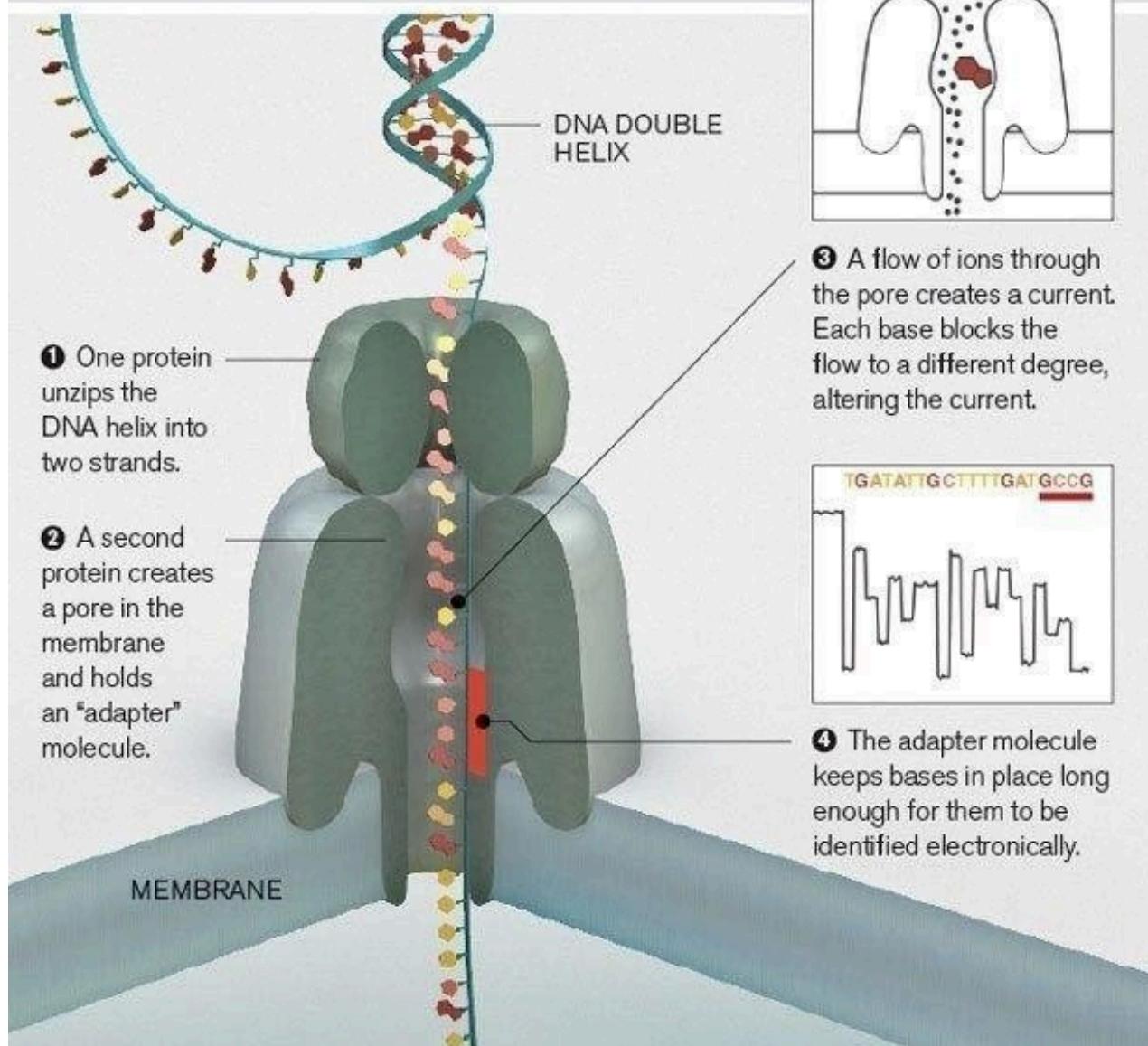
# Illumina Sequencing



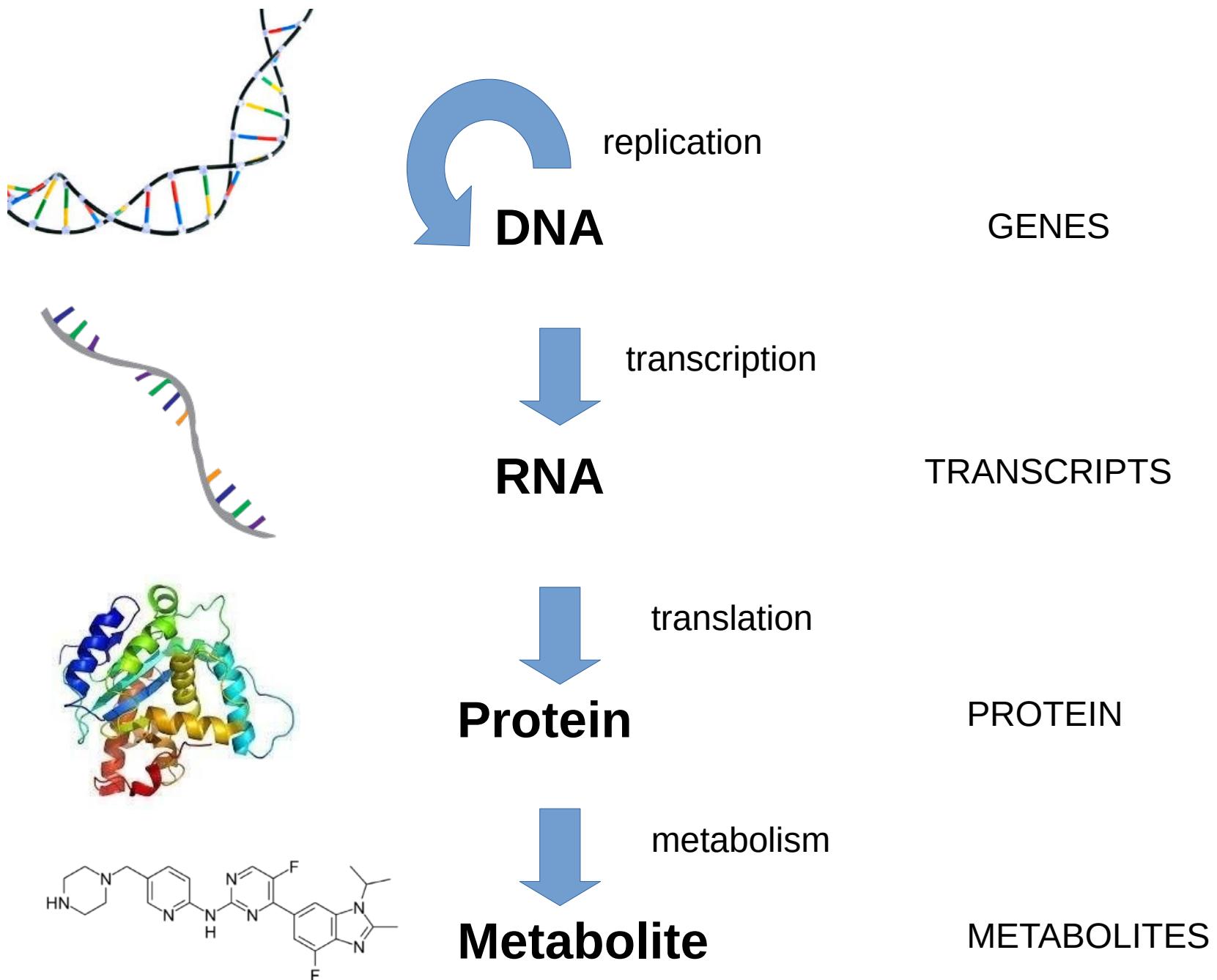
# Nanopore Sequencing



DNA can be sequenced by threading it through a microscopic pore in a membrane. Bases are identified by the way they affect ions flowing through the pore from one side of the membrane to the other.



# Information flow in biology



# A primer on language

Moving from **gene** to **genome** to **metagenome** is a matter of scales.  
We go from the single gene to the whole genome to the genomes of  
the entire community

The suffix (**omic** and **meta**) are used also for the other words in the central dogma, so we have **transcriptomic**, **metatranscriptomic**, **metametabolomic**, etc..

The same suffix have been applied to a number of other words, like **metallomics**, **mobilome**, **secretome**, etc...

Not all the techniques referred to with omic suffix are about sequencing. Proteomic and metaproteomic require the use of mass spectrometry, metabolomic requires the use of mass spectromic and/or nuclear magnetic resonance. These techniques are usually combined with sequencing

# A brief history of Metagenomic

## Norman R. Pace

propose the idea of cloning DNA directly from environmental sample to analyze 16S rRNA diversity



**Mya Breitbart** used environmental shotgun sequencing to show the diversity of virus in seawater



A pilot **GOS** project in the **Sargasso Sea** shows unprecedented bacterial diversity in seawater



**Robert Edwards** published sequences generated using pyrosequencing techniques



1985

1998

2002

2003

2004

2005

2007



**Jo Handelsman** is the first to use the term METAGENOMIC referring to the analysis of community genomes



**Craig Venter** leads the *Global Ocean Sampling Expedition* (GOS) to collect metagenomic samples throughout the journey



**Stephan Schuster** publishes the first sequences generated using high-throughput sequencing.



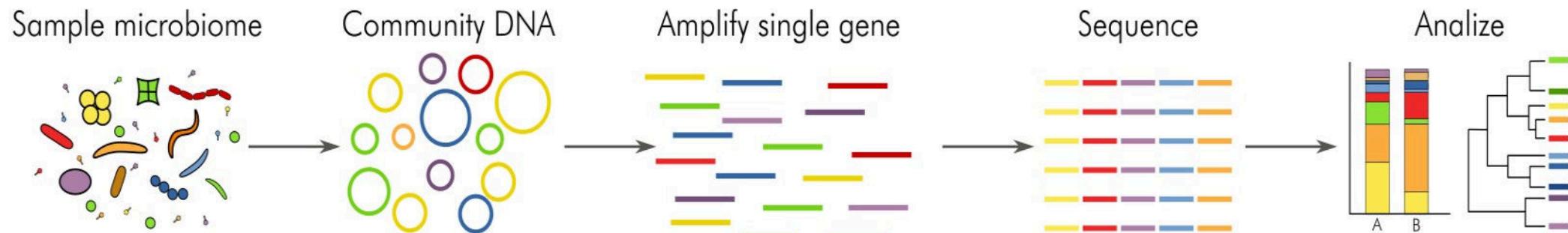


# amplicon-sequencing 16S rRNA

Amplicon sequencing (sometime referred to as tag-amplicon sequencing) is a techniques that allows to sequence the 16S rRNA (or other target genes) in a high throughput way

It is de facto one of the most used techniques to investigate microbial taxonomic diversity. It is dependent on DNA amplification through PCR, so PCR primers (often designed on cultured microbes) and the PCR can introduce significant bias.

The tag suffix, refers to the ability to use known short DNA sequences (tags) to recognize sequences coming from different samples at the data analysis stage



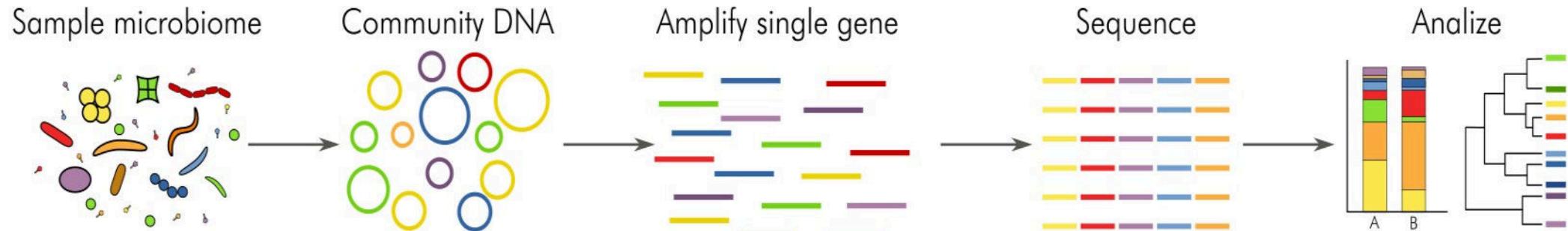


# amplicon-sequencing

Many other genes can be investigated using this techniques, both genes with taxonomic relevance (18S rRNA, ITS, mCOI) and functional genes

If the starting material is RNA (instead of DNA) this has the benefit to link the results to the active fraction of the community

The reference database is of fundamental importance for amplicon sequencing approaches, as well as the choice of appropriate primers to be used at the amplification step





# Stable Isotope Probing (SIP)

Combining stable isotopes incubation with sequencing, SIP approaches can be used to identify the fraction of the microbial community involved in specific metabolic pathways

If used in combination with amplicon sequencing, SIP has similar drawback, however SIP can be coupled to other techniques, such as (metagenomic, metatranscriptomic, etc...). It is a very powerful targeted technique if used properly linking identity to function

Main drawback is that typically you are stimulating the community and selecting for fast growers

