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# Aplications of flow cytometry in the study of microbial subpopulations
# Aplikace průtokové cytometrie při studiu mikrobiálních subpopulací.

Type of thesis

Bachelor´s thesis

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# Abbreviations

## Common abbreviations

| | |
|---|---|
| **DNA** | Deoxyribonucleic acid |
| **NA** | Nucleic acid |
| **NADH** | Nicotinamide adenine dinucleotide |
| **qPCR** | Quantitative polymerase chain reaction |
| **RNA** | Ribonucleic acid |
| **SPI-1** | Salmonella pathogenicity island 1 |
| **VBNC** | Viable but non-culturable cells |

## Technical terms and methods

| | |
|---|---|
| **AST** | Antimicrobial susceptibility testing |
| **CHIC** | Cytometric histogram image comparison |
| **CyBar** | Cytometric barcoding |
| **ELISA** | Enzyme-linked immunosorbent assays |
| **Em** | Emission |
| **Ex** | Excitation |
| **FISH** | Fluorescence in Situ Hybridization |
| **Flow-FISH** | Flow Cytometric Fluorescence in Situ Hybridization |
| **FSC** | Frontal [light] scatter |
| **SSC** | Side [light] scatter |

## Dyes, Fluorochromes, Reagents

| | |
|---|---|
| **BCECF** | 2‘,7‘-bis-(2-carboxyethyl)-5(6)-carboxyfluorescein |
| **BODIPY** | Boron-dipyrromethene |
| **CFDA** | 5(6)-Carboxyfluorescein diacetate |
| **CFDA-SE** | 5(6)-Carboxyfluorescein Diacetate Succinimide |
| **CTC** | 5-Cyano-2,3-Ditolyl-Tetrazolium Chloride |
| **DiBAC4(3)** | Bis-(1,3-Dibutylbarbituric Acid)Trimethine Oxonol |
| **DiOC2(3)** | 3,3'-Diethyloxacarbocyanine Iodide |
| **DiOC6(3)** | 3,3′-Dihexyloxacarbocyanine iodide |
| **DPH** | 1,6-diphenyl-1,3,5-hexatriene |
| **EDTA** | Ethylenediaminetetraacetic acid |
| **EGFP** | Enhanced green fluorescent protein |
| **FITC** | Fluorescein isothiocyanate |
| **GFP** | Green fluorescent protein |
| **HI** | Hexidium iodide |

| | |
|---|---|
| **PHA** | Polyhydroxyalkanoate |
| **PHB** | Polyhydroxybutyrate |
| **PI** | Propidium iodide |
| **TMA-DPH** | (1-[4-(trimthylamino) phenyl]-6-phenyl 1,3,5-hexatriene |
| **WGA** | Wheat germ aglutinin |
| **WGA-FITC** | Conjugate of wheat germ aglutinin and oregon green |
| **WGA-OG** | Conjugate of wheat germ aglutinin and fluorescein isothiocyanate |

## Abstract

This work reviews common flow cytometric methods and applications for the study of bacterial organisms. Flow cytometry is fluorescent method capable of both quantitative and qualitative analysis at the single cell level. It can offer insights about bacterial population dynamics, phenotypic heterogeneity and more. This work features a basic introduction to flow cytometry and presents some of the commonly measured variables, such as viability or membrane potential with an emphasis on the fluorescent probes used to visualize them. The difficulties of adapting flow cytometry to bacterial physiology are discussed, as well as the advantages and disadvantages of the particular probes and methods. Finally, this work seeks to demonstrate the flexibility as well as the shortcomings of flow cytometry using examples of practical applications in basic research, environmental microbiology, biotechnology, clinical practice.

Keywords: flow cytometry, microbial subpopulations, fluorescent labelling, bacterial physiology, bacterial viability

## Abstrakt

Tato práce je přehledem běžných metod a aplikací průtokové cytometrie pro studium bakteriálních organismů. Průtoková cytometrie je fluorescenční metoda poskytující jak kvantitativní tak kvalitativní data. Díky tomu je schopná informovat o dynamice bakteriálních populací, fenotypické heterogenitě v rámci těchto populací, i dalších vlastnostech. Tato práce obsahuje obecný úvod do průtokové cytometrie. Následuje seznámení s typicky měřenými vlastnostmi bakterií, například životaschopností či membránovým potenciálem. Důraz je kladen na fluorescenční sondy používané k výzkumu těchto vlastností a jejich přednosti či nedostatky. Diskutovány jsou také obtíže asociované s variabilitou bakteriální fyziologie. V závěrečné části je demonstrována flexibilita i nedostatky průtokové cytometrie pomocí příkladů ze základního výzkumu, environmentální mikrobiologie, biotechnologií i klinické praxe.

Klíčová slova: průtoková cytometrie, mikrobiální subpopulace, fluorescenční značení, bakteriální fyziologie, životaschopnost bakterií

# 1 Introduction

Flow cytometry is a well established method which has undergone much evolution since the discovery of the Coulter principle in the 1950s. While these origins appear subject to various myths, the fact remains that flow cytometry has since seen widespread use in both biology and medicine (Graham 2013). The method is capable of both quantitative and qualitative analysis of entire cellular populations based on chosen phenotypic variables. It has been particularly popular for the study of differentiated eukaryotic cells, such as immune cells, or red blood cell counting.

However, flow cytometry has also found applications in the study of bacterial cells, and in particular bacterial populations. This area of study in particular shows potential for growth. Phenotypic heterogeneity is not a phenomenon limited to fully differentiated eukaryotic cells. Even genetically identical populations of bacterial cells can show variability at the single cell level. The causes of population heterogeneity can vary from stochastic regulation to environmental effects (Davis and Isberg 2016). However, the important fact remains, that phenotypic heterogeneity plays a role in a range of phenomena from virulence in certain pathogens (Sánchez-Romero and Casadesús 2018) to product yields in commercial bioreactors (Lieder et al. 2014). The ability of flow cytometry to quantitatively observe bacterial phenotypes at the level of single cells and populations makes it uniquely suited for researching this phenomenon.

Even in genetically diverse populations, analysis of physiological variability at the population level can provide insights into the current state of a bacterial community or possible responses to stimuli—either through standard analysis or through alternative approaches such as fingerprinting (De Roy et al. 2012; Prest et al. 2013).

Finally, cytometry has been used to replace or enhance existing testing methods, namely cultivation, to determine viable cell counts. It can achieve faster analysis, while compensating for the inability of cultivation methods to detect viable cells, including pathogens, which are not dividing at the time (Moshaver et al. 2016). Flow cytometry does have shortcomings of its own, as it requires larger sample volumes for reliable detection of rare cell populations, such invading cells in bacteremia—a drawback which cultivation techniques do not have.

Flow cytometers have become increasingly affordable and flexible. Increased levels of automation have also made them more accessible to more users. Consequently, new applications for flow cytometry have been developing, and it is likely this growth will continue in the foreseeable future.

**Goals**

i. To review the methods and probes commonly used in flow cytometry to research bacterial organisms. Emphasis is placed on the merits of individual reagents over simply listing all possible options.

ii. To illustrate the possibilities of flow cytometry across different areas of microbiology via examples which may serve as basis for the design of future experiments.

## 2 An Introduction to Flow Cytometry

The primary strength of flow cytometry is its ability to rapidly evaluate thousands of cells per second and measure a number of variables for each cell. These variables depend on the fluorescent labels used, and may be different for each experiment. This chapter concerns itself with the internals of the flow cytometer itself, how it achieves its flexibility, as well as some of its limitations.

### 2.1 Basic Principles

At its most basic, the fluidic system of a flow cytometer directs the cell suspension through a laser at the interrogation point, one by one. When a cell passes through the laser, it scatters the light from the beam, and fluorescent molecules excited by the laser emit light. The optical system then measures the light scatter and fluorescence intensity of the cell, and the cytometric software records an event. In this way, every individual cell in the sample volume is measured. Flow cytometry is capable of rates of up to 40 000 cells per second, or approximately $10^6$ per minute. While practical rates may be lower, they still allow for rapid analysis of samples.

When a cell is measured, the system then records frontal scatter (FSC), side scatter (SSC) and fluorescence (FL). Fluorescence is further split up into individual light channels by bandpass filters, for example red (FL-R), blue (FL-B) and green (FL-G). The particular configuration of the fluorescence channels depends on the experiment and the fluorochromes used, however frontal and side scatter are a part of every analysis.

The system only records an event when the value of one of the above variables rises above a threshold. The goal is to measure one cell per event. Both the triggering variable and the threshold are decided as part of experiment design in order to best differentiate individual cells from the background. To achieve a single cell resolution, one must ensure a strong signal from each cell, and to ensure that only one cell is passing through the interrogation point at a given time. The size of the fluidic entry nozzle is typically configured to the size of the measured cells, allowing only one cell to pass through it at a time. Nevertheless, if two cells enter one after the other, they may be recorded as a single event—a *doublet* event. Such events need to be discarded to avoid errors. To limit the incidence of doublets, the single cell solution must have sufficient dilution that the average distance between cells in the solution (which is directly tied to the spacing between cells after they enter the fluidics system).

Particulate debris will not just cause higher backgrounds, but may also trigger events or even obstruct the entry nozzle. This places another practical limitation on flow cytometry—samples which inherently contain large quantities of debris, such as soil samples, require additional preparation, and debris may result in lowered accuracy (Bressan et al. 2015).

The scattering of the primary laser beam appears to correspond to basic cell properties such as size and density (Kerker 1983). In addition, through advanced software approaches, general cell shape may also be discerned, although with limited reliability (Jiménez-Guerra et al. 2017). Frontal scatter (FSC) and side scatter (SSC) are a basic cytometric variables gathered during every analysis, and do not require staining with fluorescent dyes as they rely on the intense light generated by the primary laser. As a result, analyses relying solely on FSC and SSC can offer relative simplicity and reductions in preparation time and cost. This makes them suitable for experiments relying on many rapid measurements.

However, data can be gathered more effectively through the use of various fluorescent dyes to track the structural or metabolic properties of a cell. Unlike fluorescent microscopy, flow cytometry does not record a detailed image of a cell and instead measures light intensity on a per-event (per-cell) basis. This means that flow cytometry can struggle to differentiate details which fluorescent microscopy may discern with ease, but excels at providing a general picture of the whole population.

The emission intensity of all fluorochromes is split into individual light channels via bandpass filters, with each filter typically configured to fit the emission maximum of one fluorochrome. This ensures that the intensity of each dye or fluorochrome can be tracked independently, facilitating the multivariate qualtiative aspect of flow cytometric analysis.

The intensities of all cells are then compared in the process of gating using specialized software, which allows the differentiation of distinct subpopulations from one another. At the most basic, simple separation of positive and negative populations for a given dye can be established. More accurate analysis may require specialized experiment design. While gating can be done by hand, modern software is capable of unsupervised gating which can significantly improve reproducibility while saving time spent on analysis.

## 2.2 General Limitations

While flow cytometry is a tool with a great deal of potential for understanding cellular populations, like all methods it is subject to limitations. Some of these have already been discussed in the previous section. Several others are listed here.

The requirement of a single cell suspension necessitates the homogenization of any analysed solid tissue, biofilms, plaques or similar. Thus, any positional information is permanently lost. Consequently, analysis of tissue-like samples such as biofilms needs to be combined with other methods to provide a complete picture. Flow cytometry still excels at quantitative analysis of populations, but in this case it only provides half the answer.

There is also a practical limit to the number of fluorochromes which can be used. Two fluorochromes cannot emit on the same frequency and be recorded independently, and compromises may need to be made. As the number of fluorochromes increases, the spectral space becomes increasingly cluttered and overlap becomes inevitable. Modern software is capable of automatically compensating for

some overlap, but avoiding overlap altogether is always a superior strategy.

Similarly, flow cytometry measures intensity on a per-cell basis. This means that localized fluorescence gets averaged over the entire cell and may in effect be too dim to register. However, combining flow cytometry with fluorescent microscopy is not uncommon, e.g.: (Nikel et al. 2015; McMullan et al. 2015; Vromman et al. 2014), as both methods can use the same fluorochromes and sample preparation. Fluorescent microscopy can observe the localization of the dye within a cell, and offers less ambiguity in interpretation. The two used in tandem can compensate for the drawbacks of either. More recently, image based flow cytometry promises the advantages of both methods combined into one. However, it requires a specialized device and as such is less readily accessible than the above (McFarlin and Gary 2017).

### 2.3 Sample Preparation

The purpose of a staining protocol is to prepare the sample for cytometric analysis before measurement. The first step is ensuring the basic requirements for a flow cytometric sample are fulfilled—a diluted suspension containing single cells that does not contain any large debris which may obstruct the fluidic system. Some samples may fulfil these requirements by default, such as drinking water, do not require preparation. To remove debris, filtration or centrifugation may be necessary. For soil or biofilm samples, detachment techniques are also required, such as scraping, vortexing or sonication.

Next is fixation, to ensure cellular structures remain together, but to prevent agglutination of cells in response to stress. Common fixatives can include formaldehyde, ethanol and others, however these are quite likely to influence final staining by modifying membrane permeability, and it is not uncommon to perform measuremens *in vivo*.

Given the diversity of bacterial species, morphologies, metabolisms and physiological states, there are some common differences in staining efficiency which must be noted. The most common example is the outer membrane of *gram* negative bacteria, which limits the entry of molecules ranging from antibiotics to dyes. This can negatively impact staining efficiency and create artefacts. In this case, Shapiro suggests employment of EDTA to chelate the outer membrane. This permeabilizes it, but can also impact the physiology of the bacterium which may be undesirable (Shapiro 2000). Different groups of bacteria, and even physiological states can similarly impact analysis, and care should be taken to ensure that a given staining protocol is applicable to an analysed species.

# 3 Fluorescent Visualization

The main strength of a flow cytometer comes from its ability to fluorescently visualize a large variety of cellular variables. Without fluorescent dyes, the repertoire of flow cytometry would be severely limited. Consequently, this chapter will introduce some of the more common variables measured and the dyes used for the purpose. This will include not just dyes specific to particular classes of molecules such as nucleic acids or lipids, but also methods of visualizing membrane integrity and membrane potential or fluorescent substrates capable of tracking enzymatic activity and more.

While the selection is by not exhaustive, it attempts to introduce characteristic examples. As fluorescent methods are often very versatile, many included items will not be conventional dyes – for example GFP expression systems. However they all serve the same ultimate purpose – to allow one to see the inner workings of the cell.

## 3.1 Cell Viability

First, a brief introduction to a very common concept which is surprisingly difficult to define at the cellular level – death. While the particulars of the various dyes used to tell apart living (viable) and dead (unviable) cells will be discussed throughout this chapter, the basic framework needs to be explained first.

Dyes used to stain unviable (or viable) cells are as diverse as the definitions of cellular death. In theory, the topic can be discussed at great length. In practice, a cell is presumed to be dead when it can no longer form colonies, when its metabolism is rendered inactive, or when its cytoplasmic membrane can no longer separate the cytoplasm from the outside medium. These criteria are tested for by observing the cells divide, detecting metabolism-dependent catalytic activity such as the generation of a membrane potential, or using dyes incapable of passing through the cellular plasma membrane if the cell is intact (membrane-impermeant dyes).

The above are shorthands, as exceptions to them exist. Certain viable but non-culturable cells (VBNC) can be fully functional, yet simply not divide. Cells can cease all metabolic activity and (spores, non-dividing cells) and are overall not quite as straightforward as distinctions between life and death in larger organisms. For a full review, see (Emerson et al. 2017). The choice of the best indicator in a given situation depends on the researcher. In practice, membrane integrity is the most commonly used proxy in cytometric analysis.

As the dyes used for the determination of viability are varied in their principles and mode of operation, this subchapter serves only to provide a basic introduction. Individual examples are introduced in their respective chapters, namely – nucleic acid dyes, membrane potential dyes and indicators of enzymatic activity.

## 3.2 Nucleic Acid Dyes

Nucleic acid (NA) dyes are among the most commonly used dyes in flow cytometry. As nucleic

acids are present in virtually all cells, DNA-based universal staining makes it very easy to differentiate cells from other particulate debris or instrument noise. While useful in eukaryotic cells, a universal stain can be key when working with bacteria whose small size makes them difficult to differentiate by other means.

Besides universal staining, nucleic acid dyes are commonly used for viability staining. Membrane impermeant nucleic acid dyes have virtually no staining targets on the outside of the cell, and as a result will only stain cells with compromised membranes. Due to the simplicity, accessibility and relative reliability of the method, nucleic acid dyes are very popular in this role.

Naturally, nucleic acid dyes have other uses as well, such as tracking relative nucleic acid content, monitoring cell cycling or growth rate (Lieder et al. 2014). However, these applications appear to be uncommon and will not be further discussed.

Similarly, while many DNA dyes are currently available, only a few of them see common usage in the available literature. This appears to be both a consequence of "natural selection", but also of standardization thanks to the proliferation of commercial kits such as the LIVE/DEAD BacLight kit discussed below.

One important consideration with most of the dyes presented is the fact that they are nucleic acid dyes, not DNA dyes. This can be desirable in a membrane permeability stain, as more staining targets are presented (Zotta et al. 2012). However, when attempting to quantify DNA (or RNA) content, a dye with high specificity may be required.

### 3.2.1 Propidium iodide and SYTO-9

The most commonly used dye combination for staining cells is propidium iodide (PI) and SYTO-9. These two nucleic acid dyes form the commercial LIVE/DEAD BacLight kit. Despite both being nucleic acid dyes, each serves a different purpose; SYTO-9 is a membrane-permeant nucleic acid dye, and serves as a universal stain. On the other hand, propidium iodide is a positively charged membrane-impermeant dye, and as a result only stains cells with damaged membranes—a viability dye. This combination can readily serve as the foundation upon which subsequent analysis is based, hence its popularity. However, it is not without flaws.

Berney et al. 2007 describe the staining pattern as clear and distinctive, however they note difficult to interpret intermediate states which appear to be neither alive, nor fully dead. Bereney et al. suggest that these states relate to progressive levels of membrane damage. They also report that the SYTO-9 has some difficulties staining *gram* negative cells (*E. coli, Salmonella, S. flexneri*) despite being classified as membrane-permeant. This is likely a consequence of the protection afforded by the outer membrane of *gram* negative bacteria. It can be alleviated by application of EDTA, however this is likely to severely alter other properties of the cells as well and may not always be an option. The authors note that another DNA dye, SYBR Green, provided more homogeneous and reproducible staining patterns than SYTO-9 in their freshwater bacteria samples (Berney et al. 2007). This is corroborated by Stiefel et al., who note differences

in the staining of live and dead *gram* negative cells (*Pseudomonas aeruginosa*) by SYTO-9, and the rapid photobleaching of SYTO-9 (Stiefel et al. 2015). Given the spectral proximity of SYTO-9 and SYBR Green, only a few alterations to a staining protocol may be needed to accommodate exchanging one for the other.

Of note is that propidium iodide has an affinity towards RNA comparable to affinity to DNA, while most nucleic acid dyes described here tend to prefer DNA. As described prior, this does not limit the usefulness of propidium iodide due to its use as a membrane probe (Suzuki et al. 1997).

Perhaps more concerning are the findings of Shi et al. that PI was capable of staining both live and dead *Sphingomonas sp.* and *Mycobacterium frederiksbergense* in the early stationary phase. The authors concluded that this was tied to fluctuations in membrane potential, suggesting that at sufficient membrane potential, even the positively charged propidium iodide becomes capable of entering the cell (Shi et al. 2007). This is corroborated by Kirchhoff and Cypionka (Kirchhoff and Cypionka 2017). This phenomenon may extend to other ionic dyes, and may need to be kept in mind when artefacts are encountered.

Documented is the tendency of PI to reduce SYTO-9 fluorescence. This seems to be a consequence of both dyes sharing the same target molecules. The mechanism is described as a combination of quenching, fluorescence transfer as well as propidium iodide simply replacing already attached SYTO-9 (Stocks 2004). This phenomenon may account for some of the difficult to explain test results observed by Berney et al.

### 3.2.2 Other Nucleic Acid Dyes

A variety of other SYTO dyes exist, such as SYTO-13, SYTO-62 (red). These dyes appear to share common properties such as being membrane permeant, while differing in their fluorescent properties. As such, it is quite possible to use them as universal stains much like SYTO-9.

They SYTO dyes are not to be confused with SYTOX Green. Despite the similarity in name, it is a membrane impermeant DNA dye which can be used as an alternative to PI. In fact, SYTOX Green has been tested on *Escherichia coli* and evaluated as superior to PI in terms of fluorescence intensity and separation (Roth et al. 1997). It has been used with a number of other species including *Pseudomonas aeruginosa*, *Streptococcus agalactiae* and *Staphylococcus aureus* and others (Defraine et al. 2018; McKenzie et al. 2016; Gerits et al. 2016).

Another group of relatively common NA dyes is the TO-PRO family. For the purposes of viability staining, TO-PRO 3 is of particular interest due to its membrane impermeant nature. In a comparative study performed by Kerstens et al., TO-PRO 3 was found to be a reliable indicator of viable cell count, correlating well with viable cell counts and offering adequate separation of live and dead subpopulations. The results were compared to viable plate counting as reference. The dye was successful in staining *Bacillus subtilis, Staphylococcus aureus* and *Escherichia coli* but not *Pseudomonas aeruginosa*. This was attributed to intrinsically different outer membrane composition of that species (Kerstens et al. 2014).

In the same vein, a comparison between propidium iodide and TO-PRO 3 was performed by D.J. Novo et al., who found the two to be functionally identical in their study of *Staphylococcus aureus* and

*Micrococcus luteus* (D. J. Novo et al. 2000). It can be concluded that, an alternative viability stain using TO-PRO 3 is possible. Notably, TO-PRO 3 requires a different excitation laser (yellow-red instead of UV) than PI, which may complicate panel design, or be of benefit in multi-laser panels.

As described in the introduction, many dyes have affinity towards both RNA and DNA due to the significant structural similarities between the two. The fluorescence intensity of a dye is likely to differ depending on whether it is bound to a DNA molecule, an RNA molecule or is free in a solution. As described earlier, propidium iodide has a relatively high affinity to RNA, making it unsuitable for quantitative or even comparative analysis of DNA content without RNAse treatment. Conversely, DAPI has relatively high specificity for DNA, making them better suited for such analyses. The comparison between the dyes was performed on eukaryotes. It is listed here under the assumption that general staining trends will not be impacted by physiological differences (Suzuki et al. 1997). The DNA affinity of DAPI was exploited by Lieder et al., who used DNA content measured by DAPI as a proxy for growth rate, as DNA content above the 2N equivalent reflected the nesting of replication in cells with faster growth speeds (Lieder et al. 2014b).

While DAPI does stain DNA reliably, it also appears to have a stronger impact on target metabolism. Toepfer et al. found DAPI can lower the motility of *Pseudomonas*, reducing the mean swimming speed by more than 50% (Toepfer et al. 2012).

Overall, it cannot be said there is one nucleic acid dye to suit all purposes, whether in its binding properties or its fluorescence profile. Therefore, the individual properties of dyes play an important role in experiment design. Naturally, many more potential dyes exist. However, the strengths and weaknesses of the dyes described above may serve as a rough guide for the evaluation of these other dyes as well.

### 3.3 Membrane Potential

Membrane potential dyes, as the name implies, stain cells to different degrees based on their membrane potential. As membrane potential is predicated upon both membrane integrity and cellular metabolic activity, these dyes can also be used as an indicator of viability. This can be particularly useful when cells are being exposed to a type of stress which does not inherently result in membrane damage, for example starvation (López-Amorós, Comas, and Vives-Rego 1995).

A number of dyes exist for measuring membrane potential. Membrane potential indicators used in flow cytometry mainly include so called slow response dyes, the response time of which is measured in minutes rather than fractions of a second. Any membrane potential oscillations taking place over the minimum measurement period are averaged together (Yamada et al. 2001). Several general categories of slow response dyes exist. According to the Thermofisher molecular probes handbook , these dyes can be grouped into three categories: carbocyanine dyes, rhodamine derivatives and oxonol derivatives. The most common representatives from each category are, in order: 3,3′-dihexyloxacarbocyanine iodide (DiOC6 (3)), Rhodamine 123 and Bis-(1,3-Dibutylbarbituric Acid)Trimethine Oxonol (DiBAC4 (3) or Oxonol).

Carbocyanine and rhodamine dyes are cationic and lipophilic in nature. As such, they are attracted to

the negatively charged interior of cells with a polarized membrane, where they accumulate in lipids and proteins. They have been linked to uncoupling or inhibition of respiration in mitochondria (Howard and Wilson 1979; Schneider 1980). Other ultimately cytotoxic effects may be observed as a consequence of dye accumulation inside cells.

On the other hand, oxonol dyes are anionic in nature and have a reverse staining pattern - they only accumulate inside depolarized cells, as they are repulsed by the negatively charged interior. As such, oxonols have less of an opportunity for direct cytotoxic action. However, when oxonol enters depolarized cells that are not dead, the same complications may arise.

A dual staining procedure utilising rhodamine and „bis-oxonol“ (presumably DiBAC4 (3)) has been utilised by Comas and Vives-Rego, who note the speed and reliability of oxonol staining compared to Rhodamine. They also describe a combination of DNA dyes, namely PI/SYTO-13 (a likely analogue to the BacLight Kit) as superior to a combination Rhodamine and DiBAC4 (3) membrane potential staining in its ability to discriminate cells from micelles and other non-cellular debris (Comas and Vives-Rego 1997). There do not appear to be other more recent studies that would further corroborate the above combination of membrane potential dyes.

Rhodamine 123 has been found usable for environmental samples by Diaper and Edwards in their study of *Staphylococcus aureus* survival in lake water and correlated with viable cell plate counting (Diaper and Edwards 1994).

The dye DiOC2 (3) has been used by D. Novo et al. for ratiometric measurement of membrane potential. This ratiometric method offers greater accuracy than regular membrane potential dyes, which the other authors describe as having poor separation between positive and negative populations.

The basic principle requires DiOC2 (3) concentration to reach very high levels (the authors applied the dye at 1000 μM instead of 10 μM), at which standard green fluorescence (530 nm) becomes independent of membrane potential, remaining dependent only on particle size. Simultaneously, in areas of high polarization, aggregates form which exhibit red fluorescence (600 nm). The fluorescence of these aggregates is dependent on membrane potential and size. A ratio between these two colours compensates for the effect of size, and allows for accurate measurements of membrane potential. The authors report an accurate range between -50 mV -120 mV, (D. Novo et al. 1999).

According to Shapiro, among non-ratiometric approaches, oxonols provide the best degree of separation. However, this is still evaluated as inferior to ratiometric techniques, which can distinguish differences of as little as 10 mV. One possible reason to use oxonols over the above method is that they can enter *gram* negative bacteria without prior EDTA treatment (Shapiro 2000).

### 3.4 Indicators of Enzymatic Activity

This category encompasses two commonly used substrate dyes; 5-Cyano-2,3-Ditolyl-Tetrazolium Chloride (CTC) and 5(6)-Carboxyfluorescein diacetate (CFDA). Both are not fluorescent themselves in their

native form, but are converted into a fluorescent product in a reaction catalysed by an enzyme. This allows the visualization of enzymatic activity.

### 3.4.1 Respiratory Activity

The fluorogenic substrate 5-Cyano-2,3-Ditolyl-Tetrazolium Chloride (CTC) can be used as an indicator of respiration. In its default state, it is colourless, but when reduced either via environmental factors or by cellular processes, it transitions to a red-fluorescent formazan which can be measured and readily observed. According to Smith and McFeters, CTC appears to be primarily reduced by elements of the electron transport chain, namely succinate and NADH dehydrogenase complexes (Smith and McFeters 1997). CTC can thus be used as a viability indicator for cells, although it appears that it is rarely used for this purpose.

Use of 5-Cyano-2,3-Ditolyl-Tetrazolium Chloride (CTC) for visualization of respiring bacteria has first been described by Rodriguez et al. 1992. They describe the use of CTC in combination with a DAPI based DNA stain with oil immersion fluorescence microscopy. They point out that spontaneous reduction of CTC can occur in low-redox environments, which would limit its applications to aerobic environments and non-reductive medium compositions. An additional concern was raised as at high concentrations, a quenching effect was observed which suggested possible cytotoxicity (Rodriguez, Phipps, and Ishiguro 1992).

Simultaneously, Kaprelyants and Kell successfully used CTC on *Micrococcus luteus* using flow cytometry. From their use of respiratory chain inhibitors, they conclude a close interaction of CTC and dehydrogenases similarly to Smith and McFeters 1997. They also determine the optimal incubation time is between 40-45 min, after which intracellular CTC concentration peaks and begins to decrease. The authors attribute this to an inhibition effect of CTC on respiration (Kaprelyants and Kell 1993).

Fuller et al. found that CTC completely failed to stain *Comamonas sp.* cells in their environmental study (Fuller et al. 2000). Similarly, Yamaguchi et al. describe CTC having difficulties staining *Pseudomonas putida, Staphylococcus epidermidis* and two unidentified strains (Yamaguchi and Nasu 1997). Overall, this limits the use of CTC to species known to work well with CTC, or else it should be expected to underestimate the respiratory activity of some cells.

Overall, while CTC may serve as reliable indicator of respiratory activity, its tendency to be reduced in low redox environments places restrictions on sample medium. Worse yet, its cytotoxicity means use of CTC is difficult to combine with the continued survival of the tested cells, ruling out cell sorting and post-analysis cultivation.

BacLight RedoxSensor Green (RSG) is a more recent alternative to CTC. It appears to function on similar principles as CTC, albeit with green fluorescence instead of red.

It has been used by Nikel et al. as an indicator of overall metabolic activation in *Pseudomonas putida*. The authors observed differences in metabolic activity in response to different substrates,

namelysuccinate, glycerol and glucose (Nikel et al. 2015).

When evaluating RSG as part of their study, Kalyuzhnaya et al. did not observe any effect on cellular growth as a consequence of RSG staining after one hour of incubation, suggesting that RSG has a far lesser impact on cellular metabolism than CTC (Kalyuzhnaya, Lidstrom, and Chistoserdova 2008).

### 3.4.2 Esterase Activity

Similarly to CTC, 5(6)-Carboxyfluorescein Diacetate (CFDA) relies on cellular enzymatic action to produce fluorescent product within the cell, which can then be measured. Carboxyfluorescein diacetate relies on nonspecific esterase activity to cleave the acetate groups, releasing the fluorescent 5(6)-carboxyfluorescein in the process.

Yamaguchi et al. have used a CFDA and CTC stain to discriminate metabolically active bacteria in river water based on esterase and reductase activity. In addition, PI was used for viability staining on the basis of membrane integrity. Comparisons were made between polluted and non-polluted river water. When tested, the combination yielded high PI signals and low carboxyfluorescein signals in active cells, and vice versa for fixed cells. The authors noted difficulties with higher backgrounds, which were attributed to nonspecific cleavage of CFDA. Overall, CFDA was found to stain cells more reliably than CTC, perhaps making it more suitable for environmental studies (Yamaguchi and Nasu 1997).

A different carboxyfluorescein derivative, 5(6)-Carboxyfluorescein Diacetate Succinimide (CFDA-SE) is also commonly used. While the esterase mechanism of CFDA-SE is likely similar to CFDA, the succinimidyl group also facilitates covalent binding to intracellular protein, significantly increasing retention of the molecule. In a comparative study between CFDA and CFDA-SE, Hoefel et al. claim that this retention mechanism results in the fluorescent labelling of all cells regardless of enzymatic levels, which would make CFDA-SE unsuitable for measuring of cellular activity (Hoefel et al. 2003).

## 3.5 pH Indicators

Dyes measuring pH alter their fluorescence intensity depending on their protonation. As such, they can only be expected to measure pH in a limited range around their pKa. This means that multiple probes are necessary if one wishes to cover the full range pH. Measurements of pH via cytometry appear to be far more common among eukaryotic studies than prokaryotic ones. Nevertheless, the commonly used pH probes serve as an example of indicators whose function relies on them altering their fluorescence in response the properties of the surrounding environment.

A commonly used fluorescein derivative, 2‘,7‘-bis-(2-carboxyethyl)-5(6)-carboxyfluorescein (BCECF), allows pH measurements between 6.5 and 8.5 by altering its intensity. However, the protocol requires an acidic environment to transport the probe inside of cells, and the probe is subsequently subject to active efflux which may impact measurements (Molenaar, Abee, and Konings 1991).

Breeuwer et al. have tested a ratiometric method for measuring pH within bacterial cells (*Lactobacillus lactis, Bacillus subtilis*) via CFDA-SE. This method is proposed as a more robust alternative to BCECF for bacterial pH measurements. The intensity of light emitted at 490 nm is dependent on environmental pH, while the intensity of light emitted at 440 nm is not. The ratiometric method compares the two wavelengths. This eliminates the effect of cell size and/or dye concentration on the measurement, and can help compensate for cellular efflux of the dye. (Breeuwer et al. 1996). In the available literature, this method has not been extensively used with cytometry.

Ultimately, while they do not appear commonly used in the available literature, they are an example of a class of dyes which rely on the modulation of their own fluorescence to yield results, rather than altering intracellular concentrations.

### 3.6 *Gram* Staining, Cell Wall Staining

*Gram* staining is a technique used to differentiate between *gram* positive and *gram* negative cells in conventional microscopy. While this technique cannot be used in flow cytometry, equivalent fluorescent methods can be used to achieve the same effect.

There are two types of staining discussed in this section. Both rely on the impermeability of the outer membrane of *gram* negative bacteria. One is hexidium iodide (HI) to stain the nucleic acids of cells, while the other uses wheat germ aglutinin (WGA) to actually target the peptidoglycan in the cell wall of these cells.

As stated, hexidium iodide is a fluorescent DNA dye which stains only *gram* positive bacteria. It has been used by Yan et al. as a single stain to identify *gram* positive *Staphylococcus aureus* in a macrophage phagocytosis assay (Yan, Ahn, and Fowler 2015). A similar technique combining HI and SYTO-13 was also used by Mason et al. on a range of bacterial strains including *Staphylococcus aureus, Bacillus spp*., *Staphylococcus saprophyticus, Enterococcus faecalis, Actinobacter spp.* and more. Clear separation between *gram* positive and negative cells was achieved, but the authors note that treatment by EDTA or alcohol fixation make HI staining of *gram* negative cells possible, effectively making HI a universal stain (Mason et al. 1998).

By contrast, staining by wheat germ aglutinin (WGA) relies on WGA agglutinating N-acetyl-D-glucosamine residues commonly contained in the cell wall. It will label the cell walls of *gram*-positive bacteria in this way, while *gram* negative bacteria with intact outer membranes will be unaffected (Sizemore, Caldwell, and Kendrick, n.d.). Mutant strains such as *S. aureus* 52A2 may not be bound by WGA (Lotan, Sharon, and Mirelman 1975). Wheat germ aglutinin is not fluorescent, and cannot be used for cytometric analysis by itself. Instead, conjugates with conventional dyes must be used. For example, a wheat germ aglutinin-fluorescein isothiocyanate conjugate (WGA-FITC), where FITC is a fluorescein derivative capable of covalently binding amine or sulfhydryl groups such as the ones found in proteins (Biancalana et al. 2017). WGA-Oregon Green (WGA-OG), a WGA conjugate with another covalently binding protein dye, and HI were used by Holm and Jespersen for cytometric *gram* determination. *Gram* positive (*Staphylococcus aureus*) but not *gram* negative (*Escherichia coli*) were stained by WGA-OG. By contrast, it appears HI was

used as a general stain, as EDTA pre-treatment rendered all cells permeable to it. The authors describe how of WGA-OGs ability to correctly identify cells degrades as a consequence of sample age and cell degradation. (Holm and Jespersen 2003).

Overall, it would appear HI is more sensitive to outer membrane damage than WGA. This makes sense, as hexidium iodide is effectively a membrane permeability dye and likely inherits many shortcomings from propidium iodide. On the other hand, WGA has a more specific mode of action. However, it is unclear to what degree the amount of peptidoglycan matters compared to the integrity of the outer membrane preventing its binding in *gram* negative bacteria.

### 3.7 Lipid Dyes

Lipid dyes have two primary targets in cells; cell membranes, and intracellular granules. Dyes intended for membranes tend to be amphiphilic and include labelled membrane lipids which carry a common fluorophore. These dyes can situate themselves at the interface between hydrophobic and hydrophilic environments, mimicking the behaviour of membrane lipids. By contrast, nonpolar dyes can situate themselves deeper inside a hydrophobic environment, potentially making them more suitable for staining hydrophobic targets with a lower surface to volume ratio.

Lipid stains which target membranes do not appear to be commonly used in cytometric analysis, as membrane fluidity assays are typically performed using spectrofluorimetry. Instead, the more common use for lipid dyes in cytometry appears to be the staining of nonpolar granules within cells, including commercially used bacterial products such as polyhydroxyalkanoate (PHA).

Quite popular for this purpose is Nile red. Nile red is a lipophilic dye with poor water solubility with very low fluorescence in water, but high fluorescence in nonpolar environments such as including lipids (Jose and Burgess 2006).

It has been used for tasks such as the assessment of polyhydroxyalkanoate (PHA) content within cells and selection of optimally-producing bacteria (Vidal-Mas et al. 2001),

Ostle and Holt have used Nile blue, the more water-tolerant precursor to Nile red, as a PHA stain in *Azotobacter chroococcum* and *Bacillus megaterium*. They point out that staining of PHA is likely achieved by Nile blue spontaneously oxidizing into Nile red (a.k.a. Nile blue oxazone, Nile pink) in aqueous solution (Ostle and Holt 1982). Nile blue has since been used more recently by Song et al to characterize PHA accumulation in *Pseudomonas putida* (Song et al. 2008).

Pinzon et al. have tested a method for rapidly screening bacterial strains for production of hydrocarbons and ketones and found Nile red to be suitable for this purpose. The testing was performed by comparing differences in fluorescence on a fluorescence spectrophotometer, not a cytometer. However, given the use of flow cytometry to detect PHA accumulation in bacteria using the same dye, it is likely cytometry can be used for this purpose as well (Pinzon et al. 2011).

An alternative to Nile red is the boron-dipyrromethene (BODIPY) family of lipid dyes. The

commonly used variants function as nonpolar lipophilic dyes, similarly to Nile Red. According to Gocze and Freeman, they may stain larger granules more than membranes than Nile red (Gocze and Freeman 1994).

While, as stated, measurements of membrane fluidity are typically not performed via flow cytometry, it appears a method capable of this has in fact been recently tested by Bouix and Ghorbal. A modified flow cytometer was necessary for the experiment. The measurement of fluidity was combined with a viability stain to allow the comparison of membrane fluidity between live and dead cells. The study utilized the nonpolar 1,6-diphenyl-1,3,5-hexatriene (DPH) and its positively charged aliphatic derivative (1-[4-(trimthylamino) phenyl]-6-phenyl 1,3,5-hexatriene (TMA-DPH) (Bouix and Ghorbal 2017). The primary difference between the two dyes is the aliphatic nature of TMA-DPH whose positively charged anchor (TMA) can limit the ability of the molecule to pass through membranes or in between membrane domains (Le Grimellec, Friedlander, and Giocondi 1988).

### 3.8 Autofluorescence

All cells show some levels of background autofluorescence caused by molecules innate to the cells themselves, as opposed to molecules introduced through a staining protocol or other experiment-related manipulation.

A variety of common molecules can contribute to autofluorescence. For example, nicotinamide adenine dinucleotide (NADH) and its phosphorylated derivative, NADPH gain additional fluorescent properties in their reduced forms, reaching spectral maxima at 340 nm excitation (Ex) / 450 nm emission (Em). Other sources of autofluorescence include flavins (360-450 nm Ex / ~520 nm Em), aromatic amino acids (260-280 nm Ex / 280-350 nm Em) and porphyrins (cca 400 nm Ex / 625 nm Em). Of particular note here are electron transporters such as NADH and NADPH, as well as light gathering pigments such as the various chlorophylls. See Monici 2005 for a review (Monici 2005; Wagnieres, Star, and Wilson 1998).

Often, autofluorescence can be a complication for analysis of fluorescence, as certain wavelengths often have high backgrounds, which can limit their usefulness for fluorescent analysis unless compensated for. However, understanding autofluorescence and using it to track cellular properties can also be advantageous, as all measured cells will effectively come pre-stained with no cytotoxic or altering effects.

The following studies appear to either measure the autofluorescence of a particular fluorochrome with an easily discernible or strong signal, or rely on unspecific autofluorescence to perform their measurements.

Unspecific green autofluorescence and red chlorophyll autofluorescence have been used as a viability indicator by Schulze et al. in cyanobacteria (*Synechocystis*) in combination with an automated fluorescence microscope. This approach can likely be used with other fluorescent techniques, including flow cytometry, and it provides a possible alternative to the more common viability indicators such as membrane integrity (Schulze et al. 2011).

Li and Dickie have utilised autofluorescence of various light capture pigments to assign cells to

broad groups while assessing plankton composition. Separation was performed on the basis of chlorophyll and phycoerythrin autofluorescence (W. K. W. Li and Dickie 2001). Similarly, photosynthetic organisms were differentiated on the basis of on red chlorophyll and chlorophyll-like fluorescence by Danza et al. (Danza et al. 2017).

Lambrecht et al. report distinguishing methanogenic bacteria on the basis of cofactor F420. Previously unknown subpopulations were found by the technique. The authors elaborate on the dependence of F420 autofluorescence intensity on the metabolic and oxidative state. In addition, the authors describe partial quenching caused by SYBR Green I staining of DNA. It is quite possible this is not the only instance of a quenching interaction which may affect measurements (Lambrecht et al. 2017).

Renggli et al. describe changes in nonspecific autofluorescence of *E. coli* in response to bactericidal antibiotic treatment. They observed both increases in autofluorescence, attributed to cell elongation as a consequence of antibiotic action, and decreases in overall autofluorescence as a consequence of cell lysis (Renggli et al. 2013).

### 3.9 Fluorescent Proteins

The introduction of reporter genes into the genome is a common technique, which can be combined with flow cytometry through the use of fluorescent proteins. In particular, the green fluorescent protein (GFP) and its derivatives are commonly used for this purpose. They can also be used to track the expression of a gene.

Much like autofluorescent molecules, these fluorescent reporters are produced within the cell itself and are not inherently cytotoxic, unlike many dyes discussed above. However they can still impact host metabolism if expressed in great quantity. They may also potentially interfere with any cellular proteins they are fused to.

The labour required to modify a strain to correctly express a fluorescent protein is considerable in comparison with most staining protocols. This limits its uses to working in a controlled environment on a limited number of bacterial strains.

A study tracking gene expression by Nuss et al. when studying *Yersinia* virulence. An enhanced green fluorescent protein (EGFP) reporter was put under the promoter of a virulence controlling molecule, RovA. This variant of GFP exhibits higher (enhanced) fluorescence intensities. The authors were subsequently able to track changes in subpopulations of RovA positive and RovA negative cells in response to a temperature increase (Nuss et al. 2016).

A similar fluorescent protein is mCherry. It reaches an emission maximum in the red spectrum circa 600 nm, rather than green, allowing for parallel use of both GFP and mCherry as was done in a study of *Salmonella enterica* virulence, which was similar in principle to the study by Nuss et al. Gene fusions of three key genes from the *Salmonella* pathogenicity island 1 (SPI-1) were created as reporters on the degree of SP-1 activation. Used reporters included both GFP, and mCherry. This allowed the authors to track the

number of SP-1 positive and negative cells relative to to the ability of the overall bacterial population to infect eukaryotic HeLa cells (Sánchez-Romero and Casadesús 2018).

While the use as an expression reporter is the most beneficial application for fluorescent proteins, it is not the only one. The low impact of fluorescent proteins makes them of interest to stain cells constitutively in studies concerned with host-pathogen adhesion.

For example, conditional GFP expression in a modified *Chlamydia trachomatis* was used by Vromman et al. to quantitatively observe its life cycle. Two fluorescent strains were created, each using a different expression vector. Both exhibited simple constitutive expression of GFP. This served to facilitate the detection of very small cells (chlamidial elementary bodies) infecting a larger host cell. While this strategy was successful, it only enabled completely distinguishing the populations of infected host cells from uninfected cells 18 hours after initial infection. By contrast, automated fluorescence microscopy could detect individual bodies within host cells much sooner and observe infection kinetics (Vromman et al. 2014). This illustrates that even GFP-based approaches cannot entirely escape the difficulties of high backgrounds. While GFP is ordinarily able to overcome cellular autofluorescence, the very small size of the GFP-carrying chlamydial elementary bodies (circa 0.3 μm) meant that a larger number were required. To facilitate earlier detection, a fluorescent protein with a higher quantum yield may have been used.

A similar approach for visualizing intracellular parasite infection was used by Drennan et al. with *Legionella pneumophila* and its host *Acanthamoeba castellanii* (Drennan et al. 2013). Similarly to above study, the incubation period required for distinction of infected and uninfected cells was *circa* 18 hours.

Finally, GFP was also used as a model protein for the testing of a heavily modified *Pseudomonas putida* strain intended for heterologous protein production in a bioreactor. The protein was expressed from an IPTG-inducible promoter, allowing comparisons between the productivity each strain (Lieder et al. 2015).

### 3.10 Protein Dyes

Some fluorescent dyes covalently bind epitopes commonly found on proteins. The groups or epitopes targeted by these dyes vary with the dye used, but are all commonly found in the side chains of proteins. When applied directly to cells, such dyes can provide stable, long-term staining of cells due to their tendency to bind covalently.

The previously mentioned carboxyfluorescein derivate, 5(6)-Carboxyfluorescein Diacetate Succinimidyl Ester (CFDA-SE) covalently binds NH2 groups. It has been used for long term tracking of nondividing cells for up to 28 days, without any observed impact on the cellular metabolism (Fuller et al. 2000).

In a different study, CFDA-SE labelling of dividing *Lactobacillus plantarum* allowed the tracking of cellular proliferation for up to eight generations through gradual dilutions of the dye (Ueckert et al. 1997).

Another fluorescein derivative, fluorescein isothiocyanate (FITC), has been used to track the antimicrobial capabilities of various antibiotics. The preference of FITC towards binding nucleophilic groups

was not directly utilized in the study (Durodie et al. 1995).

A different use for these covalently binding dyes can be found in Alexa Fluor hydrazide. Hydrazide dyes can covalently bind aldehyde and ketone groups, which are often a consequence of cellular stress (Dukan and Nyström 1998). In addition Alexa Fluor hydrazide is membrane impermeant, which may lead to its inability to stain healthy cells. Alexa Fluor hydrazide has been tested by Saint-Ruf et al. as a viability stain for both G- (*E. coli*), G+ (*Bacillus subtilis, Deinococcus radiodurans*) bacteria and yeast (*Saccharomyces cerevisiae*). It was used in combination with SYTOX Green as a membrane permeability indicator (C. Saint-Ruf et al. 2010) . This staining method had been further used in related studies (Surre et al. 2018; Claude Saint-Ruf et al. 2016).

Another way of using the above dyes is to conjugate them with other nonfluorescent molecules, such as wheat germ aglutinin, immunoglobulins (antibodies) or otherwise. This opens up a large number of possibilities, much like genetic fusion. Due to the large variety of roles these probes fill, protein-dye conjugates and similar reactants are not described as a group. Instead, their uses are shown in the examples when relevant to a particular application.

### 3.11 Flow Cytometric Fluorescence in Situ Hybridization

While sequence-specific study of DNA does not appear common, flow cytometric fluorescence in situ hybridization (Flow-FISH) has been successfully applied to bacteria.

Much like conventional fluorescence in situ hybridization (FISH), Flow-FISH uses the hybridization of fluorescently-labelled probes to detect the presence of a target sequence. Only abundant sequences, such as those of 16S rRNA, can be targeted, otherwise too few fluorochromes be present in a cell to allow for their detection. This is particularly important in bacteria, due to their smaller size.

This method, despite apparent obscurity in microbiology, is of particular interest, as it holds the potential of quantitatively genotyping bacterial populations using flow cytometry. This approach potentially offers greater flexibility than approaches relying on phenotypic signs of taxonomic allegiance, such as the presence of particular molecules.

Friedrich and Lenke have used 16S rRNA Flow-FISH and quantitative polymerase chain reaction (qPCR) to quantify bacteria in dairy starter cultures. The probes were fused to either Oregon Green protein dye or the orange Cy3 DNA dye which is typically not used in bacteria. The authors report successful detection, albeit not without difficulties such as lower-than-expected fluorescence of the hybridized probes (Friedrich and Lenke 2006).

The feasibility of the method is corroborated by Nettman et al. who describe their development of a 16S rRNA Flow-FISH protocol. In the study, Flow-FISH is used for the enumeration of anaerobic microbial communities in a bioreactor. The hybridization procedure was combined with CTC staining, allowing the tracking of metabolic state on a per-taxa basis. All Flow-FISH probes used were labelled with the Alexa 488 fluorescent dye (Nettmann et al. 2013).

# 4 Practical Applications

Flow cytometry has found a variety of interesting applications in microbiology, ranging from basic research to applications in rapid clinical testing. In some cases, specialized cytometers have been built with specific applications in mind, such as the Sysmex UF-1000i cytometer for automated urine diagnostics, or the SeaFlow cytometer capable of rapid continuous sampling for weeks at a time – both discussed below. Other techniques rely on a novel approach rather than specialized hardware, and may be more readily transferable to other areas of study This chapter will therefore discuss the uses of flow cytometry across different areas of microbiology, both in laboratory and industrial applications.

Overall, flow cytometry is capable of generating significantly larger datasets for analysis, potentially gaining information – in this sense, quantity has a quality all its own. However, as analyses become more complex and the fields more applied, cost becomes a consideration and will remain a common theme across the following chapter. Especially for long term monitoring applications, a minimalistic approach to staining is often beneficial. For example, the SeaFlow with its very high sampling rate uses no staining protocol at all (Swalwell, Ribalet, and Armbrust 2011).

While this text is about the uses of flow cytometry, it cannot be ignored that other methods compare favourably in some aspects. Fluorescent microscopy has higher resolution than flow cytometry and may discern intracellular structures, however it also has lowered ability to evaluate whole populations. Solid phase cytometry, while outmatched in terms of overall throughput, has far lower detection limits for rare populations (Lemarchand et al. 2001). Even classical plate counting methods have better detection thresholds than flow cytometry, as even a single cell can produce a readily detectable signal, where flow cytometry may require a cluster of ten or a hundred events to be certain. Lastly, qPCR has been found to be more suited for the enumeration of species in a sample than Flow-FISH, albeit unable to combine this enumeration with other analysis like flow cytometry (Friedrich and Lenke 2006).

## 4.1 Environmental Cytometry

Flow cytometry has been extensively utilized for the study of natural microbial communities, such as ones found in natural habitats. In the analysis of these communities, the main interest appears to lie in describing and monitoring actual microbial composition. While measurement of soil samples is possible, many of the following examples come from studies of aquatic systems. This is because cytometry itself analyses samples in the liquid phase, making it especially well suited for such tasks. Included are also examples of applications in water quality control, as the presence of viable but non-culturable bacteria in drinking water is a health concern (Byrd, Xu, and Colwell 1991).

One of the major benefits of flow cytometry in this regard is its ability to detect viable cells, regardless of whether they are dividing or not (Allegra et al. 2008).

The examples in this section will be focused on introducing the general concept of automated (online) cytometry as well as its applications for environmental studies. However, even without automation,

flow cytometry can prove to be a powerful tool in the study of freely living bacteria.

For instance, in a decade spanning study performed by Li and Dickie, the authors describe measuring the weekly changes in plankton composition of a single coastal inlet (Bedford Basin) in a time period between 1991 and 2000. The final dataset combines population data with other environmental readings such as salinity or temperature. Subpopulations were distinguished on the basis of size as measured by FSC, nucleic acid content (see below) and autofluorescence of pigments found in photosynthesising plankton (phytoplankton). Only a single DNA dye was used in sample preparation, yet even this basic setup allowed the authors to identify distinct subpopulations such as viruses, bacteria and phytoplankton.

It was further possible to identify the cyanobacterium *Synechococcus* and Cryptophyte algae on the basis of both sharing orange phycoerythrin autofluorescence but differing in average size. The authors further noted that it would have been possible to identify phycocyanin-rich cells as well, however their experimental setup relied on a 488 nm cyan laser while phycocyanin is excited by red light near 600 nm. Overall, the fluorescent properties of photosynthetic pigments make the organisms carrying these pigments uniquely suited for dye-less cytometric analysis.

The nucleic acid dyes used for universal staining were changed over time, starting with TOTO-1 following with SYTO-13 and finally ending with SYBR Green I. The authors claimed these changes led to improvements in signal sensitivity and ease of preparation. While no data is presented, this appears to be in accordance with other reports mentioned in Chapter 2.

Overall, this neatly demonstrates the potential of minimalistic flow cytometry for water monitoring applications. The study combined a basic universal stain with innate autofluorescence and scattering properties, and was able to identify a considerable variety of subpopulations. It was also able to and track the changes in total and relative counts over time, both on week to week, and on an annual scale (W. K. W. Li and Dickie 2001).

While is not a medium as easily sampled as water, analysis of soil samples is nevertheless possible. Bressan et al. describe a method for measuring bacterial counts in soil samples using SYBR Green DNA staining in comparison to heterotrophic plate counts. Partial cell loss due to some cells remaining attached to soil particles was described. Weak staining of soil particles with SYBR Green was noted (Bressan et al. 2015).

Another test of applying flow cytometry in high-sediment environments was performed by Frossard et al. on a variety of samples of soils, sediments and sludge. The authors compared the results with epifluorescence microscopy and ATP estimation. In almost all samples, flow cytometry was found to underestimate the overall cell counts in comparison to epifluorescence microscopy, and this difference varied with the type of soil measured. This was likely a consequence of partial cell loss as before. Ultimately, flow cytometry still correlated reasonably well with other methods (Frossard, Hammes, and Gessner 2016).

It can be concluded that particulate debris, such as the kind found in some environmental samples,

can complicate cytometric protocols. In addition, adherent bacteria must be separated from larger particles to produce a single cell suspension. This leads to a reduction in the reliability of quantitative measurements due to cell loss. In addition, detachment steps can walk a fine line between transferring cells to solution and cell damage. Overall, while applicable, flow cytometry appears to encounter some complications when analysing soil samples.

## 4.2 Online Flow Cytometry

While weekly measurements have been proven feasible, in order to track population changes happening on shorter time scales such as days, or even hours, more regular measurements are required. These in turn rapidly increase the overall amount of labour required to perform measurements. One possible solution is automation, which speeds up the overall process. Modern flow cytometers can often automate some aspects of analysis or measurement. Some are even capable of automated staining, for example the Sysmex UF-1000i, a purpose-built cytometer for rapid evaluation of urine samples (Broeren et al. 2011). At the pinnacle of automation is online flow cytometry, which is capable of retrieving a sample, staining it and then measuring it – unsupervised. While the term may imply a specialized box, for the purposes of this text, any cytometric setup capable of functioning without direct human input will be referred to as an online flow cytometer.

One of the earlier online-cytometric setups is a proof of concept described by Hammes et al. The authors describe testing an online flow cytometry setup for aquatic applications. In the experiment, both forward and side scatter were used. An automated staining chamber permitted single- or double- staining. The study used a simple viability stain combining SYBR Green I and/or Propidium Iodide (both single and double staining were tested).

While the authors conclude the overall system functions reliably, they describe several difficulties; staining efficiency was shown to depend on temperature. When the cytometer was drawing samples from a very cold source, staining efficiency decreased, introducing error into the measurements. This required adjustments of the automated temperature control. A reported 10% of all measurements were found to be outliers, reporting far higher cell concentrations than possible. The cause of this was uncertain, however it necessitated a data filtering step. The detection range of the setup was also tested, as it was unable to automatically increase sample dilution when cell concentrations approached the detection limit, although such functionality could be added (Hammes et al. 2012).

Overall, while the final product was somewhat flawed, the study demonstrated the feasibility of building an online flow cytometer. A number of more advanced studies utilizing the concept have been published since, particularly concerning the related topic of drinking water quality.

Maintaining the quality of drinking water, namely its microbial content, is a serious public health concern, as well as a factor in water taste and odour (Zhou et al. 2017). As in environmental applications, flow cytometry remains of particular interest thanks to its ability to detect viable but non-culturable organisms which may be found in water distribution networks (Byrd, Xu, and Colwell 1991).

Besmer et al. describe a setup capable of automatically adjusting its sampling rate based on precipitation. This is an answer to the sampling frequency dilemma – while weekly, or even lower frequency sampling is sufficient for dry seasons, precipitation events have been linked influxes of bacteria into the water supply on a much shorter timescale (see e.g. (Auld, MacIver, and Klaassen 2004; Howe et al. 2002)). Therefore, increased sampling rate is necessary to detect these incursions. However, increasing the sampling rate universally would significantly increase the cost of operating an online cytometer. Hence, the ingenious solution – a system which automatically adjusts the sampling rate (Besmer et al. 2017). The potential of a variable-rate self-adjusting online flow cytometer is self-evident. Such a setup, if properly configured, can be cost effective, while increasing its ability to measure desired phenomena. This is in part a consequence of the reduction in work hours required, as technology is often less expensive than human labour.

While automation and variable rate measurements are one approach to sampling frequency problems, another alternative has been developed – the SeaFlow cytometer. The cytometer is largely autonomous and capable of performing measurements at very short 3 minute intervals for weeks at a time. Long operating periods with no maintenance required are in part a consequence of its sheathless design, which prevents fouling. This design has been put into practice by the authors in to observe plankton distributions across the sea surface. Measurements are performed by simply sailing the cytometer across the sea, letting it sample the water surface as it travels. This creates massive bacterial density datasets which can complement data gathered from coastal monitoring stations (Swalwell, Ribalet, and Armbrust 2011).

Due to the sheer size of the datasets being produced, ordinary analysis software has been found inadequate for subsequent analysis. The authors have instead developed their own software capable of processing large-volume high-frequency data. Of note is its ability to detect patterns across multiple samples. This is relevant for the SeaFlow, as its very high frequency of measurements borders on continuous, and may become relevant for other cytometers in the future (Hyrkas et al. 2016).

### 4.2.1 Online Cytometry Data Analysis

While current flow cytometric software can handle typical cytometric samples, for the analysis of repetitive routine cytometric data still leaves some room for improvement. Namely, cytometric fingerprinting. Cytometric fingerprinting refers to a loose collection of holistic approaches which allow for comparisons between individual snapshots of a bacterial community in a relatively fast and reproducible way.

The principle of fingerprinting is to take in the positioning and size of measured clusters (subpopulations) as a whole (a fingerprint) and then calculate the differences between individual fingerprints across several measurements. This pattern recognition approach allows quickly discovering shifts from the normal composition of a bacterial community. These changes may be associated with outside effectors such as contamination, changes in the environment or similar. It could be said, that the approach is more focused on correlation than causation (Prest et al. 2013).

Koch et al. (Koch et al. 2014) perform a comparison of four different fingerprinting approaches—

Dalmatian plots (Bombach et al. 2010), Cytometric Histogram Image Comparison (CHIC) (Koch, Fetzer, Harms, et al. 2013), Cytometric Barcoding (CyBar) (Koch, Fetzer, Schmidt, et al. 2013), and the Flow FP software (Rogers and Holyst 2009). In summary, one general approach is a graphical one, utilizing ImageJ or similar software to calculate overlap/differences between the actual graphs (Dalmatian plots, CHIC) and then quantifying the differences via the R Software Environment. The seemingly main disadvantage of such an approach is the loss of information identifying which cell clusters are primary contributors to changes detected in the final statistic. The other approach is to group events (cells) together either via manual gating or via automated grid which are then applied to all datasets to compare event distribution across all bins/gates (CyBar, FlowFP).

Important differences also arise depending on whether clusters are defined by the user (Dalmatian plot, CyBar) or by software (CHIC, FlowFP). The latter methods likely feature a far higher level of reproducibility, and are more readily usable with automated flow cytometers, although some degree of sensitivity may be lost as a result.

All four tools were found to yield comparable results of acceptable quality. However, Dalmatian plots showed higher variability likely due to the aforementioned human element, CHIC and Flow FP were fastest with highest reproducibility, making them optimal for online flow cytometry, and CyBar was found suitable for more detailed analysis of causality with fewer individual measurements, such as comparing different kinds of environment (Koch et al. 2014).

It appears that aquatic microbiology trends towards shorter, simpler staining protocols which both reduce costs and simplify their automation. Perhaps it is the understanding of regular oscillations in bacterial populations that drives this trend. Cyclical variations in population composition can occur both in natural and man-made aquatic environments, and rapid sampling cytometry is well suited for understanding them. However, fluctuations in bacterial populations are not unique to just environmental populations, and it is possible that as online cytometers become more standardized and affordable, new unexpected applications may appear in other fields.

## 4.3 Industrial applications

Industrial applications have some aspects in common with environmental applications. They often work with only loosely defined cellular cultures. This chapter will mainly focus on the running of bioreactors. However, included are also food industry applications as an example of a less bio-technologically oriented industry.

### 4.3.1 Food Industry

In the case of food quality monitoring, the priority is simply detection of bacterial contamination, much like in water quality control. A number of food-borne pathogens exist that need to be tested for. Unlike the testing of water, however, testing for food is preferably done on a per-batch basis. Perhaps it is because of this that advances in online flow cytometry do not appear to be common for this purpose in the literature.

While the prevention of any bacterial presence in food is the ideal scenario, ensuring that bacterial cells do not multiply before the time of consumption can often suffice. For this purpose, cell proliferation studies are performed. For example Ueckert et al. studied the effects of heat shock treatment on *Lactobacillus planarum* cultures. A covalently-binding CFDA-SE was used to tag cells and measure cell proliferation, while propidium iodide was applied as a viability stain. Through regular measurements, the authors were able to observe the timescale of a gradual transition of bacterial cells from heat shock or lag phase into proliferation (Ueckert et al. 1997).

Detection limits of flow cytometry strongly depend on background noise and triggering strategy, especially when analysing samples containing large amounts of particulate debris of sizes comparable to bacteria. This can be a complication given the low infectious dose of certain pathogens (Wilkes et al. 2012).

This was corroborated by Subires et al., who used anti-*E.coli* goat antibodies conjugated with fluorescent molecules of R-phycoerythrin to target *Escherichia coli* in ready to eat salad. However, even with this approach, they encountered detection limits between $10^5$ and $10^7$ events. The authors themselves admitted that these limits are quite high, and the method would require an enrichment step in order to be applicable for food quality control (Subires, Yuste, and Capellas 2014).

Flow cytometry method is used in some countries to detect bacterial contamination in raw milk. Namely, the BactoScan FC by Foss Analytics (https://www.fossanalytics.com/en/products/bactoscan). According to the Dairy Microbiology Handbook (Robinson 2002), the BactoScan FC utilizes ethidium bromide to stain the DNA of all cells. As ethidium bromide, much like PI, is membrane-impermeant, it can be presumed that sample preparation involves a permeabilization step, despite this does not appear explicitly mentioned in the available sources. The method has been found superior or comparable to common plating techniques. Its main limitation stems from a stated detection limit between $10^4$ and $10^5$ events (Ramsahoi et al. 2011).

### 4.3.2 Bioreactors

The main purpose of cytometry in bioreactors is again monitoring, but also optimization of cultures to maximize bioproduct yields. Products can include lipids, biodegradable bacterial polymers (polyhydroxyalkanoates) as well as heterologous proteins expressed in genetically modified cells. As a result, dyes specific to all of the above are commonly utilized to determine product yields. Optimization of individual strains generally relies on one-time studies. On the other hand, monitoring of bioprocesses, while possible by hand, can greatly benefit from some form of automation, including online cytometry.

Bioreactor yields are often dependent on phenotypic heterogeneity within the bioreactor population. Different fractions of cells vary by overall productivity, even in genetically homogeneous population. Phenotypic heterogeneity can be caused both by differences in genotype or environment (as seen in larger bioreactors), but can also exist in clonal populations under very similar conditions (such as in smaller size laboratory experiment). Flow cytometry is one of the methods which can help understand the benefits, disadvantages and overall dynamics of population heterogeneity (Delvigne et al. 2014).

An example of an offline flow cytometry assay would be a study performed by Lieder et al. to investigate the relationship between cell cycling and polyhydroxyalkanonate (PHA) production in a chemostat-grown culture of *Pseudomonas putida*. Cell cycling rate in individual samples was determined by staining of DNA with DAPI and fluorescence quantification and compared with per-sample poly hydroxyalkanoate measurements obtained separately (Lieder et al. 2014b).

In a separate study, an investigation of heterologous protein expression between *Pseudomonas putida* strains was performed. A comparison was made between modified but wild type-sized genomes and artificially reduced-size genomes. Heterologous GFP expression was used to simulate the expression of a theoretical industrially produced protein, allowing a comparison of overall growth rates as well as PHA yields per-cell (Lieder et al. 2015).

Analysis of genetic expression systems *Escherichia coli* has also been investigated by Patkar et al. as via heterologous GFP expressions. This allowed observing GFP expression in a range of bacterial phenotypes found in a particular culture, rather than averaging the results. The authors were able to estimate the leakiness of a given system, as well as plasmid stability. This was done by monitoring GFP expression of cells in the absence, and in the presence of an inductor (Patkar et al. 2002). Naturally, the investigation of genetic expression systems on a population could be used to investigate overall cellular regulation.

Unlike previous examples, biogas reactors tend to rely on a poorly defined combination of species responsible for biogas (methane) production. In order to increase efficiency, first it is necessary to understand which bacterial species actually contribute to the production process. Such a study was conducted by Nettman et al. In the study, fluorescently labelled DNA oligomers targeting 16S rRNA were used to discriminate between different bacterial taxa, while CTC was used to track general metabolic activity. This made it possible to determine, which bacterial taxa are metabolically active during methane generation, and as such the most likely to be contributors to the overall process (Nettmann et al. 2013). This study represents an ingenious step towards understanding the inner workings of complex natural bioprocesses.

In a standard expression system for a heterologous protein, protein expression is triggered by the addition of an inducer. However, introduction of excessive amounts of inducer can place excessive metabolic stress upon the producer cells, and ultimately lower product yields (Lin 2000).

Brognaux et al. tested an approach utilizing online cytometry to track cellular stress via a GFP reporter and limit over-induction. The reporter was fused to a promoter believed to be sensitive to substrate limitations (*fis*). An online cytometer then performed measurements in 15 minute intervals, staining the samples with PI to rule out unviable cells. The authors were able to establish an experimental setup, as well as an evaluation approach which may allow for the creation of an automated (unsupervised) feedback control system in bioreactors (Brognaux et al. 2013).

For hydrophobic products such as PHA, the dual-staining method by Karmann et al. could form a basis. As described above, the method allowed for quantifying PHA inside cells via the BODIPY 493/503 lipid dye in combination with a SYTO 62 universal stain. This would make a combination with online flow

cytometry feasible in a system similar to the one proposed by Brognaux et al. 2013. In this capacity, the staining protocol may for instance help determine when to utilize carbon source injections or the optimal time for PHA harvesting (Karmann et al. 2016).

## 4.4 – Clinical applications

Just as flow cytometry finds many uses across others fields of clinical practice, such testing of as organ transplants (Maguire et al. 2014) to leukemia treatment (Matarraz et al. 2017) it has also found applications in clinical microbiology. Among the included examples, two general groups can be observed. Simple screening protocols, which are focused on the detection of pathogenic organisms in samples, and testing of the pathogens‘ properties, such as antibiotic resistance.

### 4.4.1 – Screening

It is not surprising that the benefits of flow cytometry over classical cultivation techniques remain the same as before. Speed of evaluation becomes even more relevant as there is potential to improve patient outcomes, and even with longer staining times, flow cytometric analysis can be accomplished on a shorter time scale than cultivation methods.

A review by Li et al. describes a large number of human pathogens capable of entering the viable but non-culturable (VBNC) state (L. Li et al. 2014). These pathogens are alive and capable of infection, but require specific conditions to begin multiplying – conditions typically not present in conventional cultivation media. However, these cells remain detectable by flow cytometry as flow cytometry does not require culturability to detect cells – a clear advantage (Allegra et al. 2008).

As flow cytometry has a natural advantage in the analysis of fluids, so do many clinical cytometry protocols rely on analysis of body fluids and look for signs of infection in them.

While the detection of bacteria in blood may seem like an ideal role for flow cytometry, the opposite may the case. During bacteremia, only low numbers of bacteria per millilitre can be found in blood – as low as 1 CFU per millilitre (Wain et al. 1998). As has been pointed out several times already, flow cytometry suffers from relatively high detection thresholds, and studies seem to report thresholds of between $10^4$ to $10^5$ per millilitre or higher (Schmidt et al. 2006; Subires, Yuste, and Capellas 2014; Ramsahoi et al. 2011).

A study performed by Mansour et al. reported surprisingly low detection thresholds of as little as 1 event per millilitre, however even if this very low detection threshold could be reliably achieved, it would still be two whole orders of magnitude away from the goal. This once again shows, that without enrichment procedures, flow cytometry is poorly suited for the task – and enrichment procedures which may be difficult to perform given the access to blood is typically limited (Mansour et al. 1985).

Cytometry has been suggested for screening the derivatives of transfusion blood. Platelet transfusion concentrates in particular are susceptible to bacterial infection due to being stored at warm temperatures. This induces a shorter shelf life, and makes testing for contamination by conventional cultivation methods suboptimal. Schmidt et al. tested a cytometric method utilizing a thiazole orange RNA stain to detect bacteria

in platelet concentrate. Samples were intentionally contaminated with six different species of bacteria at different concentrations in order to test the detection threshold of the method, yielding thresholds between $10^5$ cells $mL^{-1}$ and $10^3$ cells $mL^{-1}$. While this was still too high to be satisfactory, a short incubation was able to enrich bacterial populations without the requirement of additional transfusion concentrate. Incubation steps of up to 8 were found adequate (Schmidt et al. 2006). While this type of enrichment is a rather ingenious solution which can ultimately cut incubation times, it may not be generally applicable due to its dependence on cellular division.

Another application of cytometric analysis is screening of urine samples in patients with suspected urinary tract infection. Much like blood, samples are already in liquid solution. Unlike blood, urine may not require lysing or otherwise removing erythrocytes which may interfere with the analysis. The ability to accurately quantify cells is highly relevant. While presence of bacteria in urine is not uncommon, rise of bacterial concentration above a certain threshold is taken as an indicator of urinary tract colonization.

While urine samples may be analysed by conventional cytometers, specialized cytometers are quite common for applied areas. The automated Sysmex UF-1000i was developed for urine analysis. In their evaluation of the cytometer García-Coca et al. performed measurements on a large number of clinical samples (n=17483) utilizing the ability of the flow cytometer and software to measure not just bacterial counts, but also blood cells and epithelial cells. Morphology was determined using the BACT-morph software, classifying bacteria as either rods or cocci/mixed based on forward and side scatter. This is of interest, as it demonstrates that flow cytometry can still provide additional information about the type of infection even without additional reagents increasing overall costs (García-Coca, Gadea, and Esteban 2017).

Broeren et al. similarly evaluated the Sysmex UF-1000i for pre-screening of urine samples before cultivation. Staining was achieved using a „fluorescent poly methylene dye“ in an automated protocol. The importance of false negative rates in this „ruling out“ approach were discussed by the authors in depth as well as actual cost-benefit calculations. Ultimately, it is concluded that flow cytometric pre-screening of urine samples would increase, rather than decrease, the cost of analysis, however it is acknowledged that labs which experience more negative samples overall may still see an improvement (Broeren et al. 2011).

Conversely, Moshaver et al. compared culture techniques with a flow cytometry protocol utilizing SYBR Green DNA stain after EDTA treatment on the Accuri C6 flow cytometer. High agreement between the two methods was found, with 99% likelihood of correctly ruling out urinary tract infection, resulting in a 36% decrease in number of samples which needed to be cultured. This particular study praises the flow cytometric approach, declaring it to be more cost effective than cultivation alone. (Moshaver et al. 2016).

The evaluation of the same cytometer by professionals appears somewhat unclear. Differences in the definition of the „gold standard“ value for diagnosis of bacteriuria are a possible explanation, as it appears to vary between studies and the needs of a given laboratory.

### 4.4.2 Antimicrobial Susceptibility Testing

While further studies on cytometric detection of bacteremia do not appear to have been conducted, there remains a continued interest in the development of rapid antimicrobial susceptibility testing (AST) techniques after initial bacterial cultivation. Flow cytometry is less disadvantaged in this stage of analysis, as very low bacterial concentrations are no longer an issue. A review by Maurer et al. describes a range of existing AST techniques ranging from conventional culture based techniques, with results available in 38-48 hours, to a variety of rapid culture techniques as well as novel but often untested AST approaches with results available within as few as 1-8 hours (Maurer et al. 2017).

Generally speaking, conventional viability tests can be readily used for antibiotic susceptibility testing. For instance, Roth et al. successfully used both SYTOX Green and propidium iodide for antibiotic susceptibility testing. Testing was performed both on *gram* positive and *gram* negative bacteria—namely, ampicillin sensitive and resistant *Escherichia coli* strains as well as *Staphylococcus aureus* and *Bacillus cereus*. The antibiotics used included mainly beta-lactams ampicillin, amoxicillin and penicillin G, but also vancomycin. This provides a basis for ASTs using membrane permeability assays, although, of course, not all antibiotics impact membrane integrity, limiting the ubiquity of such an approach. The authors conclude that SYTOX Green is superior to propidium iodide due to providing better distinction between positive and negative populations. However, in practical applications and standardized kits, the price of a dye may be need to be compared with the benefit provided (Roth et al. 1997).

In more recent developments, Costa-de-Oliveira et al. have tested their proprietary FASTinov flow-cytometric AST kit. While details are unavailable, the available material seems to imply some type of membrane permeability staining. As such, the kit likely works on a similar basis as conventional viability staining. Perhaps more relevant to medical workers is that the kit is reported to yield results within approximately 2 hours – an analysis time that can compete with other modern AST approaches (Costa-de-Oliveira et al. 2017).

While rapid methods for AST may prove more expensive, there is a potential to reduce the overall number of patients receiving drugs to which their infection is resistant, thus lowering mean hospital stay and ultimately resulting in cost savings. The amount saved however depends on the ratio of resistant strains present, which may significantly vary among hospitals, with larger hospitals seeing larger numbers of antibiotic resistance (Perez et al. 2014).

A flow microsphere immunofluorescence assay has been tested on Helicobacter pylori utilizing a serological approach. Microspheres of defined size were coated with Helicobacter antigens, exposed to patient serum containing human antibodies and then exposed to fluorescent-labelled anti-human antibodies and measured cytometrically. Bead fluorescence was found to be a reliable indicator of presence of immune response to Helicobacter as the results were strongly correlated with the results of known enzyme-linked immunosorbent assays (Best, Haldane, and Malatjalian 1992). While this method doesn't necessarily play to the main strengths of flow cytometry, it demonstrates the flexibility of the method.

Ultimately, it appears that the uses of flow cytometry for the purposes of clinical microbiology are limited. This is most commonly due to its high detection limits, as well as cost considerations. Despite this, attempts to use flow cytometry to improve bacterial screening procedures exist, as its speed is superior to the culturing techniques commonly used today. However, a full replacement seems unlikely to happen unless the detection thresholds for flow cytometry are reduced by several orders of magnitude.

## 4.5 Basic Research

As basic research tends to have the greatest flexibility in its experiment design, time constraints and the cost of individual experiments, many of the examples listed above can be readily used for its purposes. However, the opposite is not the case, and many creative uses of flow cytometry are unique to it, as well as some areas of study flow cytometry is particularly well suited for. This section concerns itself with some of them.

### 4.5.1 Study of Adhesion, Endocytosis and Intracellular Parasitism

Among the less usual applications of flow cytometry is the ability to detect and quantify cell adhesion or endocytosis. While adhesion of cells together during analysis is generally undesirable, coincidence of two or more cells in the same event as a result of adhesion or endocytosis can and has been utilized for research of related phenomena.

Some pathogenic bacteria are intracellular parasites capable of living inside eukaryotic cells and utilizing nutrients from the cytoplasm. The life cycle of *Chlamydiae*, like that of many parasites, is relatively elaborate, and its obligate intracellular parasitism provides a unique challenge to attempts at quantification.

Vromman et al. have investigated the life cycle of *Chlamydia trachomatis* cells modified to express heterologous GFP. Observation was performed by both cytometry and microscopy. In host-pathogen interactions, GFP fluorescence inside host cells was taken as an indicator of infection and GFP fluorescence intensity as an indicator of progress of the infection. The possibility of measuring infection rates in non homogeneous populations was suggested. It was noted, that a similar adhesion assay may allow for screening of anti-chlamydial compounds (Vromman et al. 2014).

Similarly, Boullier et al. used flow cytometry to quantify the effectiveness of different treatments at inhibiting adhesion of enteropathogenic *Escherichia coli* to HeLa cells. All *Escherichia coli* cells were modified to express GFP, thus the green fluorescence intensity of measured HeLa cells reflected the number of attached bacteria. Different antibody sera were added to the bacterial solution and quantitative changes in adhesion levels were observed. The correlation of cytometric results with those of enzyme-linked immunosorbent assays (ELISA) as well as the overall sensitivity of the method for study of humoral responses was highlighted (Boullier, Tasca, and Milon 2003).

Many free-living amoebae serve as hosts to pathogenic bacteria which may be found in drinking waters and elsewhere. Goñi et al. reviewed known interactions between amoebae and bacteria, most notably the ability of some amoebae to aid bacteria survive bactericidal agents. This is of relevance in development

of both sanitation procedures and novel bactericidal agents. The ability of an agent to reliably kill a bacterium in vitro may not fully translate due *in vivo* to the influence of endocytosis. Flow cytometry is one of the methods which can facilitate the quantitative understanding of such mechanisms (Goñi, Fernández, and Rubio 2014). For instance, Douesnard-Malo and Daigle used flow cytometry to observe the development of a co-culture of *Acathamoeba castellani* and a *Salmonella enterica* serovar Typhi. The *Salmonella* strain was modified to exhibit constitutive GFP expression. Flow cytometry was subsequently able to provide both total cell counts, as well as to estimate the number amoebae-containing bacteria. This was done, as with previous studies, on the basis of green fluorescence within amoeba cells (Douesnard-Malo and Daigle 2011).

In another study, Flannagan and Heinrichs have been able to track the cell division of GFP-expressing *Staphylococcus aureus* inside of macrophages. First, *Staphylococcus aureus* cells were stained with the eFluor 670 cell proliferation dye and only then co-incubated with macrophages. Macrophages with a constitutive GFP signal but no eFluor 670 signal were considered to have dividing bacteria inside of them, and their total numbers were quantified by flow cytometry (Flannagan and Heinrichs 2018).

### 4.5.2 Study of Population Heterogeneity

Population heterogeneity is a phenomenon in which phenotypic differences between cells in a population emerge on the basis of stochastic or environmental stresses even despite genetic homogeneity. Examples of this can range from uneven distribution of nutrients in a solution to leaky regulation or regulator bistability. The latter is of particular interest in the study of virulence.

As many of these studies investigate *in vivo* processes by extensively relying on GFP expression in the host cells, it is important to ensure minimal metabolic effect on the researched organism. In a study performed by Cooper et al. several variable-strength synthetic promoters and vectors were used to transform *Salmonella*. Fine-tuned vectors were created, which allowed study of pathogenic phenotypes while limiting or eliminating negative impact on the pathogen due to overexpression. The study included variants expressing GFP or mCherry, whose expression was then investigated at the single cell level using flow cytometry (Cooper et al. 2017).

Flow cytometry has been utilized to observe a GFP-fused RovA virulence regulator in *Yersinia pestis*. Thanks to flow cytometry, it was possible to gradually visualize cells shift between GFP positive and negative populations in response to temperature shifts as a consequence of a temperature-dependent negative feedback loop, clearly demonstrating the temperature dependence and kinetic aspects of the regulator's function. Different RovA variants were then investigated, and the expression profile observed via flow cytometry was related to different degrees of virulence and persistence in mice (Nuss et al. 2016).

Similarly, Nikel et al. used BacLight Redox Sensor Green staining to evaluate the metabolic activity of *Pseudomonas putida* grown on different substrates. They found that a bimodal distribution formed when the cells were grown on glycerol, but not on other carbon sources. This suggested a bimodal stochastic switch governing the commitment of *Pseudomonas putida* to glycerol as part of a bet hedging strategy, with implications for the use of *Pseudomonas putida* in industrial applications (Nikel et al. 2015).

An investigation of another pathogen, *Salmonella enterica*, likewise showed bistable regulation of the *Salmonella enterica* pathogenicity island 1 (SPI-1). Most interestingly, it was found that a combination SPI-1 positive and SPI-1 negative cells, as found *in vivo*, had the highest efficiency in penetrating barriers, suggesting a cooperative nature of virulence through cell differentiation. The expression profiles of individual genes were tracked through GFP fusion and flow cytometry. Through cell sorting, the invasive capabilities of pure SPI-1 positive or SPI-1 negative populations as negative controls. Visualization using fluorescence microscopy was used as a complementary method (Sánchez-Romero and Casadesús 2018).

#### 4.5.2.1 Persistence

A particularly fruitful area of study related to population heterogeneity is the research of bacterial persistence. Bacterial persistence is a physiological state characterized by dormancy and multidrug tolerance that is not dependent on genetic factors (Lewis 2007). The topic is of high interest, as persisting bacterial pathogens are a likely source of reinfection, are more likely to survive sanitary measures and may lead to the development of antibiotic-resistant strains (Windels et al. 2019). Flow cytometry can be used both for identifying persister cells and the studying the mechanisms of persistence.

One approach to identifying persisters has been described Shah et al. The authors used a GFP reporter under a growth-linked promoter (*rrn*BP1) in *Escherichia coli* as a dormancy indicator. Thus, GFP would only be highly expressed only in non-dormant cells. These non-growing cells were suspected to be persisters, given the general metabolic down-regulation observed in persister phenotypes. Using a fluorescence activated cell sorter, cells exhibiting low GFP intensity were separated and their antibiotic tolerance was tested. The low-transcribing cells were found to have significantly higher survival rates upon subsequent cultivation, suggesting higher overall persister content (Shah et al. 2006).

Ratcliff and Denison have used flow cytometry to observe spontaneously forming subpopulations not caused by differences in regulation, but by heterogenic segregation of cell mass upon division. *Sinorhizobium meliloti* uses polyhydroxy butyrate (PHB) as an energy reserve substrate. The amount of PHB was estimated based using the lipophilic dye Nile red, while viability was tracked using PI. In the study, it was found to form high-PHB and low-PHB subpopulations in response to stress. Further analysis using GFP expression as a marker of metabolic activity showed high-PHB cells to have lower overall GFP expression and metabolic activity, higher antibiotic resistance and slower response to stimuli compared to low-PHB cells marking them as persisters (Ratcliff and Denison 2011).

An investigation of DNA damage in *Escherichia coli* in response to ofloxacin was conducted. Similarly to previous examples, GFP and mCherry were transcriptionally fused to various SOS promoters (P*recA*, P*recN*, $P_{sulA}$ and P*tisB*). The expression of SOS genes was used to track DNA damage. Fluorescence activated cell sorting was used to separate cells by SOS promoter induction, and survivor counts were compared. It was found that persister frequency was not affected by the degree of SOS activation. Instead, they concluded that paradoxically, the mechanism behind survival of ofloxacin treatment relies on repairs after the removal of the antibiotic, not the prevention of damage (Völzing and Brynildsen 2015).

# 5 Conclusion

Flow cytometry is a flexible modern method which can offer unique data in the study of bacteria. In particular, flow cytometry is capable of analysis at the single cell level, without averaging data across the entire population. This makes it uniquely suited for studying bacterial population dynamics and phenotypic heterogeneity. It is capable of both quantitative and qualitative measurement at comparatively high speeds. Notably, this allows it to observe relative frequencies of cells in different subpopulations. It also has an advantage over cultivation techniques, in that it is able to detect viable but uncultivable cells, including persisters. This makes it a particularly good choice when screening for bacteria, as many pathogens do not divide outside of hosts.

While flow cytometry is widely used for eukaryotic cells, adapting the method for bacteria introduces new challenges. The small size of bacteria makes detection difficult. The physiological diversity of bacterial species can cause difficulties when applying the same protocol to different species. For example, the outer membrane of *gram* negative bacteria can obstruct the entry of dyes into the cell. Specific species have also shown resistance to staining, such as *Pseudomonas aeruginosa*. Consequently, care must be taken when adapting techinques to different taxa. While there are often known workarounds, for example treating *gram* negative bacteria with EDTA is known to permeabilize the outer membrane, they can also introduce artefacts.

Similarly, many of the commonly used, despite their popularity, have flaws which need to be considered. The membrane integrity probe propidium iodide, which is commonly used to track cell viability, can enter live cells if their membrane potential reaches a very high level. Other dyes, such as CTC, can have difficulties staining only some species, and may have cytotoxic effects on cells. Fortunately, the market has developed to the point where there are newer dyes available, which can fulfil the same role and may not share the same drawbacks. As a consequence, an informed researcher can choose the reagent most suitable for the job and avoid compromising the results of their study.

In addition, modern fluorescent labels such as fluorescent antibodies, fluorescent NA probes (Flow-FISH), or fluorescent protein expression systems open new possibilities. While GFP-based methods are common, whether the other methods will see significant proliferation in this area remains to be seen. A part of the problem could be the lack of bacteria-specific reagents available on the market, however even with higher availability, their higher monetary and labour costs may still disadvantage these methods.

Despite the above problems, flow cytometry appears to be proliferating across various areas of microbiology, and as new applications continue to be developed. It is apparent that the method has not yet reached its full potential. In basic research, the method has been used to study phenotypic heterogeneity within bacterial populations, which includes phenomena such as persistence, bistable regulation of virulence or the quantification host-pathogen interactions. Its ability to observe phenotype at the single cell level helps it detect populations which other methods may be unable to. Understanding population dynamics of bacterial

pathogens via flow cytometry has the potential to bring significant improvements to the medical fields.

Flow cytometry has encountered some difficulties in clinical microbiology. The high detection limits of flow cytometry have in some cases proven insufficient to reliably detect the presence of bacteria. Methods utilizing short pre-cultivations have been tested as sufficient to facilitate detection in platelet concentrates. These methods are still faster than some conventional approaches. As a result, flow cytometry may be able to fill a niche in pre-screening by providing same-day screening results combined with antibiotic susceptibility testing. As bacterial cultures typically require overnight cultivation, if properly adapted, flow cytometry may be able to both improve patient outcomes through timely diagnosis. In addition, these partial cultivation techniques could be used to improve the sensitivity of flow cytometric protocols in other fields.

Flow cytometry has been the mainstay of environmental microbiology. Initially established as a tool for screening water bodies, the method has experienced significant growth characterized by the development of online flow cytometry. Online flow cytometers are capable of performing regular measurements unsupervised for extended periods of time. While the applicability of these machines is limited to sampling liquid bodies, the prospect of observing bacterial population dynamics change over time is an intriguing one. The automation means, that labour investment is significantly lower, and significantly improves the feasibility of such studies.

Flow cytometry has found uses for both monitoring of bioreactors and understanding of population heterogeneity within these reactors. With proper understanding of population dynamics and bioprocess optimization, there is potential to make biodegradable bacterial polymers such as PHA viable market alternatives to traditional plastic polymers.

Ultimately, flow cytometry is a modern method with many unique applications in bacterial study, especially in understanding bacterial population dynamics and phenotypic heterogeneity. Available methods range from both well established to novel and untested. Despite limitations, there appears to be sustained interest in continued development of the method due to the many advantages it has, and it is likely that flow cytometry will continue to proliferate across microbiology in the foreseeable future, both in basic research and in the applied fields.

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