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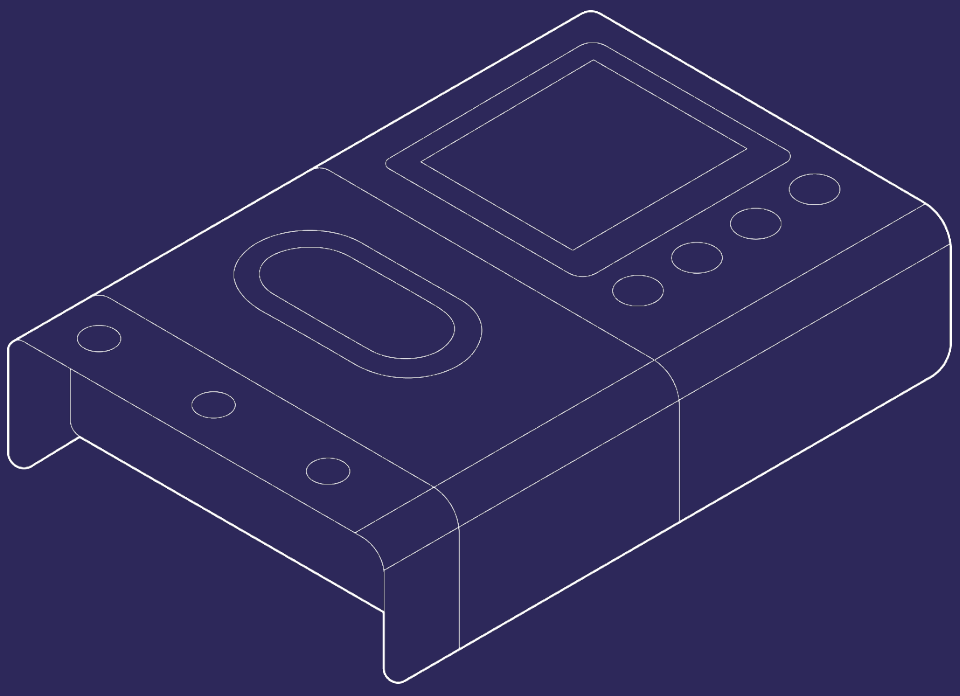
Application Note



Fluorescence Flow Cytometry vs BactoBox

Abstract

Fluorescence Flow Cytometry (FFC) is a versatile, culture-independent technique for measuring bacteria. FFC is capable of monitoring cell potency, metabolic activity, and membrane intactness for a heterogenous group of bacteria. Nonetheless, implementing FFC represents significant barriers in terms of price, training, and optimization of protocols to isolate end stain bacteria. Impedance Flow Cytometry (IFC) is a novel approach to flow cytometry for bacteria based on detection of intact lipid membranes. While being less versatile, IFC is stain independent and aimed at providing fast and actionable answers for bacterial concentrations in relatively crude samples. In this blog post prepared in collaboration with Probiotical S.p.A. and Advanced Analytical Technologies Srl, we show that IFC gives good agreement with FFC in terms of bacterial concentrations and live/dead ratios. IFC was recently introduced to the market in the form of the stand-alone instrument BactoBox®. At a fraction of the price and with very easy implementation, BactoBox strongly reduces the barriers for implementing precise and fast flow cytometric detection of bacteria. This unlocks real time measurements for control-in-process samples during probiotic production. In addition, easy access to reliable bacterial concentrations makes it possible to improve standardization of scientific experiments.

Keywords

Flow cytometry, impedance flow cytometry, BacLight™, fluorophore, fluorescence, stain, staining, probiotics, freeze-dried, cytometry, gating, propidium iodide, live/dead, viability, viable but non-cultureable, VBNC, membrane integrity, impedance spectroscopy, dielectric spectroscopy, flow cell, PAT, process analytical technologies, TFU, AFU.

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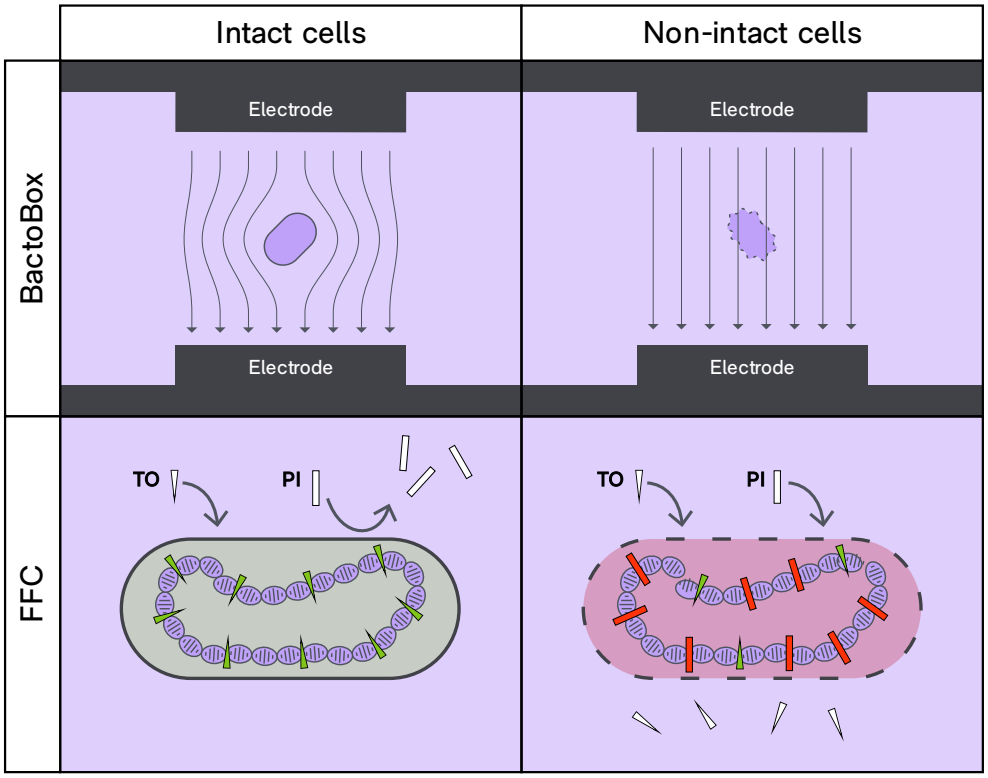
BactoBox is a novel approach to flow cytometry for bacteria

The principle of Flow cytometry relies on the possibility to study a single cell within an heterogenous population. Conceptually, fluorescence flow cytometry (FFC) and the technology employed in BactoBox, impedance flow cytometry (IFC), are very similar. Both techniques are cultivation-independent and detect cells moving in a flow of liquid. The main difference is the detector element. As illustrated below, BactoBox and IFC is based on a change in the electric field when an object passes between the electrodes in the measurement channel. By probing at several electrical frequencies, it is possible to distinguish bacteria with intact and non-intact lipid membranes. For FFC, detection is based on lasers that reveal fluorescence emission from fluorophore-tagged bacteria. A wide selection of stains for bacteria and FFC exist, but some of the typical fluorophores used for live/dead FFC rely on a combination of a stain that can penetrate virtually all cells (in this blog post, Thiazole Orange, TO) and a stain that will mainly enter cells with compromised membranes (in this blog post, Propidium Iodide, PI). When unbound, both stains display very low fluorescence intensity, but when bound to DNA/RNA the fluorescence intensity increases dramatically. TO will stain bacteria green-fluorescent, while PI will displace TO from DNA/RNA, and also quench TO-fluorescence. In effect, bacteria with compromised membranes will mainly be red-fluorescent, while the viable bacteria can be found by subtracting the dead bacteria from the total green cells.



Principles for detecting membrane intactness with BactoBox IFC and FFC: BactoBox exploits impedance flow cytometry at different electrical measurement frequencies to probe for intact membranes. An intact lipid membrane will impose a pronounced hindrance to electricity. On the other hand, a dead bacterium with a non-intact bacterial cell will be a good propagator of electricity. The BD Bioscience cell viability kit exploits stains and Fluorescence flow cytometry (FFC) to probe membrane intactness 1. Thiazole orange (TO) can penetrate intact cell membranes and upon binding to DNA, TO will fluoresce green upon excitation at 488nm. Propidium iodide (PI) on the other hand can only enter cells and bind to DNA if the membrane is compromised; PI will fluoresce red upon excitation at 488nm.

In essence, both techniques show if the membrane is intact. Membrane intactness is one of the typical indicators for bacterial viability. Worth to mention as other accepted general parameters in assessing bacterial viability are also i) the ability to reproduce as measured by e.g. colony forming units, and ii) indicators of active metabolism as measured by ATP-production, active enzymes, and respiration [2,3]. In addition to membrane intactness assessed by BactoBox and FFC with TO/PI staining, the present dataset includes reproduction as a parameter for viability. Each technique uses its own parameter set for definitions. For simplicity, in this blog post, the term “viable bacteria” is used to cover the three individual definitions of viability for FFC, BactoBox and plate counts:

- FFC: Viable (or active) bacteria are defined as bacteria with an intact lipid membrane that blocks entry of PI stain. Appropriate gating of the fluorescence channels is done to ensure proper differentiation of PI-positive and PI-negative bacteria. Total Fluorescent Units (TFU) reflect the total number of green-fluorescent bacteria while Active Fluorescent Units (AFU) reflect the TFU minus the PI-positive cells.
- BactoBox: The electrical properties are used to distinguish bacteria with intact and non-intact membranes. Intact membranes represent a hindrance (impedance) to electricity and therefore the presence of an intact membrane will lead to a delay for the electricity. Gating thresholds are based on previously recorded datasets of growth curves for bacterial monocultures. Total Particle Concentration (TPC) reflect the total particles detected from ~0.5-5µm while Intact Cell Concentration (ICC) reflects the proportion of bacteria that matches the phase shift properties for intact cells.
- Plate counts: The viability indicator is Colony Forming Units (CFUs), i.e. if a single (or small group) of bacteria deposited on an agar plate lead to a visible aggregate of cells then this is classified as one CFU.

Advantages with stain independent BactoBox analyses

Conceptually, staining and FFC appears simple and robust. But nonetheless, there are several pitfalls associated with the FFC method:

- DMSO affects permeability: The live/dead stains are typically solubilized in DMSO solvent, but it is well known DMSO can alter bacterial membranes and lead to overrepresentation of PI-positive (dead) cells [4,5].
- Stains affect membranes: The dyes themselves can also affect the physicochemical properties of the membranes by changing the fatty acid profile. Alteration of membrane lipids means can lead to alteration in dye uptake and therefore, potentially, higher dye concentrations can lead to biasing of the biological systems and overestimation of dead bacteria concentrations [6].
- Penetration-enhancers affect live/dead ratios: Sometimes, enhancers like the chelators EDTA and EGTA are needed to facilitate the entrance of dyes in difficult-to-stain bacteria. But EDTA can result in a higher proportion of PI-positive cells and therefore the exploitation of EDTA is not without pitfalls [4].
- Variability: Well-defined incubation times at specified temperatures are needed for the FFC method. Nevertheless, it is difficult to completely standardize incubation steps and holding time before FFC analysis. This leads to the risk of variations in dye permeability and fluorophore intensity over time.

With BactoBox the impedance flow cytometry principle is sufficient to detect the signatures of intact and compromised bacterial membranes. No stains or organic solvents are used. This makes the procedure faster and easier than FFC. Another benefit of BactoBox measurements is that the process is gentler to the cells (no wash/centrifugation needed), and there are no artifacts from stains and solvents. Therefore, conceptually, BactoBox IFC is less invasive than the FFC method, but in some cases a drawback of IFC can be that the information is mainly limited to membrane intactness and not species-specific information and/or probing of enzymatic activity, membrane potential, oxidation, antigens etc.

Head-to-head comparison of BactoBox and FFC for freeze-dried lactic acid bacteria

In collaboration with Probiotical S.p.A. and Advanced Analytical Technologies Srl we set out to explore the concordance between fluorescence flow cytometry (FFC) and impedance flow cytometry as measured by BactoBox. Main objective was to investigate agreement between intact cells. Secondarily, we aimed to investigate if there was a consistent live/dead ratio between the two measurement principles when using membrane intactness as a proxy for viability.

Four lactic acid bacteria strains were chosen for the study, namely *Lactobacillus plantarum* (Latto 1), *Lactobacillus casei* (Latto 2), *Bifidobacterium lactis* (Bifido 1) and *Bifidobacterium breve* (Bifido 2). The rationale for choosing these was that they are known to be difficult to enumerate with traditional cultivation-dependent methods, for example *L. casei* often has a high fraction of viable but non-cultureable (VBNC) cells while *B.*

breve is prone to aggregation with consequent underestimation in plate counts. Therefore, these strains represent cases where more precise methods are needed.

To better understand the variability within different labs and between different instruments, FFC was performed in six different labs using three different flow cytometer platforms. The BD FACS uses analogue technology, hydrodynamic focusing and reference beads as an internal standard to calculate absolute concentrations of bacteria. The Beckman Cytoflex is a digital instrument which also exploits hydrodynamic focusing but the absolute volumetric counting is done without counting beads as internal standard. Finally, the Thermo Fisher Attune NxT instrument uses acoustic focusing and reference beads as internal standard to calculate absolute concentrations of bacteria. All labs were adhering to the principles of ISO19344. IFC was done using the BactoBox® instrument. Measurements were performed according to the BactoBox protocols booklet using the general principles for freeze-dried powders, except that a stomacher was used for homogenization instead of bead beating.

Workflow for head-to-head comparison of FFC and IFC

The aim of this study was to directly compare two different analytical technologies, namely the stain dependent FFC method and the stain independent BactoBox method. Therefore, measures were taken to minimize variations from other technical contributors. The sample preparation and sample workup was performed in accordance with ISO19344 [7]. As shown in the workflow illustration below, the homogenized sample and sample workup was identical for BactoBox and FFC protocols. After the initial 1:10 dilution in peptone/salt diluent (PSD) and homogenization by the stomacher technique, the sample was split and subjected to both the FFC and BactoBox method. For the FFC method, the BD™ cell viability kit [1] or SYTO 24/PI staining (Life Technologies) were used in a 15 min incubation step, while for BactoBox measurements, the samples were simply diluted in standard diluent to reach the range of quantification from 10,000-5,000,000 total particles. Of note, the sample workup for FFC takes roughly 30 min for one sample, while the entire process for the BactoBox workflow can be completed within 12 min.

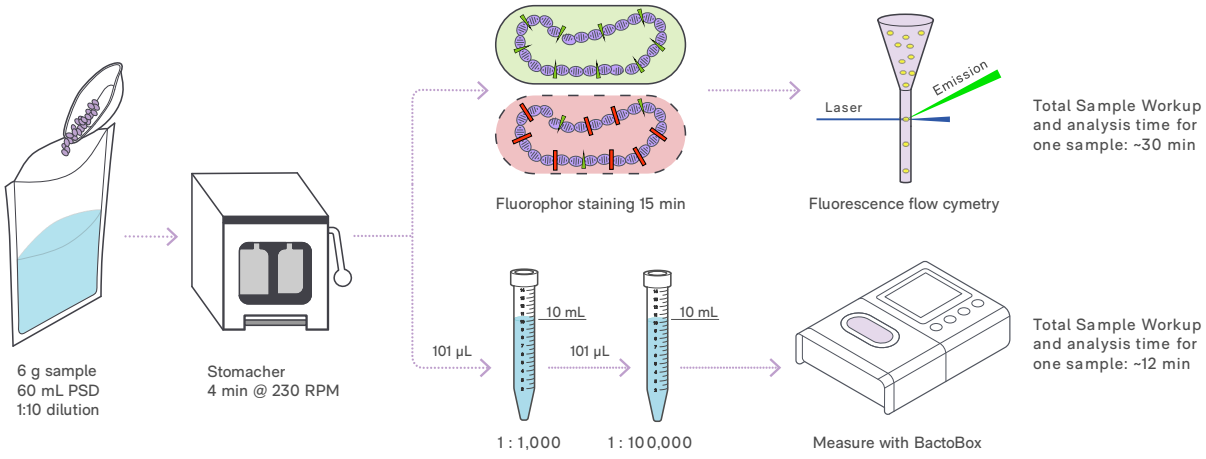
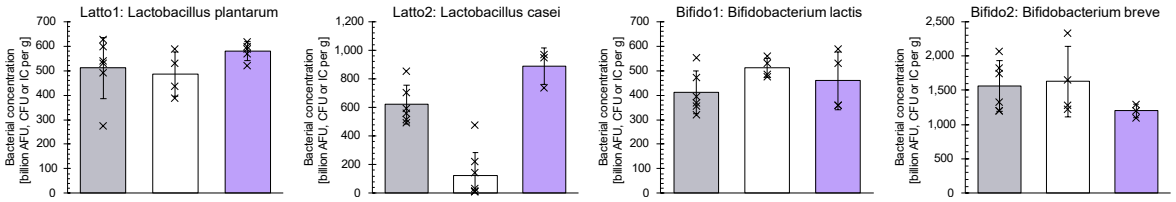


Illustration of sample workup: Initially, the freeze-dried bacterial powders were reconstituted in peptone/salt diluent and samples were homogenized by the stomacher technique. Subsequently the homogenized samples were subjected to both FFC and BactoBox sample preparation and measurement.

BactoBox and FFC viable bacterial concentration are in good agreement

The primary aim of the present study was to investigate the agreement between viable bacterial concentrations for stain based FFC and stain independent BactoBox measurements. As shown in the figure below, FFC (gray bars) and BactoBox (violet bars) match up relatively close when inspecting results for Latto1 and Bifido1. For Latto2 and Bifido2 the average concentrations are more spread, but still within the range of overlapping error bars. The variation in the FFC data is relatively high. It must be stressed here that the results represent of inter-lab comparison done at multiple sites by multiple operators and the variability can therefore be due to both sample workup and the analytical performance of the instrument. FFC performed within the same lab on the same sample and the same instrument will give much tighter error bars. BactoBox measurements was only done in one lab by a single operator, but because BactoBox measurements are practically operator-independent this is not expected to bias the results of the present study.

The plate count results (white bars) for Latto1, Bifido1 and Bifido2 are in good agreement with FFC and BactoBox. This is consistent with our other application note that compares BactoBox to CFU counts for actively growing cultures. Latto2 on the other hand shows considerably lower CFU's than AFU and ICC. It is interesting that three of the data points are in the 10^{11} range, while 5 data points are in the 10^{10} range. Probiotical S.p.A. have previously observed Latto2 plate count results to be highly variable and that freeze-dried preparation of this *L. casei* species is often associated with a high proportion of viable but non-cultureable (VBNC) cells. Both phenomena may explain the large discrepancy between AFUs, CFUs and IC's for Latto2 and clearly highlights that more precise analytical techniques like flow cytometry are necessary to complement traditional cultivation techniques.

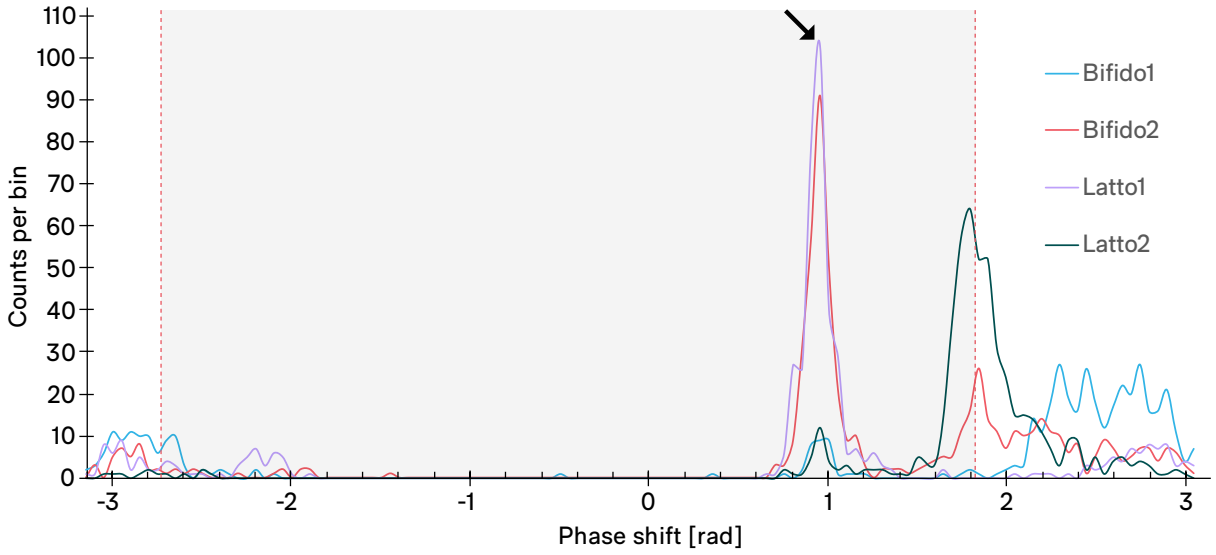


Viable bacterial concentrations are in good agreement when measured with FFC and BactoBox. The height of the grey bars represents average active fluorescent units (AFU) for the FFC method. The white bars represent plate counts (CFUs), and the violet bars represent BactoBox intact cell concentrations (ICC). Individual data points are shown with crosses. Error bars represent standard deviation.

Good agreement between non-intact particle concentrations when adjusting for spallation-induced microplastic particles.

FFC and BactoBox has several similarities, but there is one clear distinction in the fact that BactoBox detects all ~0.5 - 5 µm sized particles while FFC is gated to only detect DNA/RNA-containing objects that stain positive for either TO or PI. Therefore, BactoBox may detect some particulates that are not observed with FFC, e.g. other insoluble objects than live or dead bacteria. One of the parameters used to probe for membrane intactness is the phase shift at a 7 MHz electrical frequency. Phase shift is a concept from electrical engineering which is best explained as a "delay" of the electrical signal as it passes through objects, e.g. bacteria and other particles. The phase shift histogram below shows a BactoBox measurement for each of the four strains. The x-axis represents the phase shift properties, while the y-axis gives the counts for the objects with a given electrical phase shift.

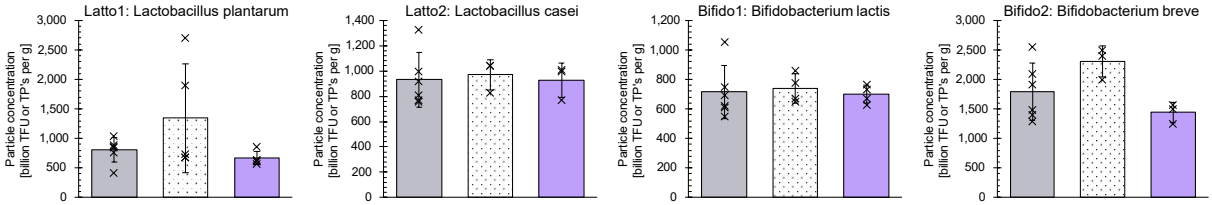
The gray region corresponds to objects with other particulates than intact cells. The white region corresponds to the intact cells. When inspecting Latto1 (violet curve) and Bifido2 (red curve) they both have a pronounced population at +1 rad (arrow). Usually, this phase shift corresponds to objects in the sample that are non-conductive like silica particles or polystyrene beads. But there is another source of non-conductive objects, namely the peristaltic pump in the BactoBox. A peristaltic pump drives flow by massaging peristaltic tubing with multiple rollers. These rollers will result in release of small rubber particles, a phenomenon known as spallation [8]. At low concentrations of bacteria, the measurement cycles are relatively long (2 minutes) and therefore the spallation-induced microplastic particles accumulate in the sample over time and will make up a non-negligible proportion of the particles in the sample. At higher concentrations of bacteria, the measurement time is shorter as statistical confidence for the concentration can be established more rapidly, and therefore relatively few microplastic particles will released from the pump and introduced to the sample. Practically, the contribution from microplastic particles will be negligible. This is the case for the Latto2 histogram where the +1 rad particles make up a much smaller proportion of the total particles detected.



BactoBox phase shift histograms reveal presence of likely microplastic particles released from the peristaltic tubing. X-axis represents the electrical properties of objects represented by the phase shift, while the y-axis shows the counts for objects with the respective phase shift properties. The grey region from -2.72 to 1.83 are standard classification limits for non-intact bacteria and other objects, while the inverted white region are standard limits for intact cells. Latto1 (violet curve) and Latto2 (red curve) have a higher proportion of +1 rad microplastic particles than Bifido1 and Latto2 (blue and green curve, respectively).

For Latto2 and Bifido1 the total fluorescent units (TFU, grey bars) from the FFC method and total particle concentrations (TPC, dotted bars) from BactoBox measurements are in good agreement. Contrarily, for Latto1, there is a pronounced variation in the data and BactoBox measurements show higher total particle concentrations than the FFC method. The TPC data for Latto1 fall in two categories; either the particle concentration is close to that of the TFUs (680, 690 and 740 billion TPC/g) or much higher (1,900 and 2,700 billion TPC/g). Curiously, the two high measurements were done at a dilution factor of 10 million, i.e. relatively low bacterial concentrations, while the three "normal" TPC measurements were done at a dilution factor of 1 million. This is consistent with the phase shift histogram above – when measurements are done at low bacterial concentrations the measurement durations are long and the contribution from the spallation-induced microplastic particles becomes substantial. The violet bar shows the average TPC, when the +1 rad population has been subtracted from the dataset; now the adjusted TPC (670 billion/g) is much more consistent with the TFU (800 billion/g) and the variation is also much lower. For Bifido2 all three BactoBox measurements were performed at the dilution factor 10 million, where the particle concentrations were relatively low and the contribution of spallation-induced microplastic particles is pronounced. Upon subtraction of the +1 rad population from the TPC's, the difference between FFC and corrected TPCs is smaller (20% difference after adjustment compared to 28% difference before adjustment).

Overall, the bar charts show a good agreement between FFC TFUs and BactoBox TPCs when adjusted for the irrelevant spallation-induced microplastic particles. A simple take home message from this is that it is advantageous to perform BactoBox measurements at >500,000 ICC's where the contribution from microplastic particles is negligible. Alternatively, the +1 population can be subtracted from the dataset using retrospective analysis in the free software BactoBox Explorer.



Total concentrations for FFC and BactoBox are in good agreement when adjusted for spallation particles. The height of the grey bars represents average total fluorescent units (TFU) for the FFC method. The dotted white bars represent BactoBox total particle concentrations as reported on the display, while the violet bars represent BactoBox total particle concentrations (TPC) when the likely microplastic particles have been subtracted from the total particles. Individual data points are shown with crosses. Error bars represent standard deviation.

Live/dead indicators for FFC and BactoBox are in relatively good agreement

The ratio between viable and total cells is a very useful indicator to evaluate the quality of a production batch, i.e. if this ratio is close to one, this indicates that virtually all cells are viable. For FFC, this information is available as the fraction of cells that are PI-negative relative to the total TO-stained cells. For BactoBox, the live/dead information is available by calculating the ratio between intact cells and total particles (given that particles are mainly originating from live and dead bacterial cells). The table below shows excellent agreement between Bifido1 and Bifido2, while there is more pronounced difference between the live/dead indicators for Latto1 and Latto2. As the TFUs and adjusted TPC were quite similar the difference must be associated with the determination of active and intact cells. It is possible that some viable bacteria are PI-permeant, but it is also possible that customized phase shift thresholds are needed to provide more precise classification of *Lactobacillus* species. Further investigation, e.g. by metabolic staining, is warranted to understand these differences in more detail.

Live/dead indicators from the FFC and BactoBox method are in good agreement. The FFC AFU/TFU row shows the ratio between active and total cells for the FFC method, while the subsequent row shows the analogous ratio for the BactoBox intact cells relative to the total particles (when adjusted for the irrelevant spallation-induced microplastic particles).

	Latto1	**Latto2**	**Bifido1**	**Bifido2**
FFC AFU/TFU	63%	67%	58%	87%
BactoBox ICC/TPC (excluding +1rad population)	87%	96%	66%	83%

Comparing FFC with IFC performed on a BactoBox

FFC is a great technique that makes it possible to interrogate heterogenous bacterial populations with sophisticated questions for membrane intactness, respiratory activity, membrane potential, antibody-binding, probing of specific bacteria in complex communities etc. But in most cases this information can be overkill when the subject of interest is to i) know the concentration of viable bacteria, and ii) get information about the quality of the sample e.g., in terms of the live/dead ratio. When it comes to these two parameters, the above results show that the FFC and BactoBox methods line up closely. As the case of Latto2 clearly illustrates, both FFC and IFC offer advantages relative to classical plate count techniques because the FFC and IFC precision is better, and the methods unlock live/dead information that is simply not achievable by cultivation-dependent techniques.

The table below summarizes the differences and similarities between FFC and IFC (BactoBox).

	FFC	**IFC (BactoBox)**
Attainable information	Enumeration of bacteria. Live/dead ratio. Membrane potential. Enzymatic and respiratory activity. Antibody-binding. Enumeration of specific bacteria, etc.	Enumeration of bacteria with intact membranes in fresh cultures. Live/dead ratio (when non-relevant events have been subtracted)
Time-to-result	Typically 15-30 minutes for freeze-dried powders depending on the stain and incubation requirements	Typically less than 12 minutes for freeze-dried powders
Footprint	Typically 50 x 50 x 100 (L x W x H) of space required for operation and ventilation.	Size of a lunchbox. 30 x 30 x 20 cm space required for operation. Can be operated within an anaerobic chamber.
Training requirements	Extensive.	Very little.
Investment costs	€ 50k-500k	Approximately € 10k

This comparison study has shown that there is an opportunity in using impedance flow cytometry instead of fluorescence flow cytometry if the information of interest is solely related to bacteria viability and live/dead ratios. The comparison study also shows that there is still progress and developments being made within the world of flow cytometry, and further development within this field of science promises to accelerate research and product development further for anyone working with cells on daily basis.

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