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MINI-REVIEW

Reevaluating multicolor flow cytometry to assess microbial viability

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Abstract Flow cytometry is a rapid and quantitative method to determine bacterial viability. Although different stains can be used to establish viability, staining protocols are inconsistent and lack a general optimization approach. Very few "true" multicolor protocols, where dyes are combined in one sample, have been developed for microbiological applications. In this mini-review, the discrepancy between protocols for cell-permeant nucleic acid and functional stains are discussed as well as their use as viability dyes. Furthermore, optimization of staining protocols for a specific setup are described. Original data using the red-excitable SYTO dyes SYTO 59 to 64 and SYTO 17, combined with functional stains, for double and triple staining applications is also included. As each dye and dye combination behaves differently within a certain combination of medium matrix, microorganism, and instrument, protocols need to be tuned to obtain reproducible results. Therefore, single, double, and triple stains are reviewed, including the different parameters that influence staining such as stain kinetics, optimal stain concentration, and the effect of the chelator EDTA as membrane permeabilizer. In the last section, we highlight the need to investigate the stability of multicolor assays to ensure correct results as multiwell autoloaders are now commonly used.

Keywords Functionality · Protocols · Viability · SYTO · Multicolor FCM

Introduction

Flow cytometers, invented in the late 1940s, found their way into microbiology in the 1980s because of technological advances (Wang et al. 2010). The main advantage is the speed of single-cell analysis and consequently, the possibility to make a detailed analysis of the cell population and its heterogeneity. This information can be used to assess microbial viability and survival in different environments. In this respect, information about the heterogeneity within bacterial populations can improve our understanding of microbial ecology and to expand our knowledge and reduce system failures in the area of microbial technologies. A second advantage is the possibility to simultaneously asses the physiological state and growth behavior-enabling control of metabolic processes both online and offline (Boi et al. 2015; Breeuwer and Abee 2000; Lieder et al. 2016; Muller 2007; Rieseberg et al. 2001; Shi et al. 2007).

Multicolor flow cytometry is a good approach to simultaneously estimate and assess multiple features and to characterize the heterogeneity of a community in detail. However, staining bacteria is a complex interplay between dye chemistry, the target organisms, and the staining conditions. For microbiological applications, the diversity of bacterial species is challenging, as even closely related organisms are known to behave very differently, making it difficult to analyze bacteria in a standardized way (Shapiro 2000). Hence, it is important to have a reasonable amount of standardization in terms of stain

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concentration, used buffers, incubation time, need for permeabilization or fixation, and the necessary controls in order to compare different samples. In addition, a better understanding of the staining chemistry is important to estimate the reliability of a staining protocol for a specific research setup. Unfortunately, it is exactly on those aspects that many available studies lack the necessary information and where data are poor and inconsistent. An overview of the parameters important for protocol optimization are given by Hammes et al. (2011). Very few "true" multicolor protocols, where dyes are combined in one sample, have been developed in such a way for microbiological applications. In this mini-review, we will discuss the discrepancy between protocols for some popular stains and offer a way in which staining could be optimized for a specific setup. The number of available dyes is vast and summarizing them all in detail is beyond the scope of this mini-review as not all dyes are useful to determine viability. For more information, consult Hammes et al. (2011), Strauber and Muller (2010), Tracy et al. (2010), and Shapiro (2003). Furthermore, we included original data using red-excitable SYTO dyes combined with functional stains for double and triple staining applications.

Viability

The concept of viability has been introduced more than 100 years ago and was based on the capability of bacteria to grow on agar plates, a method which is still used today. However, it is now clear that only a small fraction of bacteria can grow on classic media and that they can switch between different states of persistence, cultivability, and dormancy showing variances in size, shape, or nutritional behavior (Hammes et al. 2011). In a natural habitat, bacteria can have even more different physiological states depending on their metabolic activity, which can vary within spatial and temporal distributions. Bacterial abundance, activity, and community composition also changes in response to abiotic and biotic stresses and significant differences can occur over minutes, hours, or weeks. Specific bacterial activities, contributing to the overall function of the community, need to be scaled to the number of bacteria participating in that activity in order to get a full understanding of microbial ecological behavior and viability.

Although, numerous studies indicate this uncultivability of bacteria, some advances have been made. Recovery of the uncultivable bacteria has been difficult to prove, produce, and reproduce (Amor et al. 2002; Boi et al. 2015; Falcioni et al. 2008; Gasol and Del Giorgio 2000; Hammes et al. 2011; Lopez-Amoros et al. 1995). Hence, the phenotype and physiological basis for these processes have not been defined and is still open to investigation (Breeuwer and Abee 2000; Epstein 2013; Kell et al. 1998). A good review for the range of distinct bacterial states, the viable but non-cultivable phenomena, and the difference in dormancy is given by Kell et al. (1998) and Li et al. (2014). For more information about uncultivable bacteria, we recommend the detailed review of Stewart (2012).

Defining viability is thus challenging and it requires an assessment of various cellular criteria (such as growth, DNA transcription and RNA translation, energy generation, metabolism) that constitute or relate to life and death of an organism. Examples of parameters used to measure these criteria are the following: membrane integrity, membrane potential, pH gradient, pump activity, respiration, cell morphology, enzyme activity, and reproduction. The most important function herein is maintaining the membrane potential, as bacteria need to be able to maintain an intracellular environment that supports energy generation (Breeuwer and Abee 2000; Hammes et al. 2011).

Therefore, to fully comprehend viability, tools that accurately and reliably measure these parameters need to be established (Boi et al. 2015). As mentioned, the golden standard that is used to measure viability is culturing, which is cheap and easy but a slow, retrospective and a binary tool. Other conventional methods, such as fluorometric, luminometric, or conductivity assays, have also been established to identify and characterize bacteria in a high-throughput manner. They offer a fast and simple bulk assessment of the viability of a bacterial culture. Therefore, estimating differentiation and heterogeneity within a population in relation to viability requires a combination of single-cell techniques such as cytometry, molecular characterization, or microscopy (Cangelosi and Meschke 2014; Ericsson et al. 2000; Hastings and Wilson 1976; Jameson et al. 2003). In this respect, flow cytometry is an excellent technique as it offers single-cell analysis of various parameters in a high-throughput manner with improved speed and sensitivity. Basic cell functions such as metabolic activity, respiration, and membrane integrity can easily be determined simultaneously. These multiparametric data allow estimation of population heterogeneity and distinction of different viable stages. However, these data do not offer absolute discrimination between live and dead bacteria as these parameters can vary in response to different environmental factors. In addition, many methods lack standardization, thereby, creating significant variation in interpretation and estimation of viability (Davis 2014; Nebe-von-Caron et al. 2000; Neidhardt et al. 1990; Prescott et al. 2004; Roszak and Colwell 1987). Other emerging technological developments such as single-chip analysis, gel microdroplets, microfluidics, etc. could also improve our understanding of bacterial heterogeneity and viability. As these techniques offer single-cell analysis in a controlled microenvironment, they facilitate studying the effect of certain stressors on different cellular levels simultaneously (Amor et al. 2002; Benoit et al. 2010; Hammes et al. 2011; Keer and Birch 2003). The multitudes of techniques mentioned above have both advantages and disadvantages for assessing

viability (summarized in Supplementary Table S1). In addition, by allowing the determination of complex physiological parameters, it has become more complex to determine "viability" and as a consequence the 'viable' paradigm has shifted (Falcioni et al. 2008; Hammes et al. 2011; Nebe-von-Caron et al. 2000). In general, no single technique or stain is suited for viability measurements of all species and under all conditions (Davey 2011).

Dyes and their protocols

A wide variety of fluorescent dyes are available for flow cytometry. Thus, very different aspects of microbial physiology can be assessed and monitored. However, it is important to understand how different dyes function and how they can be applied in order to draw the correct conclusions. In this mini-review, we will separately discuss cell-permeant nucleic acid stains and functional stains that give information about bacterial physiology.

Cell-permeant nucleic acids dyes

Nucleic acids carry the genetic information used in the development and functioning of all living organisms and viruses and could therefore be a very logical indicator of life. However, it is important to note that DNA can be persistent and that dead cells may still contain DNA. For example, when bacteria are killed by UV-C irradiation, lethal thymine dimers are produced that will be stained by most nucleic acid dyes (Hammes et al. 2011). Nucleic acid stains can also act as good counterstains for labeling all organisms which are present, live, or dead. Generally, nucleic acid stains can be divided in cell-permeant and cell-impermeant dyes depending on their ability to pass through the cell membrane. The latter, being unable to cross intact membranes, can therefore be considered as viability-dependent dyes. Furthermore, the difference between Gram-positive and Gram-negative bacterial cell wall structure poses additional difficulties (Berney et al. 2007; Shapiro 2003; Strauber and Muller 2010) and permeabilization of the outer membrane of Gram-negative bacteria is often necessary in order to optimize staining. Either EDTA or citrate can be used for this purpose (Marie et al. 1996). Both compounds permeabilize the outer membrane by chelating cations and stripping the LPS layer of the outer membrane (Chen et al. 2004). These cations can also be deleterious as they decrease binding efficiency of certain dyes such as 4′,6-diamidino-2-phenylindole (DAPI) or Hoechst 33342 (Marie et al. 1996). Here, we will focus on EDTA as previous results from Marie et al. (1996) showed that EDTA gave similar to those with citrate.

Many dyes can be used to stain nucleic acids and well-known examples are DAPI (stains DNA), and SYBR green I (stains DNA) and SYTO 9 (stain DNA and RNA). DAPI is excited by a UV laser (355 nm) but despite the good results, UV lasers are expensive and not available in the standard configuration of most bench-top flow cytometers. SYBR green I and SYTO 9, which are compatible with almost all bench-top flow cytometers (ex./em. 488:530 nm), have already been extensively studied and are good candidates for nucleic acid staining (Berney et al. 2007; Lebaron et al. 1998; SLMB 2012; Van Nevel et al. 2013; Zipper et al. 2004). Besides SYTO 9, many other SYTO dyes are available. All SYTO dyes share some common characteristics such as high signal to background fluorescence, high molar absorptivity, permeant to nearly all cell membranes, and high quantum yields when bound to nucleic acids (Johnson and Spence 2010). The SYTO stains differ in their affinity to bind DNA and RNA and in their spectral characteristics, making the SYTO family a versatile tool for multicolor flow cytometry. In contrast to other DNA stains, some SYTO dyes are excited by a red laser (640 nm), increasing the possibility for multicolor combinations and enabling their use as a counterstain. Red lasers are frequently used for the popular fluorochromes APC, Alexa Fluor® 700 or APC-eFluor® 780 in combination with antibodies for research with mammalian cells. Therefore, commercial bench-top flow cytometers are often standardly equipped with red lasers. However, only a few applications are known for this laser in microbiology such as DRAQ5 as cell-permeant nucleic acid stain. Since it has been reported that DRAQ5 alters morphology and decreases cell viability, it is less suitable in combination with viability stains (Silva et al. 2010). The characteristics of these red-excitable SYTO dyes are summarized in the supplementary information (SI, Supplementary Table S2).

To further scrutinize the usability of SYTO dyes, we investigated the possible application of the red-excitable SYTO dyes 17, 59, 60, 61, 62, 63, and 64 for both the Gram-negative bacterium *Cupriavidus metallidurans* CH34 and the Gram-positive bacterium *Lactobacillus brevis* LMG 18022 (Supplementary Material, Materials and Methods, Assessment of staining kinetics and assessment of optimal conditions for single stains). First, we tested the staining kinetics by measuring the change in fluorescence over time. Results indicated that dye uptake was immediate for the Gram-positive bacterium while an incubation period of approximately 15 min was required for the Gram-negative bacterium to reach maximum fluorescence intensity (Supplementary Figs. S1 and S2). For the Gram-positive population, fluorescence intensities were stable, indicating time-independent uptake. For the Gram-negative population, fluorescence intensities increased over time showing time-dependent uptake. After uptake, the fluorescence signal remained stable during the entire measurement period (30 min). This difference in uptake between Gram-positive and Gram-negative bacteria is likely due to differences in cell

membrane composition and is also observed for blue-excitable SYTO dyes (Lebaron et al. 1998). A second test was performed where different dye concentrations and the effect of EDTA were assessed for all dyes. This test was performed because the amount of cells counted (of a known cell concentration) can vary depending on the used dye concentrations and incubation time regardless of the fluorescence intensity of the cell population. Thus, different dye concentrations and the effect of EDTA were assessed at different time points during incubation (0, 15, and 30 min) for all red-excitable SYTO dyes. As a benchmark, cell counts were compared to the standard SYBR green I staining as described in the Swiss protocol for detection of bacteria in drinking water (SLMB, 2012). Results of this experiment for SYTO 60 and the SYBR green bench mark are shown in Fig. 1; the results of the other dyes are available as supplementary material (Supplementary Figs. S3–8). A stain was assessed as good, if cell counts did not deviate more than 10 % from the SYBR green benchmark. Our results showed that a final stain concentration of 0.5 μM is preferable for all red-excitable SYTO dyes and that an incubation period between 15 to 30 min depending on the organism is sufficient to obtain a reliable estimate of cell numbers (Fig. 1). Only SYTO 64 was unable to stain bacteria under these conditions, as no cells could be detected with the flow cytometer (Supplementary Fig. S8). This optimal stain concentration is comparable to that found by Comas and Vives-Rego (1997) who tested SYTO 17 at 1 μM on *Escherichia coli*. However, our protocol relied on a shorter incubation time of 15 min instead of 60 min and a higher incubation temperature. This difference in incubation temperature could facilitate dye intrusion by diffusion (Johnson and Spence 2010). As cell concentrations remained stable after the minimal incubation time, it indicates that the dyes are not pumped out of the cells. In case no bleaching occurs, longer incubation times can also be used. The addition of 5 μM EDTA did not improve staining efficiency nor signal intensity, except when lower dye concentrations (0.05 μM) were used for the Gram-negative bacterium.

Functional dyes

Cell-impermeant nucleic acid stains such as propidium iodides (PI) are not able to cross membranes because of their size and charge, and are therefore used as an indicator of membrane permeabilization (Berney et al. 2007). Since membrane integrity is vital to keep the intracellular environment stable, membrane damage can be an indication of cell death (Hammes et al. 2011). However, it is known that contact time, incubation temperature, and stain concentration are crucial factors for proper staining, which emphasizes the importance of standardization (Hammes et al. 2012; Van Nevel et al. 2013). Furthermore, a recent study on *E. coli* suggested that porins and periplasmic transporters induced by substrate limitation facilitate PI entry into cells and that staining efficiency is influenced by the physiological state (Brognaux et al. 2014). Shi et al. (2007) showed that more bacteria were stained during early exponential phase than during the early lag phase. This indicates that PI cannot be used as viability estimator and suggests the necessity for multiparameter viability determination. Besides PI, other dyes with similar mode of action are available such as SYTOX dyes, the TOTO and TO-PRO family of dyes (Shapiro 2003).

Another aspect of viability is maintaining the cell's membrane potential. All active microbial cells need to keep their membrane potential, which is produced through a functional electron transport chain. The membrane potential also powers processes such as ATP synthesis and solute-ion transport. If the membrane potential decreases, the cell will be unable to transport essential molecules, eventually leading to cell death. Membrane potential should be considered as a more conservative measurement of viability compared to membrane permeabilization because of the link between membrane potential and cell respiration (Hammes et al. 2011). $DiBAC_4(3)$, also known as bis-oxonol (BOX), is mostly used to evaluate membrane potential. It enters depolarized cells because of its anionic structure and non-specifically binds to intracellular proteins (Muller and Nebe-von-Caron 2010). In contrast, the cationic Rhodamine 123 (Rh123) only accumulates in cells with active membrane potential (Diaper et al. 1992). Since this stain can be actively pumped out by certain cells it has a limited use in standardized protocols (Tracy et al. 2010). Alternative dyes are 3,3′-dihexyloxacarbocyanine iodide (Di-OC6(3)); 3,30-diethyloxacarbocyanine (Di-OC2(3)); and 3,30-dipropylthiadicarbocyanine (DiSC3(5)) (Shapiro 2003). Various protocols exist for the use of $DiBAC_4(3)$ with final stain concentrations, incubation time, and temperatures ranging from 0.24 to 29 μM (Herrera et al. 2002; Nielsen et al. 2009), 2 to 20 min (Comas and Vives-Rego 1997; Lopez-Amoros et al. 1995; Rezaeinejad and Ivanov 2011), and room temperature to 40 °C (Linhova et al. 2012; Rezaeinejad and Ivanov 2011), respectively. Table 1 provides an overview of staining protocols and their references.

Besides membranes, other aspects of the cell can be used to assess viability or functionality. All bacteria possess housekeeping enzymes such as esterases or dehydrogenases that are linked to the respiratory activity of metabolically active cells. The inactivity of these enzymes indicates metabolic inactivity, but not necessarily cell death as these enzymes can still be active even after cell death has occurred. Measurement of cellular enzymatic activity is useful although the mentioned limitations need to be considered. Generally, dyes used to monitor enzymatic activity are cleaved upon uptake in the cell, leading to the production of a fluorescent signal (Shapiro 2003). Again, a wide variety of dyes that target different enzymatic activities are available. A popular dye is 5-cyano-2,3-ditolyl tetrazolium chloride (CTC),which is reduced by dehydrogenases to fluorescent membrane-impermeant formazan

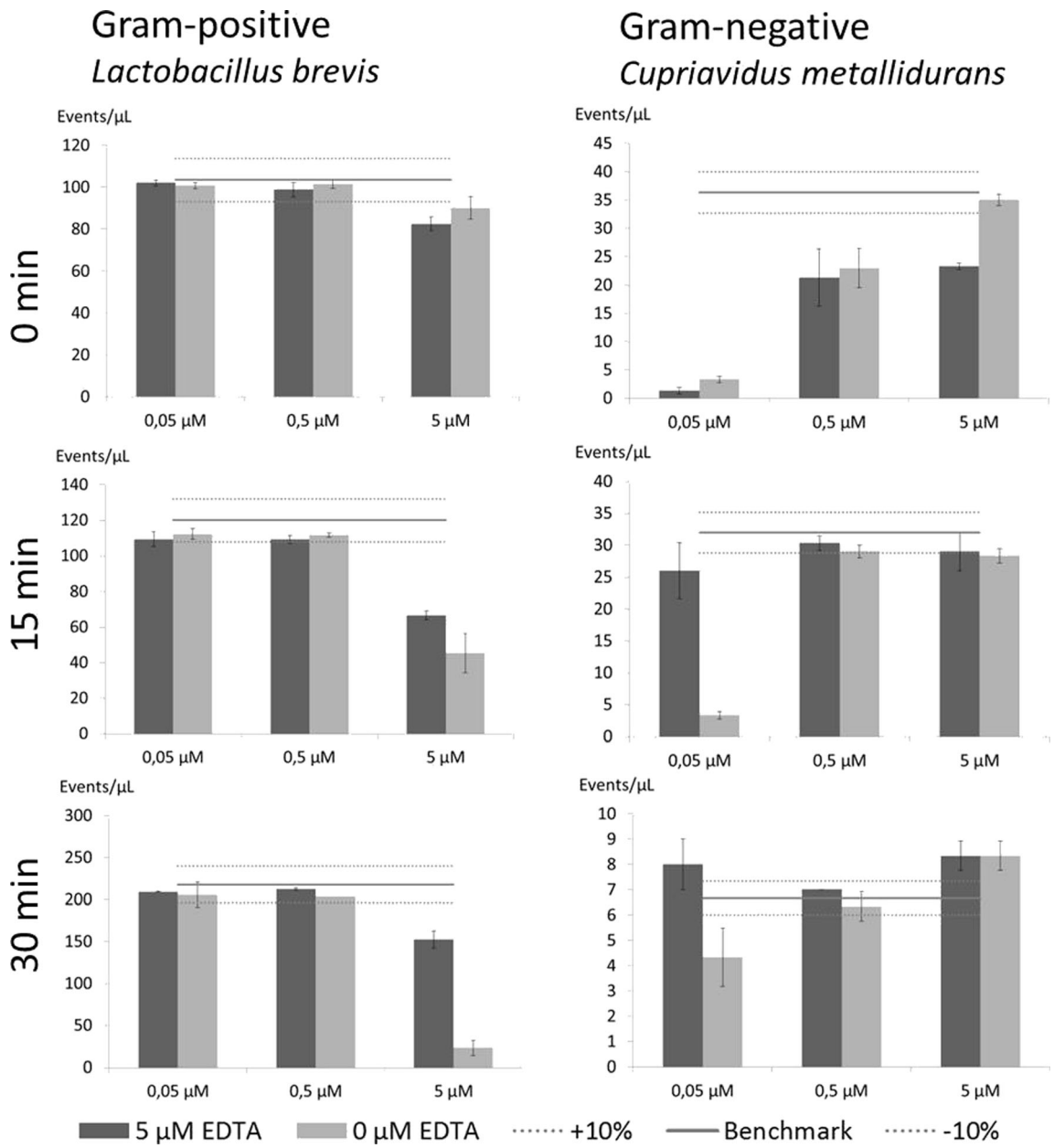

Fig. 1 Optimization of the red-excitable dye SYTO 60 for both the Gram-positive *Lactobacillus brevis* LMG 18022 (*left*) and Gram-negative *Cupriavidus metallidurans* CH34 (*right*) on three different time points: 0 min (*top*), 15 min (*middle*), and 30 min (*bottom*). Staining was performed with three different stain concentrations (5, 0.5, and 0.05 µM) with and without 5-µM EDTA. All samples were measured in triplicate. Cell counts are expressed as events/microliter and should be compared to the results obtained with SYBR green I staining as benchmark. A maximum 10 % deviation on the SYBR green I results was accepted

(Lopez-Amoros et al. 1997). Since respiratory activity is linked to the maintenance of the membrane potential, CTC reduction and $DiBAC_4(3)$ diffusion are complementary (Hammes et al. 2011). Fluorescein diacetate (FDA), another popular dye, is cleaved by esterases to release the fluorescent fluorescein. Since fluorescein easily leaks from cells, FDA modifications have been developed such as carboxyfluorescein diacetate (cFDA), with better retention kinetics, and modifications of cFDA in order to further reduce leakage such as carboxyfluorescein diacetate acetoxymethyl ester (cFDA-AM); 20,70-bis-(2-carboxyethyl)-5-(and-6)-carboxyfluorescein-AM (BCECF-AM); calcein-AM; and carboxyfluorescein diacetate succinimidyl ester (cFDA-SE). Evidently, to avoid leakage, cell permeabilization (e.g., by EDTA or citrate) to improve staining is not recommended with those dyes. Furthermore, permeabilization of the membranes can affect viability. An interesting feature of these dyes is that the fluorescence emission intensity of fluorescein depends on the pH, thereby giving additional information about cell metabolism. With a maximal emission at pH 9 and minimal emission at pH 5, changes around the

Table 1 Overview of concentration, incubation time, and temperature used in staining protocols for different dyes and organisms compared with our findings

	Concentration [μM]	Incubation time [min]	Incubation temperature [°C]	Organism studied	Reference
$DiBAC_4(3)$	5	22–40	37	*C. metallidurans* CH34, *L. brevis* LMG 18022	This study
	1	4	37	*B. adolescentis*	Amor et al. (2002)
	1	2	RT	*E. coli*	Comas and Vives-Rego (1997)
	19.4	10	RT	*E. coli*, *P. aeruginosa*, *S. aureus*	Jepras et al. (1995)
	0.24	10	RT	*P. fluorescens*, *P. ultimum*, *R. solani*	(Nielsen et al. 2009)
	0.5	15	37	*S. macedonicus*	Papadimitriou et al. (2007)
	1	20	37	*E. coli*	Rezaeinejad and Ivanov (2011)
	0.48	20	40	*L. delbrueckii* subsp. *bulgaricus*	Rault et al. (2008)
	1	2	RT	*E. coli*, *S. typhimurium*	Lopez-Amoros et al. (1995)
	1.94	7	RT	*C. pasteurianum*, *C. beijerinckii*	Linhova et al. (2012)
	29	10	RT	*E. coli*	Herrera et al. (2002)
	10	10	20	*E. coli*	Berney et al. (2009)
	3.87	10	RT	*P. pastoris*	Hyka et al. (2010)
cFDA	10	30–40	37	*C. metallidurans CH34, L. brevis LMG 18022*	This study
	50	10	30	*L. plantarum*	Bunthof and Abee (2002)
	50	10	30	*L. lactis*	Bunthof et al. (1999)
	10	10/20 and 30	RT/30/40	Lake water bacteria	Porter et al. (1995)
	10	10	37	Lactic acid bacteria	(Chen et al. 2012)
	10	30	37	*B. adolescentis*	(Amor et al. 2002)
	0.000217	10	40	*L. delbrueckii* subsp. *bulgaricus*	Rault et al. (2008)
	0.00217	10	40	*L. delbrueckii* subsp. *Bulgaricus*	Rault et al. (2009)
	25	45	37	*S. macedonicus*	Papadimitriou et al. (2007)
	10.8	10	37	*P. pastoris*	Hyka et al. (2010)
	50	60	30	*B. cereus* endospores	Cronin and Wilkinson (2008a)
	10	30	37	Activated sludge bacteria	Forster et al. (2002)
	21,7	10	RT	*C. pasteurianum*, *C. beijerinckii*	Linhova et al. (2012)
	10	15	35	*A. hydrophila*, *B. subtilis*, *E. coli*, *P. aeruginosa*, *S. epidermidis* and bacteria from environmental waters	Hoefel et al. (2003)
	21.7	30	37	*E. coli*, *P. aeruginosa*, *S. aureus*	Jepras et al. (1995)
	5	30	30	*L. lactis*	Hansen et al. (2015)
cFDA-SE	0.2	38–40	37	*C. metallidurans CH34, L. brevis LMG 18022*	This study
	0.0448	10	40	*L. delbrueckii* subsp. *bulgaricus*	Rault et al. (2008)
	100	150	25–37	Strains from aquatic environment	Fuller et al. (2000)
	10	30	35	*A. hydrophila*, *B. subtilis*, *E. coli*, *P. aeruginosa*, *S. epidermidis*, and bacteria from environmental waters	Hoefel et al. (2003)
	11	20	37	*S. pyogenes*	Hytonen et al. (2006)
	1	15	30	*Bifodobacterium*, *Lactobacillus*	Lahtinen et al. (2006)
	50	20	37	*L. casei*	Lee et al. (2004)
	10	30	37	*B. licheniformis*	Hornbaek et al. (2002)
	35	10	RT	*L. plantarum*	Fitzgerald et al. (2004)
	1	10	30	*L. lactis*	Breeuwer et al. (1996)
	44.8	10	40	*O. oeni*	Bouix and Ghorbal (2015)

Table 1 (continued)

	Concentration [μM]	Incubation time [min]	Incubation temperature [°C]	Organism studied	Reference
	50	30	30	*L. lactis*	Hansen et al. (2015)
	0.5	4	RT	*Cronobacter* spp.	Arku et al. (2011)
HE	5	40	37	*C. metallidurans* CH34, *L. brevis LMG 18022*	This study
	31.7	10	RT	*E. coli*	Herrera et al. (2002)
	10	0	RT	*Cupriavidus, Shewanella*, *E. coli*, *Deinococcus*	Baatout et al. (2005)
	0.07	4	RT	*Cronobacter* spp.	(Arku et al. 2011)
Nile Red	0.13	10–40	37	*C. metallidurans CH34*, *L. brevis LMG 18022*	This study
	10 and 100	30	RT	*Synechocystis* spp., *E. coli*	Tyo et al. (2006)
	157	10–15	RT	*P. auroginosa*	Vidal-Mas et al. (2001)
	3.1	30	RT	*E. coli*	Herrera et al. (2002)
	94.2	10	RT	*R. eutropha*	Gorenflo et al. (1999)
	60	10	RT	Environmental bacteria	Koch et al. (2013)
	0.1258	30	RT	*M. rhodesianum*	Ackermann et al. (1995)

RT room temperature

neutral pH can be detected (Johnson and Spence 2010). Again, different protocols for cFDA and cFDA-SE have been reported and generally a short incubation time at higher temperatures is necessary (Table 1).

Oxidative stress can also be assessed by flow cytometry. Reactive oxygen and nitrogen species (ROS and RNS) such as superoxide anion radical ($O_2^{\bullet-}$), hydrogen peroxide (H_2O_2), and the hydroxyl radical ($HO^{\bullet}$) are naturally occurring by-products of respiration and oxidation. To protect themselves from those toxic compounds, aerobic organisms use enzymes like superoxide dismutase (SOD) or non-enzymatic anti-oxidants like glutathione (GSH) to control the level of ROS. Environmental oxidizers, such as UV irradiation or chlorination can increase intracellular ROS levels leading to increased oxidative stress and eventually to cell death. To measure the increase of ROS, dihydroethidium (hydroethidine; HE) can be used. The oxidation of HE results in the formation of ethidium, a fluorescent compound that intercalates DNA (ex./em. 520:610 nm) (Munzel et al. 2002). Research has shown that not only $O_2^{\bullet-}$ but also cytochrome c and other reactive oxygen and nitrogen species can oxidize HE (Tarpey et al. 2004). The relative reactivities are $ONOO^- > Fe(II)/H_2O_2$ (i.e., $HO^{\bullet}$) $> O_2^{\bullet-} > H_2O_2$ (Murrant and Reid 2001) and show that HE provides an indication of both ROS and RNS production (Gomes et al. 2005). Literature on the use of HE for assessing oxidative stress in bacteria by flow cytometry is scarce and staining protocols used concentrations between 0.07 and 31 μM (Arku et al. 2011; Herrera et al. 2002) and incubation times between 0 and 10 min at room temperature (Baatout et al. 2005; Herrera et al. 2002) (Table 1).

Although lipid composition is not related to cell viability, it is an interesting parameter to investigate cell physiology. Lipid stains can roughly be divided into two groups: lipid analogs and lipophilic organic molecules. The BODIPY-labeled fatty acid analogs are often used for mammalian cells and microscopy, their use in flow cytometry applications is rather rare (Benincasa et al. 2009; Papadimitriou et al. 2007). An example of the second class is Nile red (NR), which binds selectively to non-polar lipid droplet inside cells (Johnson and Spence 2010) and can be used to detect the presence of storage lipids (PHA/PHB) in spectrophotometry (Greenspan and Fowler 1985) and flow cytometry (Ackermann et al. 1995; Gorenflo et al. 1999; Herrera et al. 2002; Tyo et al. 2006; Vidal-Mas et al. 2001). Tyo et al. (2006) showed that ionic strength of the dilution buffer influenced staining efficiency and recommended to use deionized water as dilution buffer instead of physiological saline solution (0.9 % NaCl) to improve signal to noise ratio. The authors also mentioned the need for membrane permeabilization for specific bacterial species, which reduced viability. Interestingly, they also showed that the optimal concentration of NR is species-dependent potentially because of differences in PHA contents. Protocols vary with concentrations between 3 and 100 μM (Herrera et al. 2002; Tyo et al. 2006) and incubation times ranging from 10 to 30 min at room temperature (Gorenflo et al. 1999; Tyo et al. 2006) (Table 1). Characteristics of the functional dyes are also summarized in the supplementary information (Supplementary Tables S3–6).

Similar to the cell-permeant nucleic acid dyes, we first assessed staining kinetics for the different dyes summarized in Table 2. For most dyes, it was found that dye uptake was time-dependent but after fluorescence intensity maximized, it remained stable until the end of the measurement (40 min). In contrast to the other functional dyes tested, HE showed a time-independent uptake in the Gram-positive population (Supplementary Fig. S9). For $DiBAC_4(3)$, the minimal

Table 2 Overview of the results obtained for time measurements of the different DNA and functional stains

Stain	Dihydro-ethidium (HE)	$DiBAC_4(3)$	cFDA-SE	cFDA	Nile Red	SYTO59	SYTO60	SYTO61	SYTO62	SYTO63	SYTO64	SYTO17
Time of maximum fluorescence (in min) at 37 °C												
Gram +	40	22	38	21	10	0	0	0	0	0	–	0
Gram −	42	22	26	30	5	15	15	15	15	15	–	15
Stability (in min) at 37 °C												
Gram +	40	40	40	40	40	30	30	30	30	30	–	30
Gram −	42	40	40	40	40	30	30	30	30	30	–	30
Concentration												
For both strains (in μM)	5	1	0.2	10	0.3	5, 0.5, and 0.05	5, 0.5, and 0.05	5, 0.5, and 0.05	5, 0.5, and 0.05	5, 0.5, and 0.05	5, 0.5, and 0.05	5, 0.5, and 0.05
Analysis												
	Superoxidegeneration(ROS)	Lost membrane potential	Active cells	Active cells	Polyhydroxy-alkanoates	DNA	DNA	DNA	DNA	DNA	DNA	DNA
Kinetics												
Time-dependent												
Gram +		√	√	√								
Gram −	√	√	√	√	√	√	√	√	√	√	/	√
Time-independent												
Gram +	√				√	√	√	√	√	√	/	√
Gram −												

incubation time was 22 min regardless of the type of bacteria. For the other functional dyes, a difference could be noticed between the Gram-positive and Gram-negative bacteria. Incubation times for the Gram-positive *L. brevis* LMG 18022 are 21, 38, 24, and 10 min for cFDA, cFDA-SE, HE, and NR, respectively. For the Gram-negative *C. metallidurans* CH34, the incubation times are 30, 26, 30, and 5 min for cFDA, cFDA-SE, HE, and NR, respectively (Supplementary Figs. S9 and 10). Dye concentration was determined by literature review and titration. The optimal stain concentration was chosen based on maximized signal to background distinction (Supplementary Fig. S11). A concentration of 1, 10, 0.2, 5, and 0.3 μM was chosen for $DiBAC_4(3)$, cFDA, cFDA-SE, HE, and NR, respectively. The use of EDTA was evaluated for all functional stains with the exception of DiBAC4(3) as EDTA can alter membrane permeability and thus impact membrane potential. Results showed an important increase in background fluorescence because of leakage for cFDA and cFDA-SE. For HE and NR, the use of EDTA did not improve staining significantly (data not shown). The addition of EDTA with any functional dye is therefore not recommended as it will impact the measurement. The fluorescence stability and the similar incubation conditions enable the combination of different dyes with seemingly incompatible staining protocols despite the different minimal incubation times.

Stain combinations

Combining different stains offers the possibility to simultaneously assess different physiological states of bacteria within a population, thereby improving understanding of bacterial behavior within a specific condition (Nebe-von-Caron et al. 2000; Nielsen et al. 2009; Rezaeinejad and Ivanov 2011). Several studies used and described double and triple staining protocols in order to determine different functional properties of a bacterial community (Hewitt et al. 1999; Johnson and Spence 2010). A widely used stain combination is available in the commercialized Live/Dead® BacLight kit (Thermo Fisher Scientific, USA), which uses a combination of SYTO 9 and PI to distinguish intact "live" cells from permeabilized "dead" cells. This kit has been used in numerous studies (Alonso et al. 2002; Dalwai et al. 2006; Lawrence et al. 1998; Leys et al. 2009; Mah et al. 2003; Vriezen et al. 2012). Table 3 gives an overview of genuine double and triple staining protocols and their applications. For these combinations, many multicolor protocols have been optimized in which dyes are separately added to different technical replicates of the same sample. This is different from a genuine multicolor setup in which dyes are added to the sample simultaneously.

In order to combine different dyes, it is important to choose dyes that possess the right spectral properties, to determine the incubation time, incubation conditions, and dye concentration for each fluorescent probe separately, and to assess possible interference. When combining stains, one of the most common issues is overspill, which is a consequence of the spectral characteristics of the dyes. To resolve overspill, other dye combinations can be made or compensation can be applied. A second type of interference, also related to the spectral characteristics of the dyes, is fluorescence resonance electron transfer (FRET). In this case, the emission of one dye (donor) is absorbed by a second dye (acceptor) in close proximity. As a consequence, the fluorescence intensity of the donor decreases (quenching) and the fluorescence intensity of the acceptor increases (Horváth et al. 2005). Besides FRET, the matrix can also cause fluorescence quenching and decrease in fluorescent intensity. Each dye combination behaves differently within a certain combination of medium matrix, microorganism, or instrument and needs to be tuned to determine possible compensations and obtain reproducible results (Hyka et al. 2010; Tracy et al. 2010). To test the feasibility of 'true' triple staining protocols, stain combinations were chosen on the basis of spectral characteristics. A green fluorescent stain ($DiBAC_4(3)$, cFDA, and cFDA-SE) was combined with an orange fluorescent stain (HE, NR) and a red fluorescent DNA counterstain (SYTO 60). Several important parameters such as distinguishable populations, total cell concentration, interference between stains, and overspill in other channels were evaluated and a gating protocol was established. This has been further illustrated in Fig. 2.

Four different *C. metallidurans* or *L. brevis* suspensions were made (i.e., a heat-killed, a peroxide-exposed, a stationary phase and a mixed population), which were subsequently stained either with a single stain or all combinations of double and triple stains. This approach was necessary to determine the appropriate compensation, thresholds, and gating. For all triple stain combinations, four populations could be identified with flow cytometry. The threshold and the number of events detected were always affected when stains were combined because of the increased background to signal ratio as a result of spectral overlap and compensation. (Supplementary Table S7). In addition, compensation was necessary for all green and orange fluorescent dyes, as all caused overspill in the other fluorescent channels. $DiBAC_4(3)$ caused the most spillover and a compensation of more than 100 % was necessary making this dye unsuitable in combination with NR and HE. Both cFDA and cFDA-SE showed to be more suitable for triple staining. Combinations with NR required a 25 and 15 % compensation in combination with cFDA and cFDA-SE, respectively. This is slightly higher than for HE, which required a 9 and 13 % compensation in combination with cFDA and cFDA-SE, respectively. SYTO 60 did not require compensation and allowed to distinguish cells from the background as well as correcting for false positive and false negative events. Because of this, any combination of a functional dye was possible with SYTO 60, resulting in more reliable population counts.

Table 3 Overview of double and triple combination of stains, setup of combinations and subject in which they were used

Stain	Setup	Subject	Reference
Hexidium iodide/cFDA	Double stain	Microbial fermentation	(Hewitt and Nebe-Von-Caron 2001)
cFDA/PI/DiBAC$_4$(3)	Double cFDA/PI single DiBAC$_4$(3)	Bile salt stressed bifidobacteria population	(Amor et al. 2002)
cFDA/PI	Double	Heating, freezing, low pH, or bile salt exposed cells	(Bunthof et al. 1999)
cFDA/PI	Double	Gastro-intestinal stresses on probiotic bacteria	(Chen et al. 2012)
Rh123/DiBAC$_4$(3)/PI/SYTO13/SYTO17	Double PI/SYTO13 single other stains	Gramicidin, formaldehyde, and surfactants effects on *Escherichia coli*	(Comas and Vives-Rego 1997)
SYTO9/PI + cFDA/Hoechst3342/ resazurine/SYTOX green	Double	Growth phase-related changes in *Bacillus cereus*	(Cronin and Wilkinson 2008b)
Hexidium iodide/cFDA	Double	Activated sludge bacteria	(Forster et al. 2002)
PI/DIOC$_6$	Double	Starvation response in *Bacillus licheniformis*	(da Silva et al. 2005)
Rh123/DiBAC$_4$(3)/PI	Double Rh123/PI and DiBAC$_4$(3)/PI	Starvation survival in seawater of *Escherichia coli* and *Salmonella typhimurium*	(Lopez-Amoros et al. 1995)
Thiazole orange(TO)/PI/ DiBAC$_4$(3)	Double TO/PI and single DiBAC$_4$(3)	Characterization of bacteria in microbial fuel cells	(Matos and da Silva 2013)
SYBR green/EB/ DiBAC$_4$(3)/PI	Double SYBR green/PI triple SYBR green/EB/DiBAC$_4$(3)	*Pseudomonas fluorescens* DR54 biocontrol	(Nielsen et al. 2009)
PI/cFDA/ DiBAC$_4$(3)	Double PI/cFDA single DiBAC$_4$(3)	Acid stress of *Streptococcus macedonicus*	(Papadimitriou et al. 2007)
CTC/fluorescent-antibody /DAPI	Double CTC/fluorescent-antibody single DAPI	Enumerating of enterohemorrhagic *Escherichia coli* O157:H7	(Pyle et al. 1995)
cFDA/cFDA-SE/PI/ DiBAC$_4$(3)	Double cFDA/PI single DiBAC$_4$(3) and cFDA-SE	Fermentation of *Lactobacillus delbrueckii*	(Rault et al. 2008)
cFDA/PI	Double	Viability of *Escherichia coli* O157/H7 in river water	(Tanaka et al. 2000)
cFDA/PI/CTC	Double cFDA/PI and single CTC	Natural river water samples	(Yamaguchi and Nasu 1997)

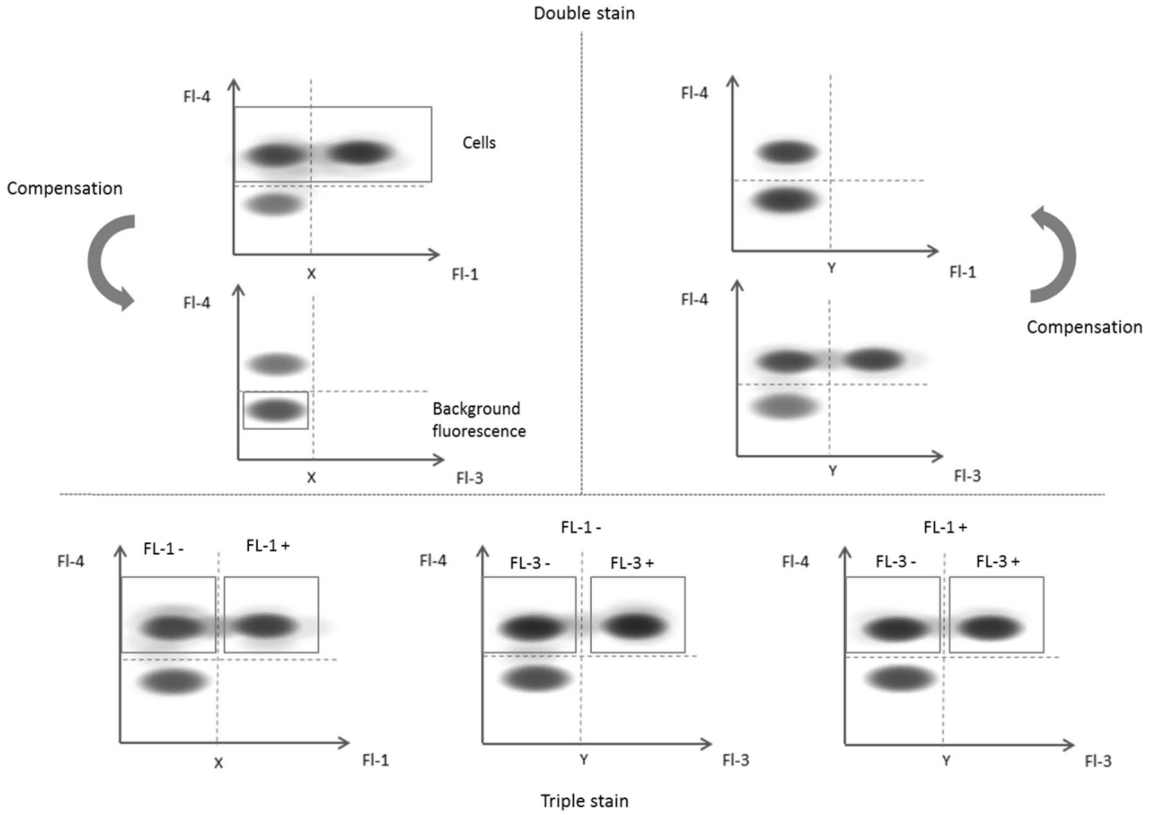

Fig. 2 Illustration of the gating protocol used to discriminate the four populations when using triple stains. The red-excitable nucleic acid stain SYTO 60 was used as a counterstain to differentiate cells from background. For the double stains with SYTO 60, first, both FL1 positive (*upper left*) and FL3 positive cells were gated versus FL4 (*upper right*). Then, both positive and negative FL1 and FL3 populations were gated in the opposite channel (e.g., the FL3 positive population was gated in the FL1 channel) to establish the necessary compensation. After the right gates and compensation were established, four populations could be distinguished with the triple stains: $FL1^+/FL3^+$, $FL1^-/FL3^+$, $FL1^+/FL3^-$, and $FL1^-/FL3^-$

It thus can be concluded that the simultaneous discrimination of certain physiological states is possible when protocols and staining conditions are optimized and appropriate compensation is set. The addition of a red-excited nucleic acid dye as counterstain reduces the background to signal ratio and improves the separation between the positive and negative populations.

Stability

An advantage of flow cytometry is the possibility to analyze samples in a high-throughput manner. The use of multiwell autoloaders has become very common and samples are often stained and incubated at the same time. However, this can lead to discrepancies as samples are not measured simultaneously. In that time frame, biological changes such as aggregation or physiological and chemical changes such as bleaching, dye extrusion, or intrusion can occur and significantly alter the results and subsequently affect the reliability and reproducibility (Hyka et al. 2010). Dye stability is therefore an important factor to take into account, particularly when measuring in high-throughput screening mode. Only Hammes et al. (2012) and Van Nevel et al. (2013) discussed this issue for the double SYBR green and propidium iodide staining. To assess the stability of the dye and the dye combination, Van Nevel et al. (2013) aliquoted a sample with a known concentration of cells in a multiwell plate after staining with their optimized staining protocol and monitored the mean fluorescence intensity and cell concentration of the detected populations. This way, the gradual changes in the results could be detected. They showed that SYBR green I staining is stable for at least 74 min, making this stain suitable for multiwell plates. On the other hand, they demonstrated that the combination of SYBR green and propidium iodide is less stable and that, albeit the number of cells in each population remained stable, the fluorescence intensity changed over time. As a consequence, care should be taken when many samples are analyzed in batch and when a fixed gating template (Prest et al. 2013) or flow cytometric fingerprinting (De Roy et al. 2012) is used to analyze the data. The authors also clearly mention that the dye stability is different when other dye concentrations or samples are tested and illustrate the usability of such tests to develop high-throughput assays.

We therefore investigated the stability of the previously described triple staining protocols (Supplementary Material,

Materials and Methods, Stability). A synthetic microbial community with different cell populations (e.g., dead, live, or oxidized cells) was aliquoted in a 96-well plate, stained, and incubated as previously described. Stability was assessed by comparing the number of cells in the pre-established gates of each sample. As all samples were measured consecutively and for a fixed time (1 min), the stability in time could be determined. For few triple stains, a satisfying degree of stability (10 % deviation in the cell counts per gate) for the different populations was found based on the analysis of a 96-well plate. While $DiBAC_4(3)$ is difficult to combine with both NR and HE, only combinations with cFDA and cFDA-SE are considered. As expected, both carboxyfluorescein stains impair the stability of triple stains due to the previously mentioned leakage. Only a maximum stability of 15 min was measured making these triple stain combinations unsuitable for high-throughput screening test requiring more than 15 min analysis time (Supplementary Material, Supplementary Table S8). Similar to the results found by Van Nevel et al. (2013), stability can be affected or even improved when other dye combinations or other dye concentrations are used.

Conclusion

Since the introduction of flow cytometry for research, it has been used to analyze cell populations through high-throughput single cell analysis. Numerous staining protocols have been developed for many applications. However, knowledge about the influence of the different methodological factors on the measurement, and its subsequent interpretation, is still lacking. This is important as they impact the reliability and reproducibility of a staining protocol. Few genuine multicolor protocols have been developed for microbiology and even fewer give an explanation why a certain concentration, incubation time and temperature were chosen or how stable the added fluorochromes were. Furthermore, few results have been published regarding the performance of multicolor protocols in multiwell assays.

We demonstrate how red-excitable SYTO dyes can be integrated as a counterstain for multicolor protocols. Moreover, we tested some functional dyes and showed that, like the SYTO dyes, the efficiency differed between the tested organisms, confirming that optimization is necessary for accurate functional measurements. For all tested dyes, we showed that their fluorescence intensity remained stable after fluorescence intensity maximized, offering the possibility to combine dyes with different minimal incubation times. However, we found that the combined dyes influence the results and that appropriate controls and compensation are crucial for a correct analysis. $DiBAC_4(3)$ was difficult to combine as it produced a lot of background fluorescence and spillover while cFDA and cFDA-SE were more suitable for combinations. Being excited by a red laser, SYTO 60 did not create spillover and was thus easily combined with other dyes. In general, the addition of a nucleic acid stain improved the results as background fluorescence could be more correctly assessed. Another parameter that influences stain performance is the dye stability during analysis, as the last sample of a batch analysis will not be analyzed at the same time as the first sample and may have undergone changes in biology (e.g., aggregation, sedimentation behavior, physiological changes) as well as in staining chemistry (e.g., bleaching or leakage). Both literature and our preliminary results clearly show that stability is important to ensure correct results.

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Author Contributions B.Byloos and B. Buysschaert contributed equally to this work. B. Byloos assessed the functional dyes while B. Buysschaert assessed the red-nucleic acid dyes and both B. Byloos and B. Buysschaert tested the stain combinations. R.V.H., N.L., and N.B. helped with data interpretation, scientific guidance, and preparation of the manuscript.

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Conflicts of interest The authors declare that there exist no conflicts of interest.

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