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Ministero dell'Università e della Ricerca

Italiadomani
PIANO NAZIONALE DI RIPRESA E RESILIENZA

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The flow cytometry and implication in food safety

28/11/2023

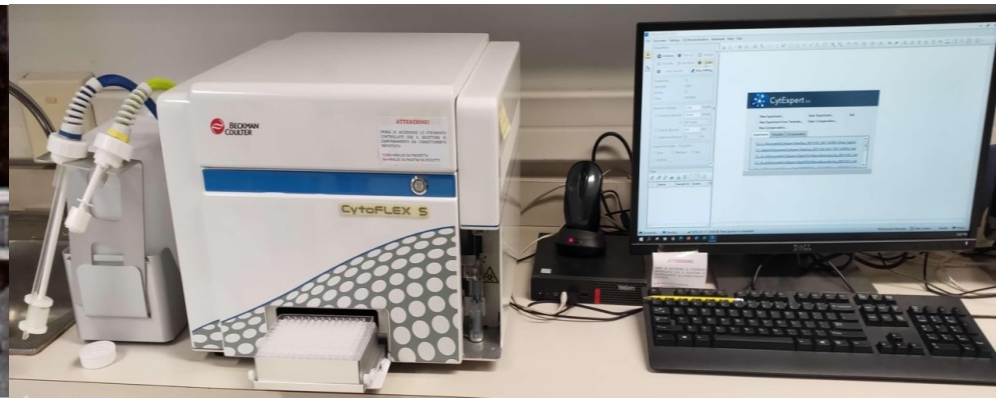
Training course SUS-MIRRI.IT

- **Flow cytometry** (FCM) is an analytical technique developed in the 1960s, closely related to light microscopy, to study the chemical and physical characteristics of eukaryotic and subsequently prokaryotic cells.

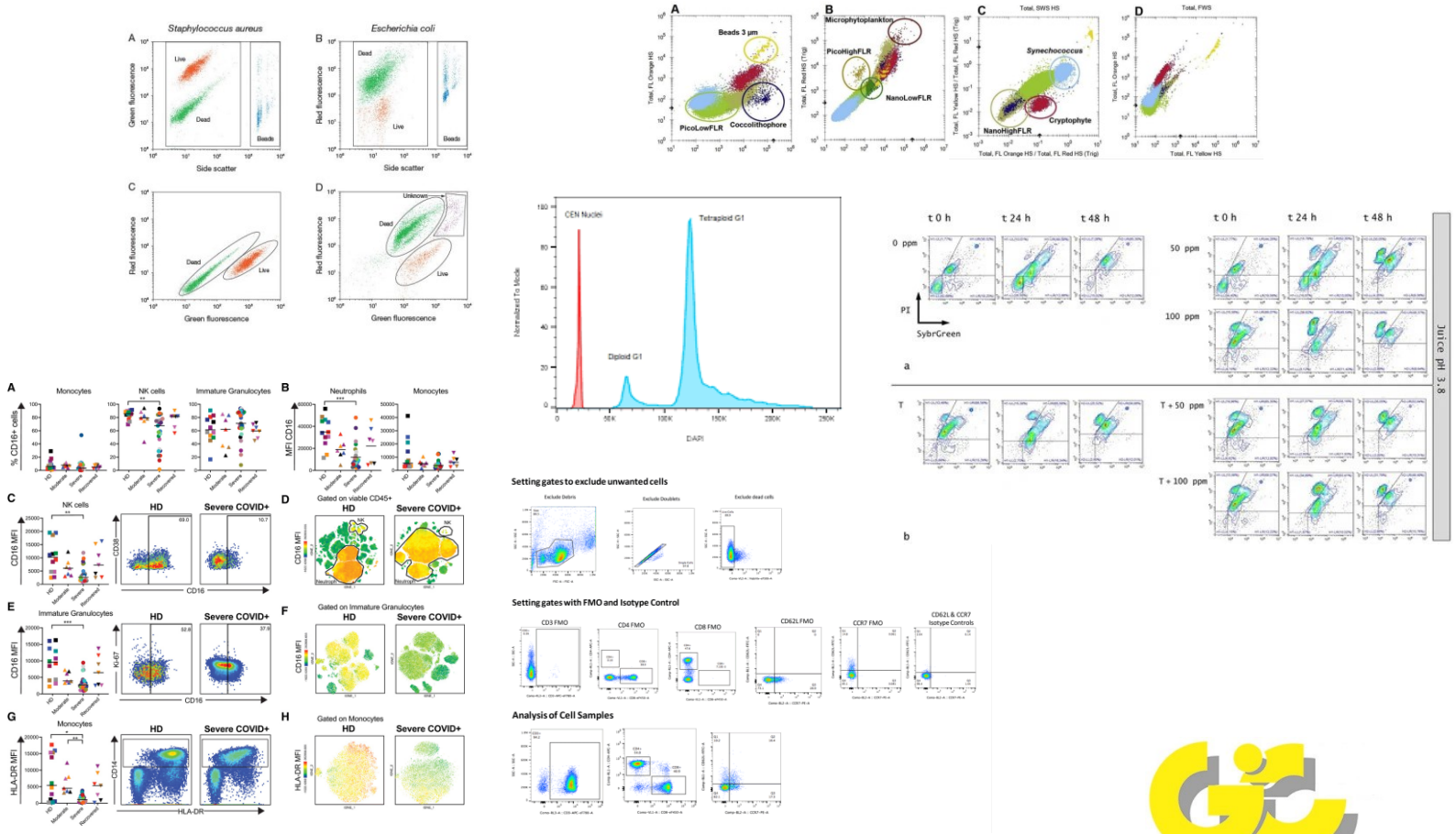
- It allows the automatic analysis of cells and cellular elements in suspension generically called **"events",** led by a conveying fluid to intercept light radiation.

- The data available for each individual event generate a matrix of analysis parameters and provide quantitative information on optical, physical and chromatic properties for each particle of interest, enabling its characterisation and physical separation for subsequent manipulation





- ✓ Allergology
- ✓ Reproductive biology
- ✓ Stem cells and cell therapies
- ✓ Cell cycle and apoptosis
- ✓ Molecular cytogenetics
- ✓ Covid 19
- ✓ Clinical and experimental haematology
- ✓ Clinical and experimental immunology
- ✓ Transfusion medicine
- ✓ Clinical and experimental microbiology
- ✓ Microscopy
- ✓ Clinical and experimental oncology
- ✓ Veterinary pathology
- ✓ Toxicology and Radiotherapy

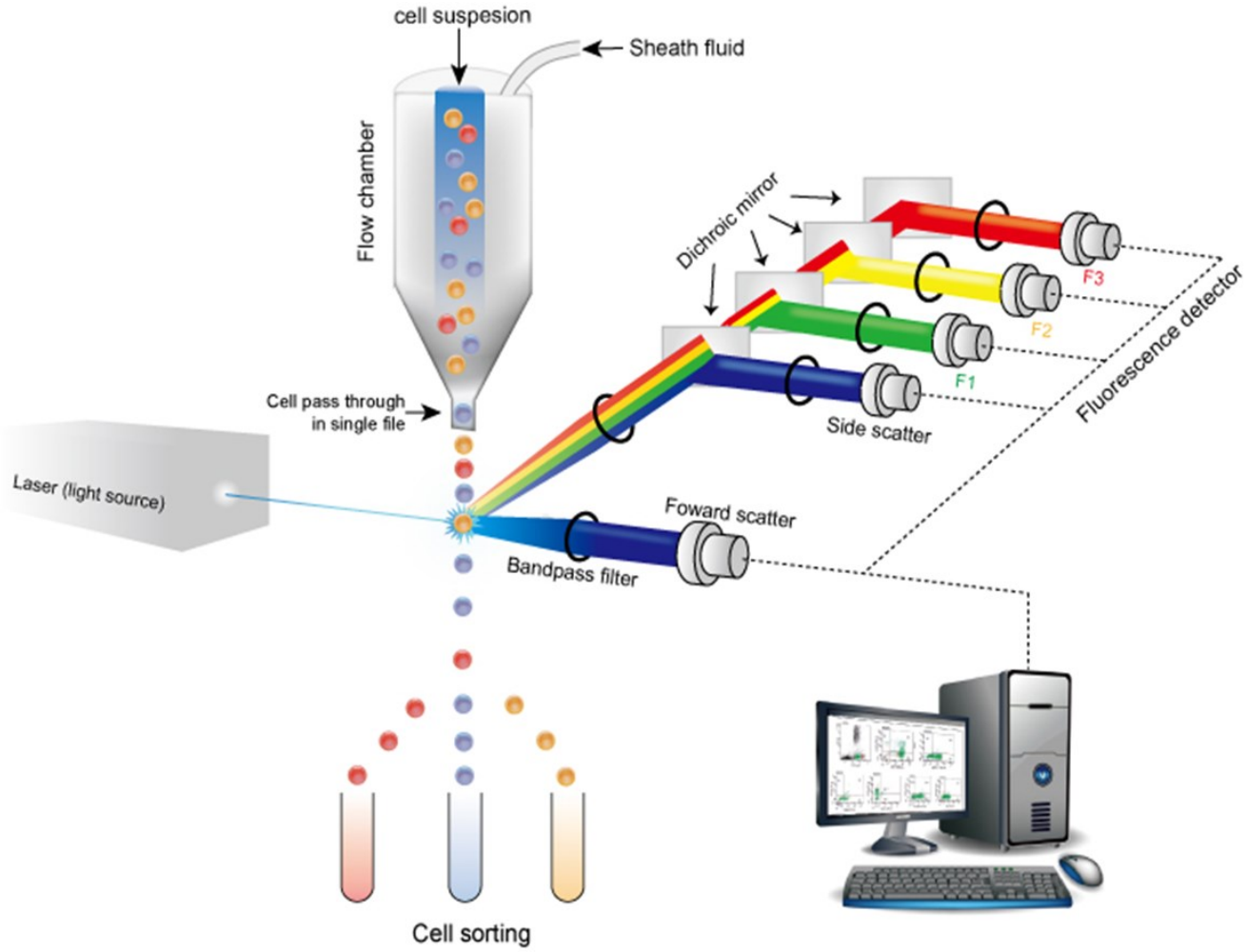


✓ **ENVIRONMENTAL CYTOMETRY → INCREASING NON-BIOMEDICAL APPLICATIONS**

Italian Cytometry Society

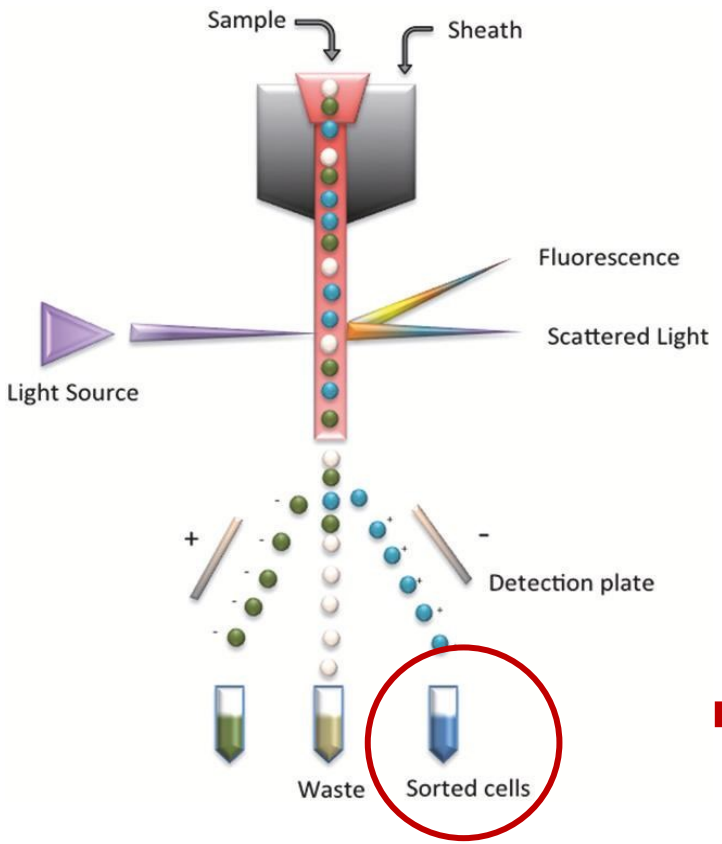
Basic principle

1. Interaction of events with the excitation source (one or more lasers)
2. Light diffraction phenomenon and fluorescence emission occurring because of the interaction
3. The emission is collected by filters that select specific signals
4. The signal is recorded, and the data are displayed as events on histograms or dot plots

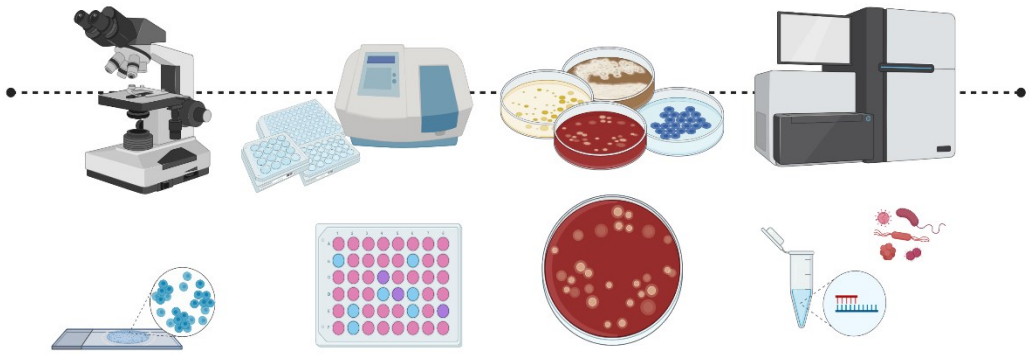


A. Adan, G. Alizada, Y. Kiraz, Y. Baran e A. Nalbant, «Flow cytometry: basic principles and applications,» Critical Reviews in Biotechnology, n. 37(2), pp. 163-176, 2017.

FACS: Flow sorting for cloning single cells and/or populations of interest

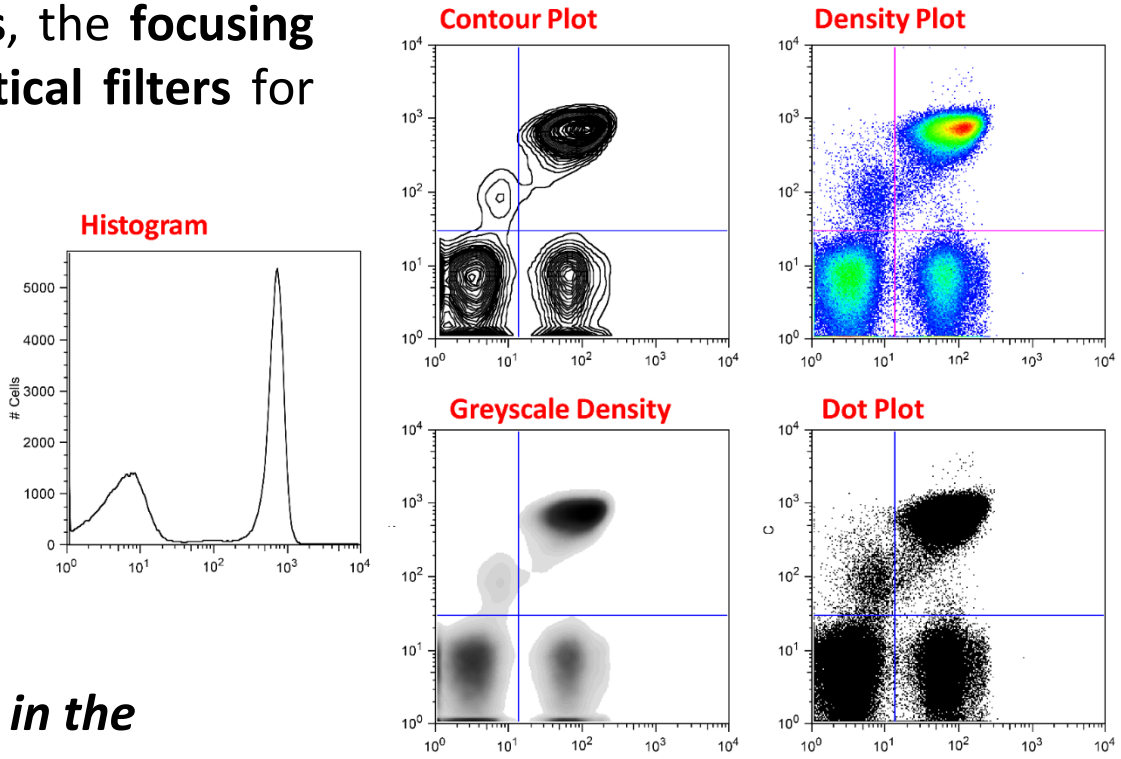


- Physical isolation of specific **subpopulations** based on cellular physiological state
- Single events are **electrostatically charged** by electromagnetic pulses and deflected by a stationary magnetic field
- Cell separation can be performed in various media for **subsequent specific analysis and manipulation**



A. Adan, G. Alizada, Y. Kiraz, Y. Baran e A. Nalbant, «Flow cytometry: basic principles and applications,» Critical Reviews in Biotechnology, n. 37(2), pp. 163-176, 2017.

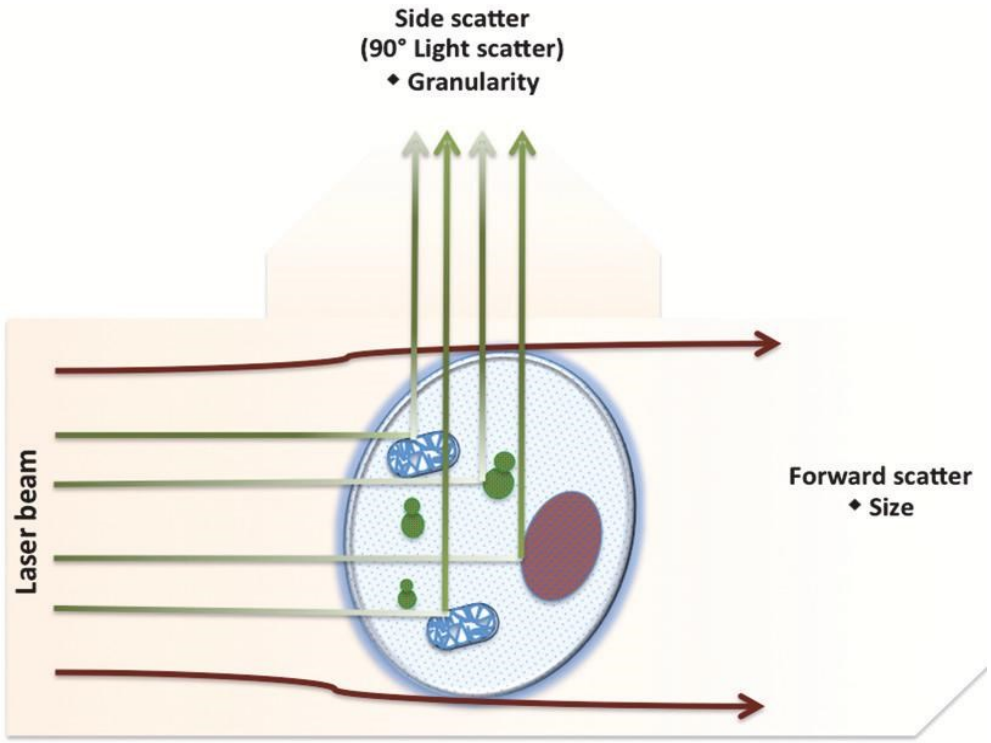
- **Fluidic component:** the sample is dragged by a *sheath fluid* that allows it to be aligned towards the laser, generating fluorescence signals and diffracted light (*scatter*).
- **Optical component:** consisting of the **excitation sources**, the **focusing lenses** capable of directing the laser beam, and the **optical filters** for selecting the emission wavelengths.
- **Electronic component:** allows the conversion of the light signal emitted by the sample into electrical signals proportional to intensity. The graphic representation of the data can be biparametric (*dot plot*) or monoparametric (*histograms*).



Real-time information on the parameter distribution in the population of interest

MULTIPARAMETRIC ANALYSIS

Simultaneous measurement of multiple parameters of individual cells



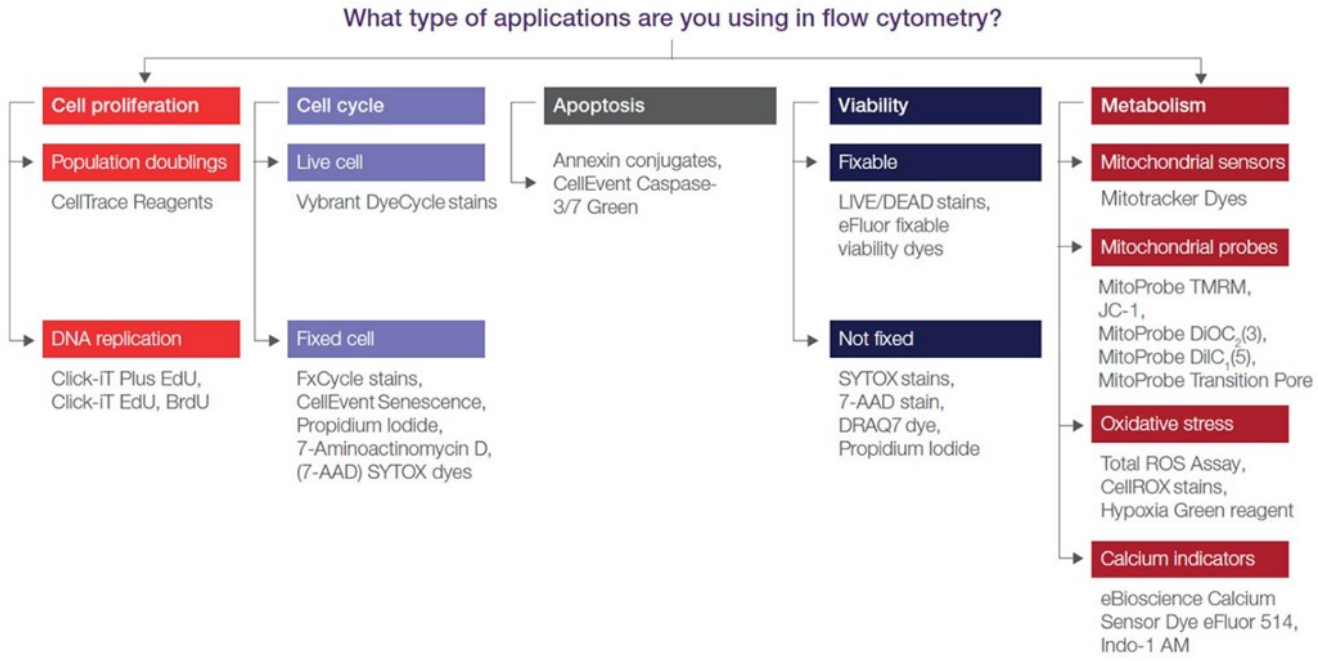
- **Relative size and volume:** *frontal diffraction* (*Forward Scatter* - **FSC**) picked up by a photodiode placed **in front of** the optical path of the laser beam.
- **Granularity or internal complexity:** *side diffraction* (*Side Scatter* - **SSC**) detected by a photomultiplier placed at **right angles** to the excitation beam, together with the fluorescence signal.
- **Fluorescence intensity:** FL1, FL2, FL3 ... using fluorescent molecules as probes.

A. Adan, G. Alizada, Y. Kiraz, Y. Baran e A. Nalbant, «Flow cytometry: basic principles and applications,» Critical Reviews in Biotechnology, n. 37(2), pp. 163-176, 2017.

Explorable parameters

- **Intrinsic:** studied without introducing probes or reagents. The signal depends only on the interactions between light radiation and the interrogated event (*autofluorescence*)

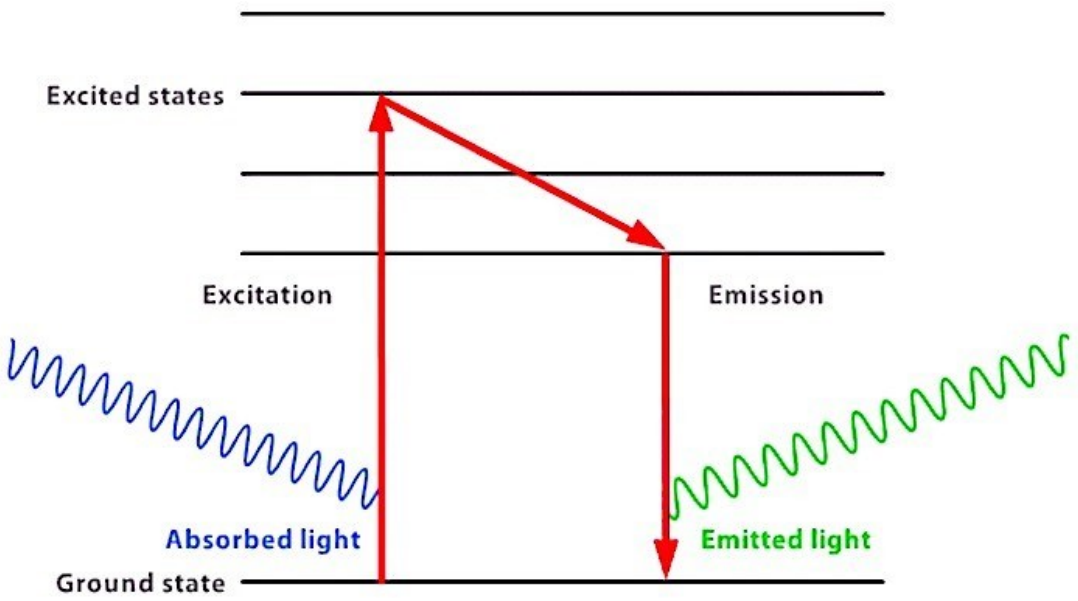
- **Extrinsic:** requires the insertion of a probe functional to the parameter being explored into the analytical system and produces a fluorescence signal



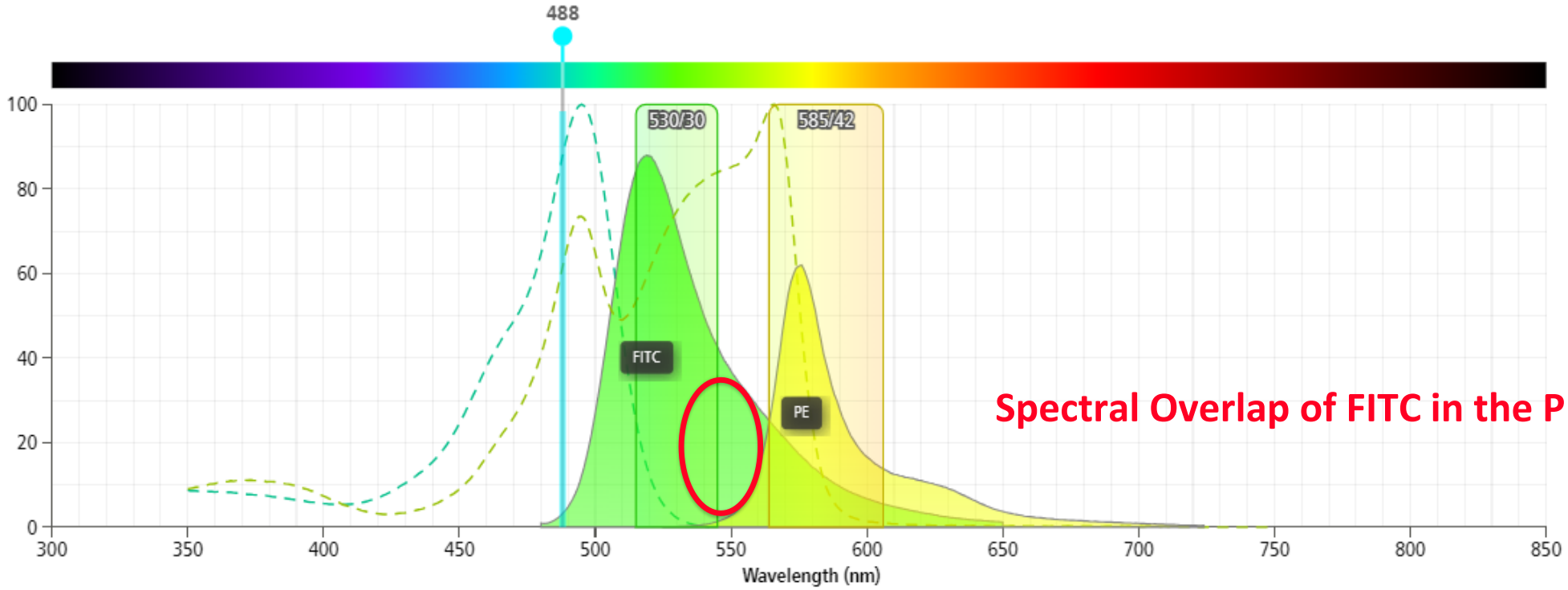
https://www.thermofisher.com/it/en/home.html

Nucleic acid-binding fluorochromes

- Fluorochromes are a group of molecules capable of exhibiting fluorescence after being excited by a light source
- Fluorochromes capable of binding to nucleic acids can provide a lot of information in the membrane permeability assessment of to generate bacterial viability information



Fluorochromes selection



Spectral Overlap of FITC in the PE detector

	Fluorochrome	%Max	Ex	Em	Filters	FITC	PE	PerCP...	PE-Cy7
1	FITC	88.0	☑	☑	530/30 ☑	47.4%	0.4%	0.3%	1.8%
2	PE	61.6	☑	☑	585/42 ☑	12.5%	70.4%	0.5%	16.4%

https://www.bdbiosciences.com/en-eu/resources/bd-spectrum-viewer-classic

PROPIDIUM IODIDE

Ext. 342 e 540nm; **em**. 615nm

It is unable to cross the membrane of intact, viable cells.
application for viability assessment:

cell permeability to PI begins already in the presence of early alterations in the organisation of the cell membrane (detection of bacterial cells in a VBNC state).

Table 1. Fluorophores for flow cytometry.

Fluorophores	Fluorescence Color	Maximal Absorbance, nm	Maximal Emission, nm	Relative Brightness
DyLight 405		400	420	3
Alexa Fluor 405		401	421	3
Pacific Blue		410	455	1
Alexa Fluor 488		495	519	3
FITC		490	525	3
DyLight 549		562	576	4
PE*		496, 546	578	5
APC		650	661	4
Alexa Fluor 647		650	665	4
DyLight 650		654	673	4
PerCP		490	675	2
Alexa Fluor 700	Infrared	702	723	2

* PE is the same as R-phycoerythrin.
APC, allophycocyanin; FITC, fluorescein isothiocyanate; PE, phycoerythrin; PerCP, peridinin chlorophyll protein.

Table 2. Tandem dyes for flow cytometry.

Fluorophores	Fluorescence Color	Maximal Absorbance, nm	Maximal Emission, nm	Relative Brightness
PE–Alexa Fluor 647		496, 546	667	4
PE–Cy5		496, 546	667	5
PerCP-Cy5.5		490	695	3
PE–Cy5.5		496, 546	695	4
PE–Alexa Fluor 750	Infrared	496, 546	779	4
PE–Cy7	Infrared	496, 546	785	4
APC-Cy7	Infrared	650	785	2

* PE is the same as R-phycoerythrin.
APC, allophycocyanin; PE, phycoerythrin; PerCP, peridinin chlorophyll protein.

Table 3. Fluorescent proteins in flow cytometry.

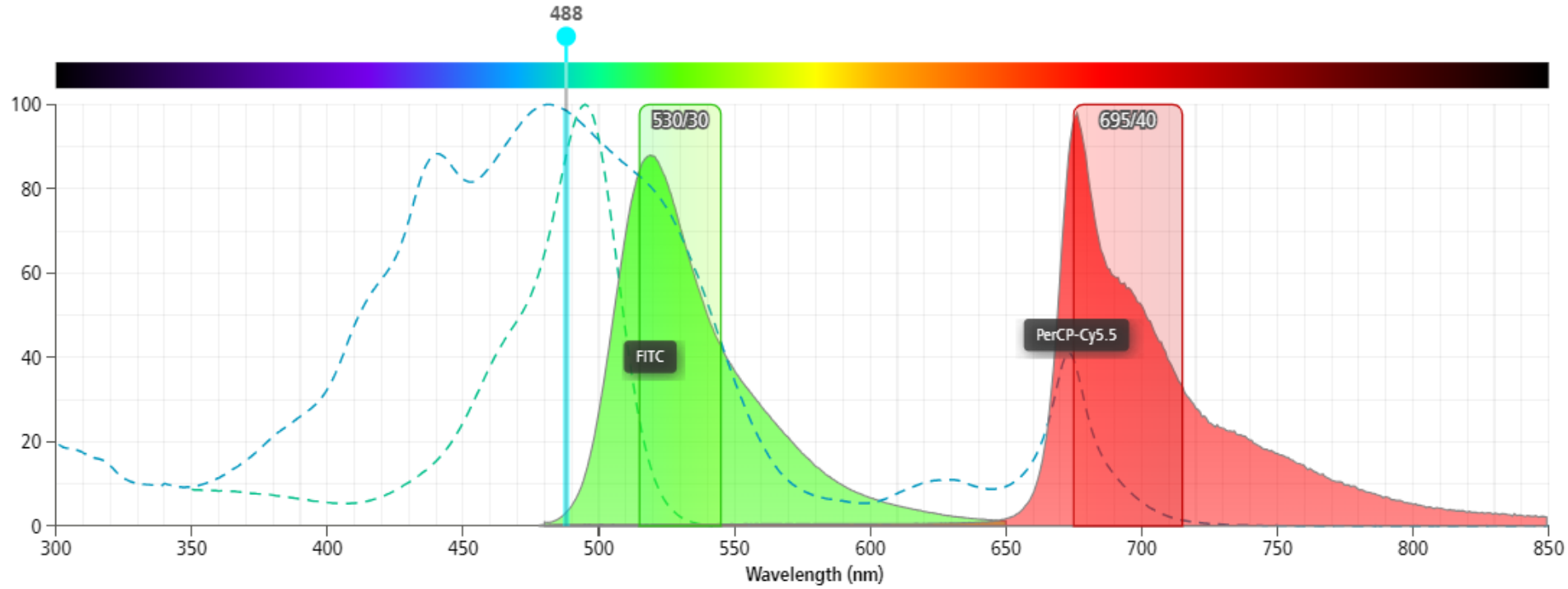
Fluorophores	Fluorescence Color	Maximal Absorbance, nm	Maximal Emission, nm	Relative Brightness
EBFP		383	445	2
CFP		439	476	2
EGFP		484	509	4
YFP		514	527	5
RFP		558	583	4
mCHERRY		587	610	3

SYBRGreenI/SYTO24

SYBRGreen I (**Ext**. 488nm; **ems**. 522nm) and SYTO24 (**ext.** 490nm; **ems.** 515nm)

Penetration capacity in intact membrane cells.

The emission spectrum allows double staining in combination with PI, giving complementary information to PI on the overall viability states of the sample



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2	PE	61.6	☐	☐	585/42	☐	12.5%	70.4%	0.5%	16.4%
3	PerCP-Cy5.5	98.4	☑	☑	695/40	☑	x	2.0%	48.4%	1.4%

https://www.bdbiosciences.com/en-eu/resources/bd-spectrum-viewer-classic

PROS

- Possibility of **multi-parametric analysis** of many cells
- **Quantitative** (optical, physical, colour properties) and **qualitative** (cell physiology) information of all suspended elements
- **Real-time** analysis: rapidity, reproducibility and statistical reliability of data
- Analysis of very **rare cells** difficult to detect by other methods
- **Physical separation** for subsequent manipulation (*flow cell sorting*)

CONS

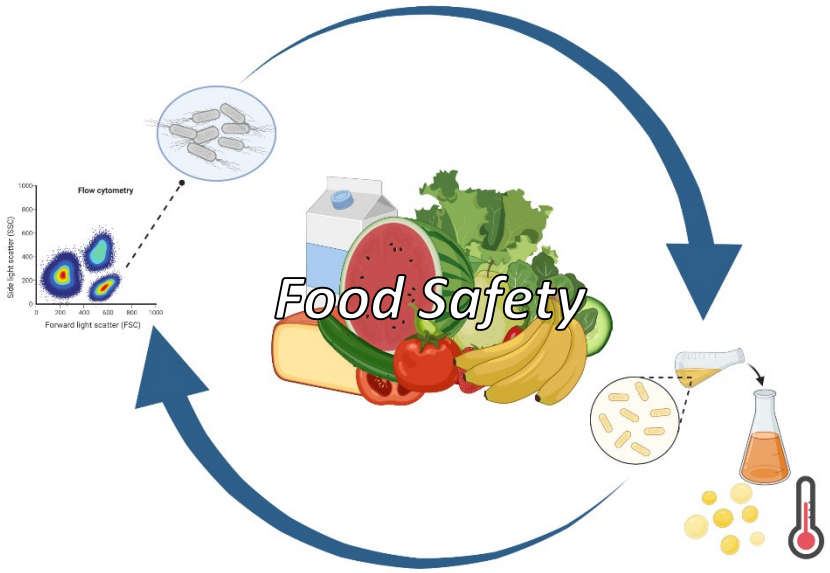
- No working in a sterile environment
- Appropriate sample treatment to reduce background noise
- No taxonomic approach

Summing up

- ✓ **The technique is applicable to any experimental model where the parameter explored can produce a specific signal detectable by the instrument**
  → applicable to various fields of agrifood and environmental microbiology (e.g. food matrix, soil, water, etc.) as an analytical complement to conventional culture-based methods

- ✓ FCM allows **rapid and accurate** analysis to assess the **physiological and metabolic states** of cells, underestimated by culture-based methods that consider **viability exclusively in terms of cultivability**

Why in food microbiology?



Contents lists available at ScienceDirect
Journal of Food Engineering
journal homepage: www.elsevier.com/locate/jfoodeng
ELSEVIER

Modelling of *Listeria monocytogenes* Scott A after a mild heat treatment in the presence of thymol and carvacrol: Effects on culturability and viability

Stefania Arioli[a], Chiara Montanari[b,*], Michael Magnani[c], Giulia Tabanelli[b], Francesca Patrignani[b,c], Rosalba Lanciotti[b,c], Diego Mora[a], Fausto Gardini[b,c]

foods
MDPI
Article

Synergistic Action of Mild Heat and Essential Oil Treatments on Culturability and Viability of *Escherichia coli* ATCC 25922 Tested In Vitro and in Fruit Juice

Luciana Di Gregorio [1,†], Alex Tchuenchieu [2,3,*], Valeria Poscente [1,4,†], Stefania Arioli [5], Antonella Del Fiore [1], Manuela Costanzo [1], Debora Giorgi [1], Sergio Lucretti [1] and Annamaria Bevivino [1,*]

microorganisms
MDPI
Review

Current and Future Flow Cytometry Applications Contributing to Antimicrobial Resistance Control

Luminita Gabriela Marutescu [1,2]

frontiers | Frontiers in Microbiology
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RECEIVED 18 September 2023

Lactiplantibacillus plantarum monolayer enhanced bactericidal action of carvacrol: biofilm inhibition of viable foodborne pathogens and spoilage microorganisms

Valeria Poscente[1,2], Luciana Di Gregorio[1]*, Manuela Costanzo[1], Chiara Nobili[1], Roberta Bernini[2], Luigi Garavaglia[3] and Annamaria Bevivino[1]*

Comparison between Flow Cytometry and Traditional Culture Methods for Efficacy Assessment of Six Disinfectant Agents against Nosocomial Bacterial Species

Richard Massicotte[1], Akier A. Mafu[2], Darakhshan Ahmad[3], Francis Deshaies[4], Gilbert Pichette[3] and Pierre Belhumeur[4]*

foods
MDPI
Review

Potential of Flow Cytometric Approaches for Rapid Microbial Detection and Characterization in the Food Industry—A Review

Elena Zand [1,†], Antje Froehling [2,†], Christoph Schoenher [3], Marija Zunabovic-Pichler [3], Oliver Schlueter [2] and Henry Jaeger [1,*]

FCM applications in Microbiology

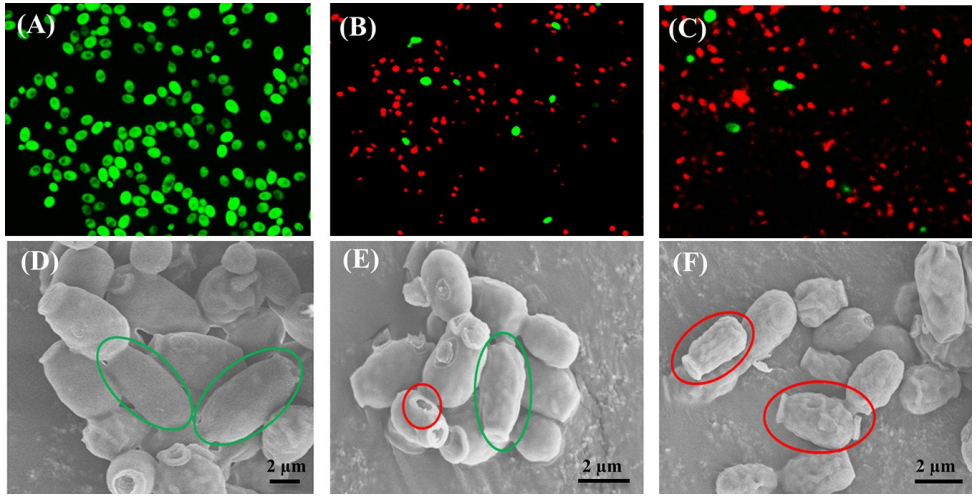
… to do what…

- ✓ Cell counting and viability of both planktonic and sessile cells
- ✓ Quality control on multi-strain formulation
- ✓ New protocol for strains isolation
- ✓ Cell-sensitivity to toxic compounds/antimicrobials
- ✓ Cell-to-cell metabolic interactions

FCM applications in Microbiology

… to do what…

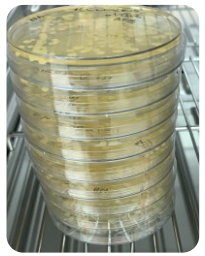
✓ Cell counting and viability of both planktonic and sessile cells



Viability is generally equated with the ability to reproduce and subsequently by cultivability. Not all bacterial cells obey this relationship and indeed, while they may lack the ability to reproduce and grow under certain conditions, they may possess many of the properties of fully functioning viable cells.

We arrive at another classification based on the term **"vitality"** or the degree to which a cell can perform various aspects of metabolic, physiological and genetic functionality and the extent of structural and morphological integrity

Detection methods of microorganisms



Sample

- **Biochemical-Microbiological Methods**
  - *Plate count*
  - *CLPP (Biolog)*
  - *FAME Analysis*
- **Molecular Methods**
  - *GC content*
  - *Microarray*
  - DGGE
  - TGGE
  - RFLP
  - T-RFLP
  - *DNA sequence analysis*
    - *Genomics*
    - *Metagenomics*

Low sensitivity and long response time:
Information on phenotype

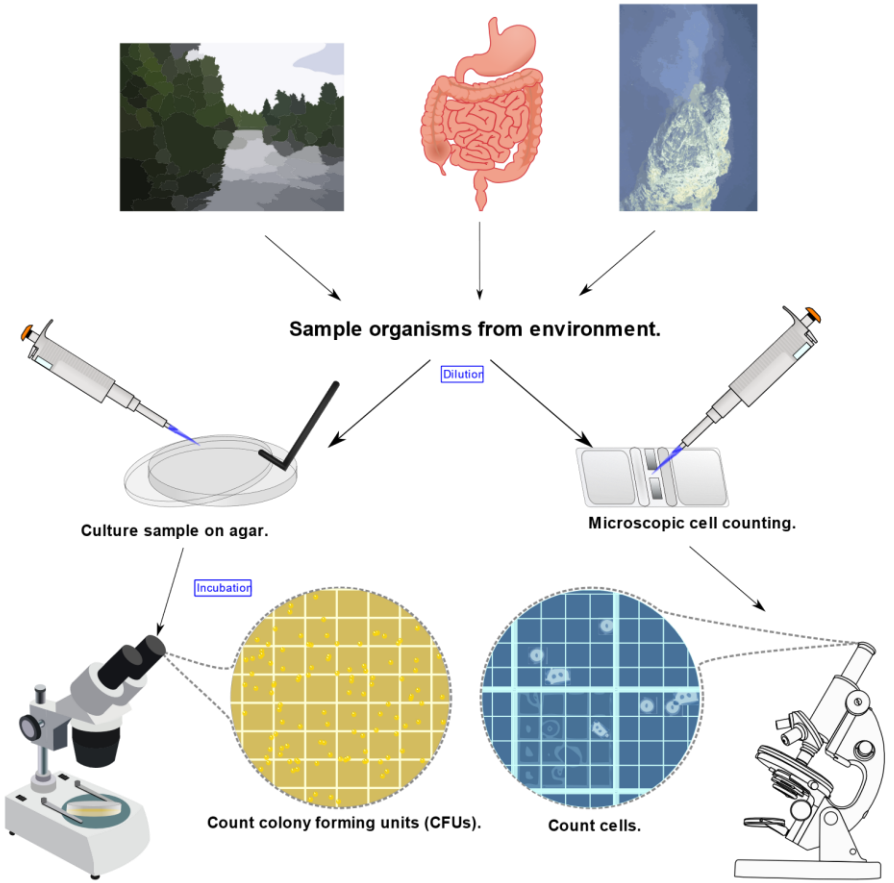
Definition of viability in terms of cultivability

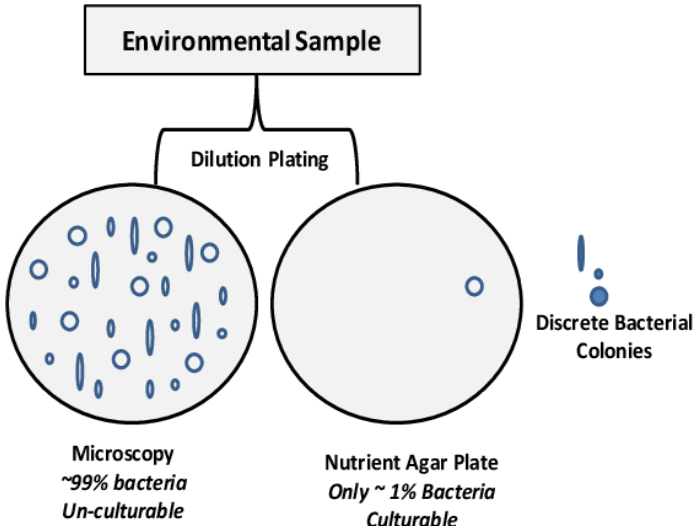
High sensitivity and rapidity:
Information on genotype

Information on the microbial genetic and functional diversity

Modify by Kamuran Ayhan, Serap Coşansu, Esin Orhan-Yanıkan, Gülcihan Gülseren. Advance methods for the qualitative and quantitative determination of microorganisms, Microchemical Journal, Volume 166,2021,106188.

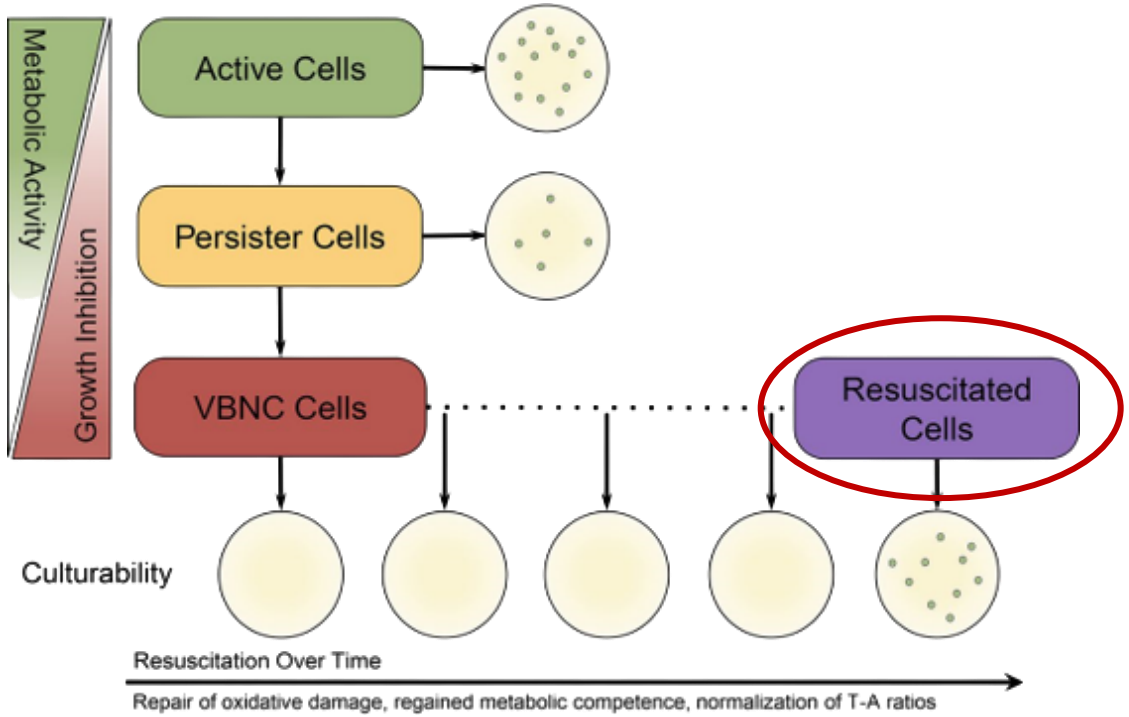
The great plate count anomaly

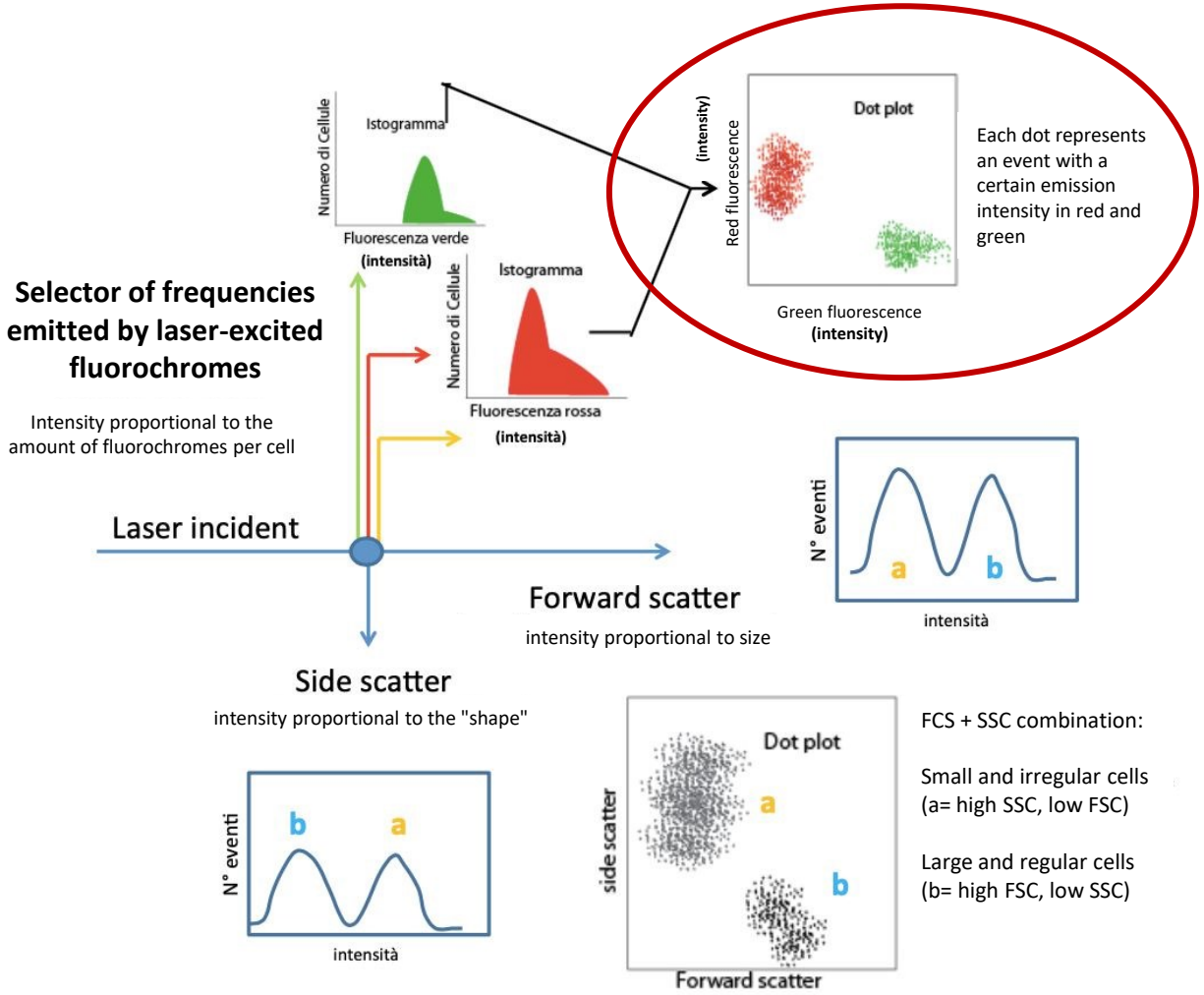




- species requiring special growth conditions or the presence of nutrients, oxygen concentrations, etc.
- species found at a low cell density
- viable but non-culturable species (VBNC)

Harwani, Dharmesh. "The Great Plate Count Anomaly and the Unculturable Bacteria." International journal of scientific research 2 (2012): 485-488



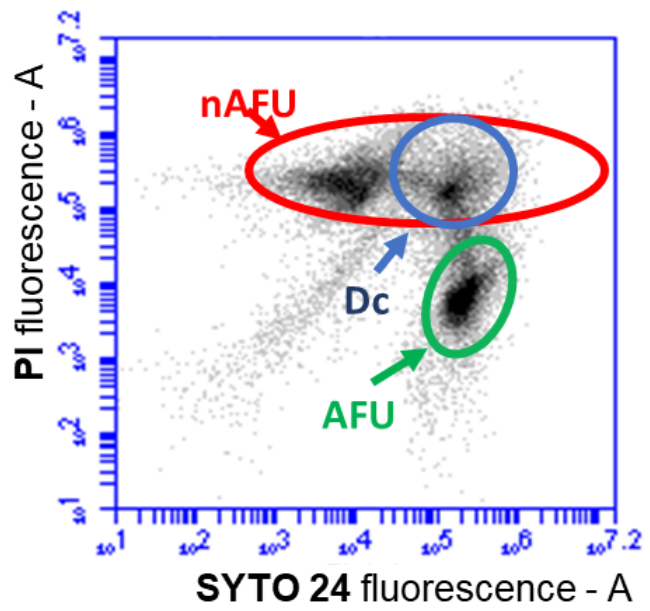


A. Adan, G. Alizada, Y. Kiraz, Y. Baran e A. Nalbant, «Flow cytometry: basic principles and applications,» Critical Reviews in Biotechnology, n. 37(2), pp. 163-176, 2017.

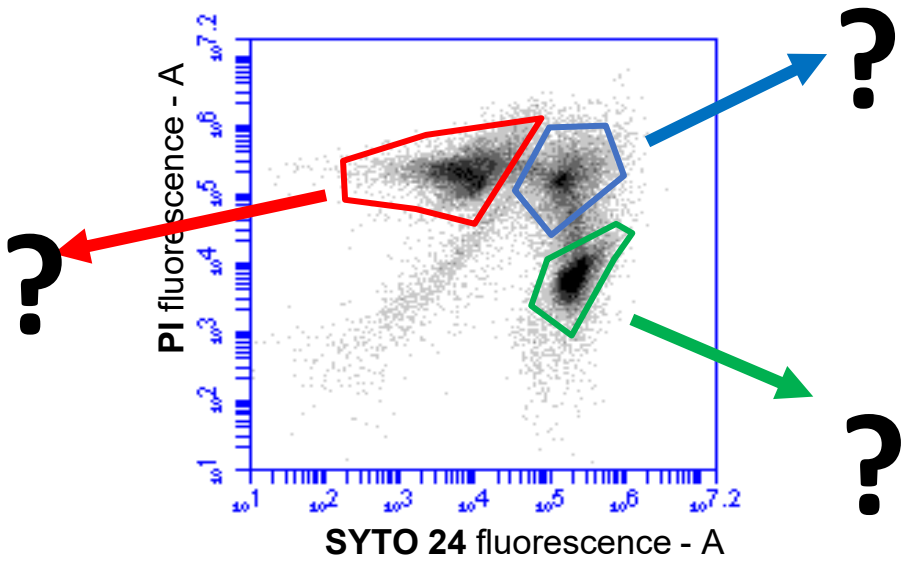
International Organization for Standardization (ISO) 19344:2015

Milk and milk products — Starter cultures, probiotics and fermented products — Quantification of lactic acid bacteria by flow cytometry

"The quantification and use of the fraction of active cells per total cells is a key feature and an important flow cytometry tool to evaluate the fitness of a given cell population. This is of special relevance for certain applications such as optimization of production process and stability assessment during shelf-life"

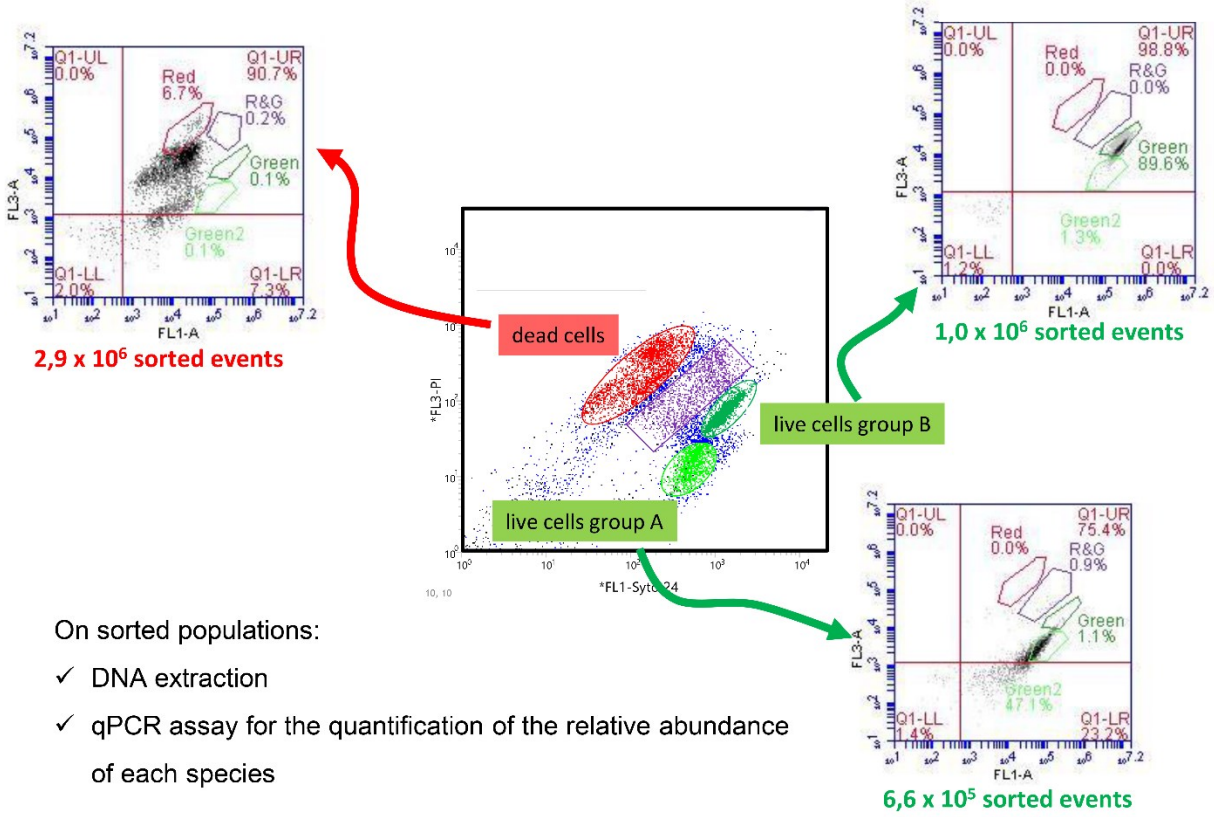


- **Active fluorescent units (AFU)**: events counted in a gate specific for scatter/fluorescence characteristics of presumed live cells, i.e. cells stained for the specific activity indicator used in the protocol
- **Non-active fluorescent units (n-AFU)**: events counted in a gate specific for scatter/fluorescence characteristics of presumed dead cells, i.e. cells damaged to an extend that they do not stain for the specific activity indicator
- **Damaged cells (Dc):** should be considered injured cells not dead and potentially able to growth
- **Total fluorescent units (TFU)**: total AFU and n-AFU



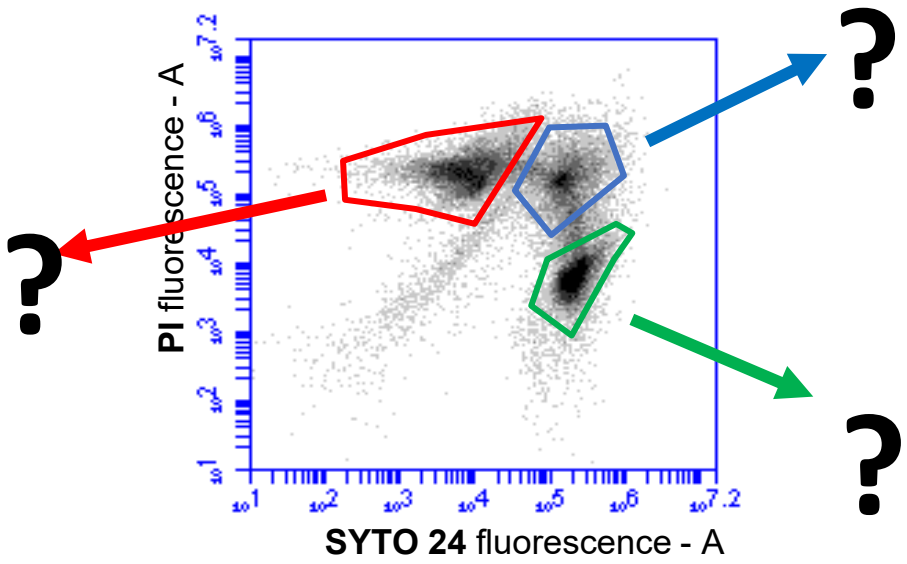
Flow cell sorting for the quantification of species relative abundance in each sorted population

- ✓ **Unknown** relative abundance of the species in each population
- ✓ **No taxonomic** information can be obtained directly by FCM



On sorted populations:

- ✓ DNA extraction
- ✓ qPCR assay for the quantification of the relative abundance of each species



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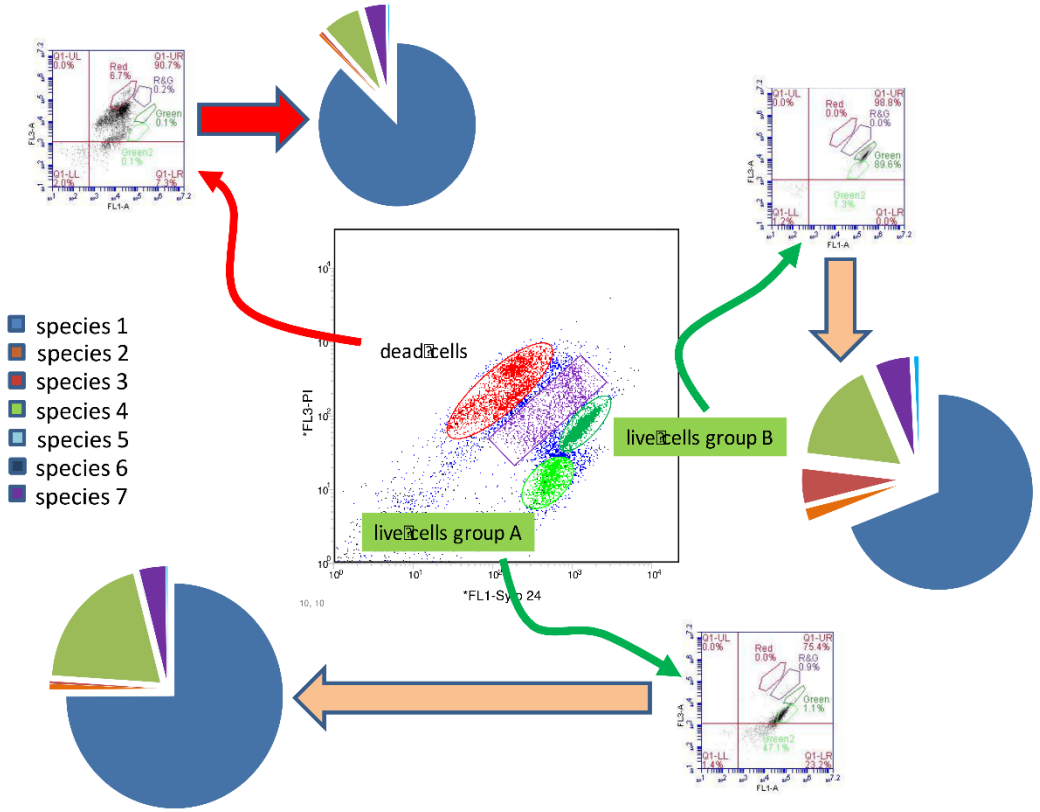


Flow cell sorting for the quantification of species relative abundance in each sorted population

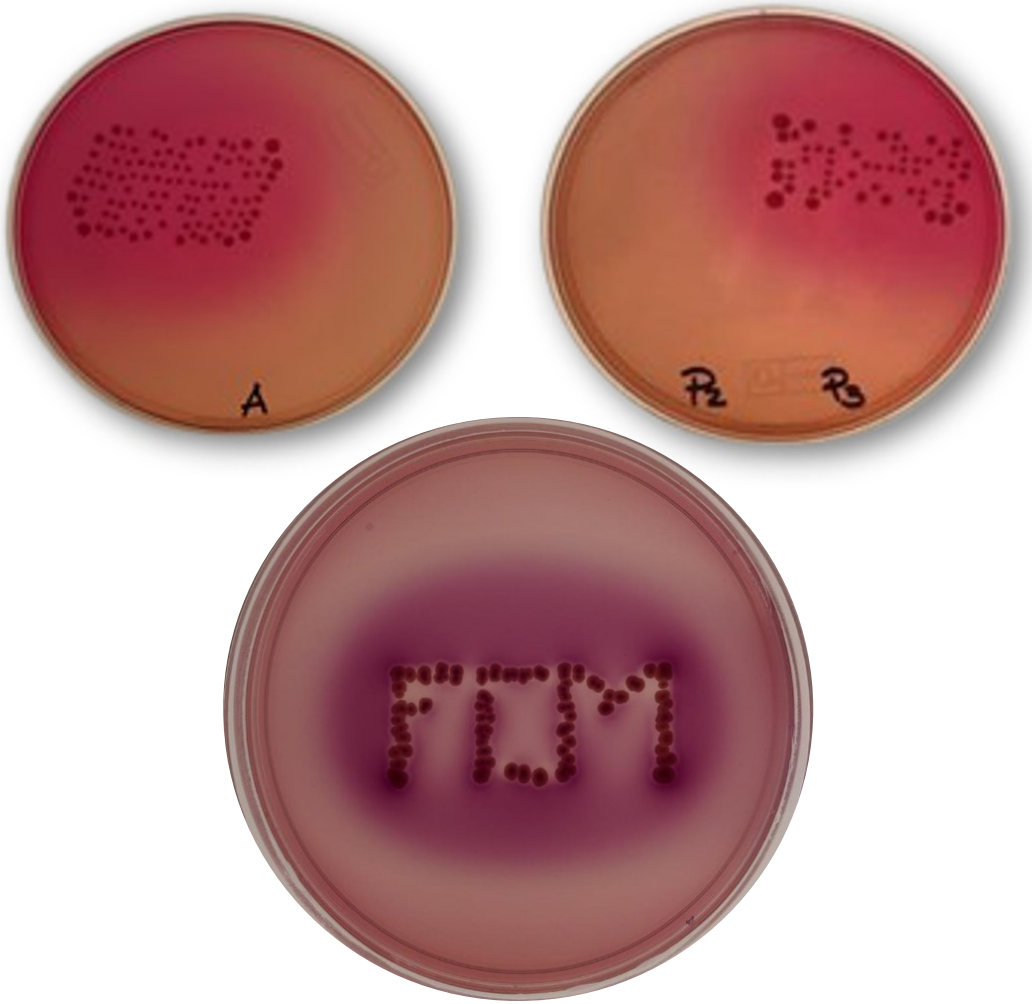
New protocols for strains isolation

Dilution and plating

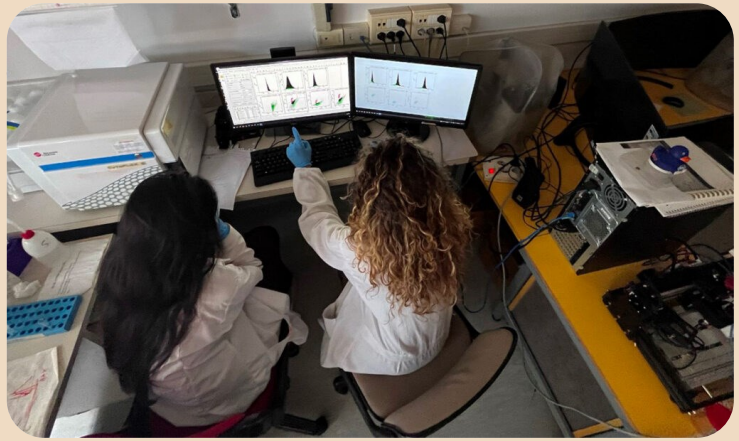
- is time consuming
- several dilutions must be plated to allow single strain isolation

FACS-based strain isolation

- rapid
- strain colony well separated
- strains could be easily screened by colony morphology



Thank you for your attention!



We look forward to seeing you at the practical lesson:
28/11/2023 – 14:00-17:00

valeria.poscente@enea.it