Two-colour fluorescence fluorimetric analysis for direct quantification of bacteria and its application in monitoring bacteria growth in cellulose degradation systems

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Highlights

- An assay for quantification of bacteria in mixtures containing insoluble substrates
- The assay uses SYBR Green I and propidium iodide dual staining with fluorimetry
- DNA standards were used to define an equivalent fluorescence DNA (EFD) unit
- Inter-lab/instrument comparisons are possible when the DNA standards are used

Abstract

Monitoring bacteria growth is an important technique required for many applications such as testing bacteria against compounds (eg. drugs), evaluating bacteria composition in the environment (eg. sewage and wastewater or food suspensions) and testing engineered bacteria for various functions (eg. cellulose degradation). Traditionally, rapid estimation of bacterial growth is performed using spectrophotometric measurement at 600 nm (OD600) but this estimation does not differentiate live and dead cells or other debris. Colony counting enumerates live cells but the process is laborious and not suitable for a large number of samples. Enumeration of live bacteria by flow cytometry is a more suitable rapid method with the use of dual staining with SYBR I Green nucleic acid gel stain and Propidium Iodide (SYBR-I/PI). Flow cytometry equipment and maintenance costs however is relatively high and unavailable in many laboratories that may require a rapid method for evaluating bacteria growth. We therefore sought to adapt and evaluate the SYBR-I/PI technique of enumerating live bacteria cells for cheaper platform, a fluorimeter. The fluorimetry adapted SYBR-I/PI enumeration of bacteria in turbid growth media had direct correlations with OD600 ($p>0.001$). To enable comparison of fluorescence results across labs and instruments, a fluorescence intensity standard unit, the equivalent fluorescent DNA (EFD) was proposed, evaluated and found useful. The technique was further evaluated for its usefulness in enumerating bacteria in turbid media containing insoluble particles. Reproducible results were obtained which OD600 could not give. An alternative method based on the assessment of total protein using the Pierce Coomassie Plus (Bradford) Assay was also evaluated and compared. In all, the SYBR-I/PI method was found to be the quickest and most reliable. The protocol is potentially useful for high-throughput applications such as monitoring of growth of live bacteria cells in 96-well microplates and in assessing *in vivo* activity of cellulose degrading enzyme systems.

Keywords
Quantification of bacteria; Fluorimetry; SYBR Green; Propidium iodide; Cell density; Cellulose

1. Introduction

Monitoring bacterial growth is essential for assessing many microbial applications. Determination of bacterial cell numbers can be done by direct and indirect methods. The commonest and oldest methods for this are turbidimetric measurements (optical density at 600 nm, OD600) and viable (plate/colony) counts (Breed and Dotterrer, 1916, Koch, 1970). Turbidimetric measurements are indirect methods which are fast and usually preferred when a large number of cultures are to be counted. The readings obtained from these measurements are a representation of the cell numbers (Koch, 1970). However to obtain definitive numbers, the readings must be correlated initially with cell number determined by other means (eg. plate counts). Plate counts on the other hand gives a direct measure of viable cells within the sample. Direct enumeration by microscopy using Petroff-Hausser counting chambers can also be performed (Treuer and Haydel, 2011). The aforementioned methods are however not universally applicable due to various limitations. Turbidimetric methods are unreliable for direct enumeration of bacteria cells in media containing insoluble substances such as food and environmental samples. Unavailability of suitable culture media and low concentrations of viable bacteria are major limitations to plate counting. To get around these challenges, different approaches based on the use of fluorochromes have been devised to investigate microbial viability and density (Barbesti et al., 2000, Caron and Badley, 1995, Diaper et al., 1992, Foladori et al., 2010, Kaprelyants and Kell, 1992, Porter et al., 1996, Tamburini et al., 2014).

Fluorochromes used in staining and enumerating bacteria cells by flow cytometry are based on membrane integrity, DNA binding and energy transfer between the fluorochromes (Barbesti, Citterio, Labra, Baroni, Neri and Sgorbati, 2000, Gregori et al., 2001, Humphreys et al., 1994, Sgorbati et al., 1996). Barbesti et al (2000) demonstrated that when DNA is simultaneously stained by SYBR-I (membrane permeant) and PI (non-membrane permeant) there is a decrease in the fluorescence of SYBR-I and an increase in the fluorescence of PI. This is due to a strong energy transfer between the two fluorochromes which facilitates

discrimination between living and dead bacteria. This transfer is due to the extremely high quantum yield of DNA bound SYBR-I complex (~0.8, Molecular Probes Inc., USA) and the overlapping of its emission spectrum (Fig. 1) with the absorption spectrum of PI. As a result, the fluorescence of SYBR-I is 'quenched' by PI when stained with both. 'Dead cells' are regarded as cells with compromised membranes. The compromised membrane integrity allows both PI and SYBR-I to permeate the cells at which point such cells will fluoresce red (PI) when excited. 'Live cells' on the other hand allows only SYBR-I to permeate and when excited, fluoresce green.

Figure 1. Excitation and emission spectra of SYBR green I and propidium iodide from Fluorescence SpectraViewer, Life Technologies

Fluorescence has been used in quantitation for a long time. Although the technique has improved over time, a major challenge it faces is standardization and references for fluorescent measurements. A special issue on "Quantitative Fluorescence Cytometry: An Emerging Consensus" published by the journal *Cytometry* identified some of these challenges (Lenkei et al., 1998). Among them were **(1)** inter-laboratory comparisons (Purvis and Stelzer, 1998, Waxdal et al., 1998, Zenger et al., 1998), **(2)** instrumentation (Purvis and Stelzer, 1998, Wood, 1998, Wood and Hoffman, 1998), and **(3)** reagent and calibration standards (Gratama et al., 1998, Lenkei et al., 1998, Purvis and Stelzer, 1998, Schwartz et al., 1998, Shapiro et al., 1998, Wood and Hoffman, 1998, Zhang et al., 1998). A common response to these challenges has been the development of a fluorescence intensity standard (FIS), the MESF (molecules of equivalent soluble fluorochrome) for use in flow cytometry (Gaigalas et al., 2001, Schwartz et al., 2004, Schwartz et al., 2002, Wang et al., 2002). The MESF is based on a comparison between the number of fluorophores in two solutions, where one solution is a standard with known values. The standard is often a suspension of labelled microbeads. Although all these standardizations contribute to making the use of flow cytometry a better platform for enumerating live bacteria than OD600 and colony counting, there are some

challenges that do not favour its wide use. First, the flow cytometers cost relatively high and not available in many laboratories monitoring and enumerating bacteria growth. Secondly, the use and interpretation of flow cytometric data require special training and expertise. Furthermore, flow cytometers require regular servicing which is not available particularly in most developing regions such as sub-Saharan Africa. There is therefore a need to adapt the SYBR-I/PI principle of differentiating and enumerating bacteria for a simple and cheaper platform such as fluorimetry. As mentioned above, a major challenge for fluorescence measurement is the inability to make comparable fluorescence intensity measurements across laboratories and between different instruments. In response to this, a FIS based on DNA stained with SYBR-I and PI was also developed. This standard, like the MESF used in flow cytometry, is based on equivalency between the intensity of fluorophores in two solutions, one standard (known concentration(s) of DNA) and the other the unknown sample. An alternative method for semi-quantification of bacteria using the total protein content of the sample was also evaluated and compared with the two-colour fluorescence method.

As the world's fossil fuel reserves deplete, there is a growing need to develop sustainable fuels (Creutzig et al., 2015, Fulton et al., 2015, Nuffield Council on Bioethics, 2011). Solar electric, hydropower (hydroelectric, tidal and ocean thermal power) and geothermal appear are safe sustainable sources of energy. However, these sources are not practically useful in the long-distance transport sector, thus emphasizing the need for liquid fuels. The only sustainable source of liquid fuels appears to be plant derived biofuels. The United States (US) and the European Union (EU) have individually set targets to expand the production and use of biofuels. The Energy Independence and Security Act (EISA) of 2007 establishes that blending of renewable fuels (Biomass diesel and cellulosic biofuels) into transportation fuels in the US should increase from 9 billion gallons in 2008 to 36 billion gallons by 2022 (Van Dyk and Pletschke, 2012). Similarly, the EU has also projected biofuel supply in transportation fuels to reach 25% by 2030 (Himmel *et al.*, 2007; US EPA, 2013). Despite the potential of plant derived biofuels to meet these targets, large scale applications are currently problematic

due to the difficulties in converting plant biomass (mostly made of cellulose, hemicellulose, lignin, pectin, and protein) into bioalcoholic derivatives and biodiesel. Naturally occurring cellulose degrading microbes use a battery of multiple catalytic enzymes to hydrolyse cellulose. We have described applications of synthetic biology that expand the technical capabilities of engineering efficient cellulose degrading enzyme systems (Duedu and French, 2016, French et al., 2015, Lakhundi et al., 2016) making it potentially easier to develop an ideal biofuel producing microorganism (IBPM) (French, 2009). Characterization and fine tuning of microbial cellulose degrading systems requires reliable methods for monitoring growth of cells on cellulose as a main carbon source. Substrates used for such experiments (eg. avicel, paper and pre-treated plant materials) are generally insoluble. This makes the use of turbidimetric methods (eg. OD600) which are fast and suitable for screening large numbers of samples unreliable. Colony counting on the other hand is an arduous process. Dual staining of samples with SYBR-I and PI has been demonstrated as a useful method for quantifying bacteria in environmental and food samples using flow cytometry. The application of this method for fluorimetry has not been reported. Here, SYBR-I/PI dual staining was used to evaluate the cell content of cultures containing microcrystalline cellulose (avicel).

2. Materials and Methods

2.1 Reagents, equipment and sample preparation

The SYBR-I/PI protocol was evaluated using DNA and cell suspensions of *Escherichia coli* strains JM109 and DH5α and *Citrobacter freundii* strains ATCC8090 and SBS197. The SBS197 strain was obtained from the Biology Teaching Laboratory of the School of Biological Sciences (SBS), University of Edinburgh. *E. coli* and *C. freundii* were chosen because they are the hosts we use in our biomass degradation experiments (Lakhundi, Duedu, Cain, Nagy, Krakowiak and French, 2016). *E. coli* and *C. freundii* were grown in 100 ml Luria Broth (LB) overnight at 37°C, 200 RPM. The cells were centrifuged at 5000 x g for 10 minutes, the supernatant discarded and the pellet resuspended in 5 ml 1 x PBS. Human genomic DNA

(200 ng μl^{-1}) was obtained from Bioline, London, UK and used as a calibration standard. Additionally, plasmid DNA was extracted from *E. coli* JM109 harbouring the BioBrick plasmid, pSB1A2-BBa_K523025 using the QIAGEN plasmid midi kit (QIAGEN GmbH, Hilden, Germany) according to the manufacturer's instructions. Dilutions of the DNA were made in nuclease free water and stained with the fluorochromes. Propidium iodide (1mg ml^{-1} stock solution) and SYBR-I (10,000 X concentrate) were obtained from ThermoScientific, Eugene, OR, USA. SYBR-I dilutions were made using Pierce™ Dimethylsulfoxide (DMSO) (ThermoScientific, Rockford, IL, USA). Fluorescence measurements were taken using the Modulus™ Single Tube multimode reader (P/N 998-9203, Turner BioSystems, Sunnyvale, CA, USA).

2.2 Fluorescence staining of cells and DNA

One ml of diluted DNA or cell suspension was stained with 10 µl each of SYBR-I (1:30 dilution of commercial stock) and PI in a cuvette (FisherBrand FB55147, Fisher Scientific, Loughborough, UK). The samples were covered with parafilm, mixed by inverting about 6 times and incubated in the dark for 15 minutes at room temperature.

2.3 Fluorescence measurement and energy transfer

Fluorescence was measured using the blue (P/N 9200-040, λ_{ex} = 460 nm, λ_{em} = 515 - 570 nm) or green (P/N 9200-042, λ_{ex} = 525 nm, λ_{em} = 580 - 640) Modulus™ fluorescence kits (Turner BioSystems, Sunnyvale, CA, USA). Measurements obtained from the instrument were given in fluorescence units (FSU). To convert the FSU to a FIS which can be compared between instruments and across labs, a standard was generated using dilutions of DNA stained with either SYBR-I, PI or both. For each standard 10 ml was prepared. Aliquots of 1 ml were transferred to labelled cuvettes in triplicates for SYBR-I, PI and SYBR-I/PI staining. All samples were measured individually in both the red and green fluorescence channels. Energy transfer from SYBR-I to PI was evaluated.

2.4 Discrimination and semi-quantification of cells

To assess the discriminatory capacity of the protocol, various degrees of damage were induced in the *E. coli* and *C. freundii* cells. To induce damage, 500 µl of cell suspension was ultrasonicated at 10 µm (amplitude) for various intervals. The MSE Soniprep 150 Plus Ultrasonic Disintegrator (MSE (UK) Ltd, London, UK) with the exponential probe was used to sonicate cells. Cell damage was assessed by performing total protein quantification on 100 µl of each sample using the Pierce Coomassie plus (Bradford) assay kit (ThermoScientific, Rockford, lL, USA). The ultrasonicated samples were then stained with SYBR-I, PI or both. Measurements were taken for all samples in both the green and red fluorescence channels.

2.5 Determination of how presence of cellulose particles affects fluorescence

To assess the effects of addition of cellulose on fluorescence, 20 mg of cellulose powder (20 µm microcrystalline powder, Sigma Aldrich, Irvine, UK) was added to cell suspensions. Two sets of cell suspensions were prepared at different cell densities. The turbidity of cells was determined by measuring the absorbance at 600 nm using the absorbance module (Model E6076, GLOMAX MultiJR, Promega, Southampton, UK) with the Modulus™ reader. To one set of cells, cellulose powder was added and the turbidity re-measured. Both sets of samples were stained in parallel with SYBR-I/PI and the fluorescence determined as described above.

2.6 Total protein assay

The Pierce Coomassie plus (Bradford) assay was used to determine the total protein concentration in cells. Reactions were set up in 1 ml volumes containing 100 µl of sample and 900 µl of Bradford reagent and mixed. To determine release of soluble protein from sonicated cell suspensions, cells were removed by centrifugation (10,000 g, 10 min) and 100 microliters of supernatant was assayed. The reaction mixture was incubated at room temperature for 10 minutes according to the manufacturer's instructions after which absorbance was measured at 600 nm using the Modulus™ reader. To measure total protein content of intact cells, 100 µl

of the cell suspension was added to 900 µl of Bradford reagent, mixed and incubated at 65°C for one hour to lyse the cells. The samples were allowed to cool for one hour at room temperature during which time cellulose particles settle at the bottom that so they do not interfere with the absorbance measurements. Absorbance values were converted to protein concentration by comparing with a standard curve prepared with dilutions of the 2 mg ml^{-1} bovine serum albumin (BSA) included in the kit.

2.7 Statistical analysis

Microsoft Excel was used for data entry, organization and generation of graph plots. Statistical tests were conducted in IMB SPSS version 21. Correlations and tests for linearity between fluorescence and DNA concentration or cell density were analysed using Pearson's product-moment correlation tests. To determine whether fluorescence depended on cell density or degree of damage, Kendall's rank correlation (tau b) was used. All experiments were performed in at least three replicates and the means plotted with standard error values.

3. Results

3.1 Staining characteristics and energy transfer

Both stains fluoresced in either channel due to the overlap between their absorption and emission spectra. Furthermore, fluorescence values recorded for SYBR-I were higher than those of PI for the same sample. SYBR-I fluorescence in the green channel was 10.06 % (SD = ± 2.16) that of the fluorescence recorded from the red channel. Fluorescence of propidium iodide in the green channel was 0.34 % (SD = ±0.25) of that in the red channel (supplementary information figure 1). Background fluorescence of PI is not likely to affect fluorescence of SYBR-I in the green channel but that of SYBR-I will potentially affect the fluorescence of PI in the red channel.

Energy transfer was assessed after compensation for SYBR-I and PI emission in the red and green channels respectively as described by Barbesti et al (2000). The energy transfer from SYBR-I to PI was evaluated as a decrease in green SYBR-I fluorescence and increase in PI fluorescence of DNA samples stained with both fluorochromes as compared to the same samples stained with only one of the two fluorochromes. Reduction of SYBR-I fluorescence was 98.6 % (SD = ±0.18) whereas increase in PI was 120.2 % (SD = ±15.5) indicating a strong discriminatory capability (table 1 and figure 2).

Table 1: Energy transfer from SYBR-I to PI

Figure 2: Differences between single and dual staining of DNA

3.2 Definition of an equivalent fluorescent DNA (EFD) unit

A fluorescence intensity standard was developed based on the fluorescence of DNA stained with either of the two fluorochromes to enable comparison of fluorescence across laboratories and instruments. An equivalent fluorescent DNA (EFD) unit was defined as the amount of fluorescence obtained from staining 1 ng μl^{-1} of DNA for 15 minutes at room temperature (25°C). A best fit line obtained for each stain was used to determine the EFD unit for that particular stain after compensating for fluorescence of SYBR-I and PI in the red and green channels (figure 3a).

3.3 Assessment of single fluorescent staining of bacteria cultures and their discriminatory capability

Quantification of bacterial cells was assessed first by staining dilutions of *E. coli* suspensions with single fluorochromes. There was no correlation between cell density (OD600) and the fluorescence obtained when PI alone was used to stain cells (figure 3b). On the other hand, a strong correlation (R^2 = 0.988) between cell density and fluorescence was obtained with

SYBR-I alone. Although SYBR green I is a green fluorescent stain, red fluorescence was detected from cultures stained with SYBR green I. This fluorescence was however 3 to 6 fold lower than the green fluorescence detected.

To investigate discrimination of live and dead cells, we needed a system where specific degrees of cell damage could be attained. Ultrasonication of cell suspensions for increasing duration of time gave increasing extracellular protein concentrations indicating release of protein from cells whereas the cell density measured by absorbance at 600 nm slightly decreased (figure 3c). Complete lysis of all cells by incubating at 65 °C for one hour post-sonication resulted in protein concentrations levels that were similar to each other irrespective of the duration of sonication. (supporting information figure 2). To confirm that this damage also resulted in release of varying amounts of DNA, suspensions were stained with both SYBR-I and PI. Fluorescence increased with staining by both stains confirming that varying degrees of cell damaged had been achieved (Figure 3d).

Figure 3: DNA standard preparation and estimation of the EFD of damage-induced and non-damaged cells stained with single fluorochromes

3.4 Discriminating between live and dead cells by dual staining

To demonstrate that simultaneous staining of cells with both SYBR-I and PI can allow quantification of live and dead cells using fluorimetry, cell suspensions of *E. coli* JM109 and *C. freundii* NCIMB11409 were sonicated to induce various degrees of cell damage and stained with both SYBR-I and PI. The population of live cells as determined by dual staining decreased with increasing sonication duration whilst the dead cells increased (Figure 4a and 4b). There was a strong direct correlation between duration of sonication and the dead cell population for both *E. coli* (R^2 = 0.99; figure 5-8c) and *C. freundii* (R^2 = 0.99 for both *E. coli* and *C. freundii*). There was an inverse correlation between the duration of sonication and the live cell

295 population, (R^2 = 0.87, *E. coli*; R^2 = 0.76, *C. freundii*). Furthermore there were strong
296 correlations between the dead cell measurements and the extracellular protein released as a
297 result of sonication for both *E. coli* (R^2=0.98) and *C. freundii* (R^2=0.99) (supporting information
298 figure 3).
299
300 *Figure 4: Dual staining with SYBR-I and PI to discriminate live and dead E. coli and C. freundii*
301 *cells*
302

303 ***3.5 Effects of the presence of insoluble cellulose on turbidity of cell suspensions***

304 To determine what effects addition of an insoluble substance will have on the cell density
305 measurement (OD600), cellulose powder was added to cell suspensions after initial OD600
306 had been measured. As expected, the addition of cellulose resulted in high OD600 values
307 (supporting information figure 4). The values however did not correlate with the values
308 obtained before the addition of cellulose indicating presence of insoluble substances will lead
309 to inaccurate OD600 values. To investigate whether leaving cuvettes to stand for some time
310 to allow avicel to settle would improve the correlation between OD600 without and with avicel,
311 OD600 measurements were taken at 5 and 15 minutes after addition of avicel. The OD600
312 did not significantly change with longer standing time. There was still no correlation with the
313 original OD due to cells alone. The values for 15 minutes wait were however lower than the
314 values for 5 minutes as a result of settling of particles (supporting information figure 5). The
315 rate of decrease was similar for all the samples tested.
316

317 ***3.6 Detecting live and dead cells in the presence of cellulose***

318 To investigate the ability of the SYBR-I and PI dual staining to estimate the amount of cells
319 present in suspensions with cellulose, correlations between OD600 and fluorescence were
320 determined from *E. coli* and *C. freundii* cell suspensions with or without cellulose. The addition
321 of avicel did not significantly affect the fluorescence measurements (Figure 5 (a)). Pearson's

correlation was used to test whether there was any correlation between OD600 before addition of avicel and the live cells (green fluorescence) before and after addition of avicel. Strong correlations were obtained for all cells whether avicel was present or not (Table 2). To determine whether the fluorescence measured depended on the OD600, Kendall tau correlation test was performed (Table 2). The results showed that irrespective of the type of bacteria used or whether there was avicel or not, fluorescence depended on the OD600 before addition of avicel.

Table 2: Significance of correlations between OD600 and fluorescence in the presence or absence of cellulose

3.7 Assessing growth in the presence of cellulose by total protein estimation

Total protein was determined using the coomassie assay on samples following incubation at 65°C for one hour as described above. There was no significant difference between total protein for cells with or without avicel (p = 0.117 (*E. coli*) and 0.600 (*C. freundii*); figure 5 (b)). Significant correlations were observed between OD600 and the total protein of *E. coli* without cellulose (p = 0.001, R^2 = 0.960) and with cellulose (p = 0.003, R^2 = 0.923) as well as *C. freundii* without cellulose (p < 0.001, R^2 = 0.990) and with cellulose (p < 0.001, R^2 = 0.984). Total protein was statistically dependent on OD600 of cells (tau_b = 1, p ≤ 0.011).

4. Discussion

There have been tremendous advances in fluorescence cytometry (instruments, fluorophores and methods) over the years which enable direct analysis and quantification of bacteria and other cells in different environments (Foladori, Bruni, Tamburini and Ziglio, 2010, Gregori, Citterio, Ghiani, Labra, Sgorbati, Brown and Denis, 2001, Lebaron et al., 1998, Lenkei, Mandy, Marti and Vogt, 1998, Melamed et al., 1972, Nunez et al., 2004, Tamburini, Foladori, Ferrentino, Spilimbergo and Jousson, 2014). In this study, it has been demonstrated that a simple two colour fluorescence fluorimetric technique can effectively be used to monitor

growth of bacteria in turbid growth cultures including those containing insoluble (eg. cellulosic) substrates. This is essential for assessing *in vivo* activity of cellulose degrading enzyme systems for biofuel production (Duedu and French, 2016, Lakhundi, Duedu, Cain, Nagy, Krakowiak and French, 2016). The total protein assay evaluated in this study was also found to be suitable for this purpose. The fluorescence technique however has some advantages over the total protein technique. Whilst the fluorescence method can be completed in about 20 minutes, the total protein will be completed in not less than two hours. Additionally, the stains used in the fluorescence technique are not regarded as harmful by European Union regulations whereas the protein reagent is harmful. Another advantage of using the SYBR-I/PI method over the total protein is that it allows direct estimation of live and dead cells making it suitable for experiments where cell lysis is suspected. With an appropriate standard curve (eg. plate count or flow cytometry), the green fluorescence values can be converted directly to cell numbers.

Barbesti et al (2000) demonstrated that there is energy transfer from SYBR-I to PI in bacterial cells stained with both fluorochromes. This study has demonstrated that the energy transfer also occurs with DNA in solution but at a slightly higher percentage. This is expected as the fluorochromes readily bind DNA and do not need to cross barriers (eg. membranes) as occurs for cells (Lebaron, Parthuisot and Catala, 1998, Melamed, Adams, Zimring, Murnick and Mayer, 1972). The strong energy transfer facilitates discrimination of live and dead bacteria and it has been shown that it is not affected by the metabolic state (eg. stationary or exponential) or the type of bacteria (gram positive or negative) (Barbesti, Citterio, Labra, Baroni, Neri and Sgorbati, 2000). The discriminatory properties of the SYBR-I/PI dual staining have been shown to be very effective, with strong correlations demonstrated between dead cells (red fluorescence) and the degree of induced damage (sonication). This also means that the fluorimetric application of the SYBR-I/PI method can account not only for cells with compromised cell membranes as happens in flow cytometry but also for completely lysed cells. Flow cytometry will count cells meaning completely lysed cells will not be accounted for.

This makes the application of two colour fluorescence staining with fluorimetry applicable in a wider context.

Obtaining conditions necessary for the ideal measurement of fluorescence intensity is in practice very difficult (Gaigalas, Li, Marti, Henderson, Vogt and Barr, 2001). The development of the MESF unit is a practical approach that seeks to take the application of fluorescence cytometry from just enumeration to actual quantitation. With this unit, quantitative fluorescence data are no longer dependent on the instrument or the environment within which cells are present (eg. media) but are standardized and comparable with others (Schwartz, Gaigalas, Wang, Marti, Vogt and Fernandez-Repollet, 2004). Although the unit has not been evaluated on other fluorescence platforms such as fluorimeters, there are some foreseeable challenges. Fluorimeters do not count cells but rather give a value for the total fluorescence obtained. The fluorescence obtained is also dependent on the amount of DNA present which these stains (i.e. SYBR-I and PI) bind to. Thus, a recombinant organism harbouring a large piece of foreign DNA will likely produce higher fluorescence than the wild type. The equivalent fluorescent DNA (EFD) unit developed in this study leverages the principles of the MESF and the foreseeable challenges of its application in fluorimetry to best serve its purpose. The EFD value obtained for a sample can be directly compared to another sample of the same constitution provided the DNA standard and samples were measured on the same instrument. Rather than using a known quantity of standard beads, the EFD can be converted to a quantitation standard when cell quantities are standardized with plate counts or another appropriate measure.

Despite the advantages highlighted, the method does have some limitations. On its own, dual staining followed by fluorescence measurement does not give absolute quantification of cells. A standard curve must be generated, for example from plate counts or other suitable method, and used to estimate the number of cells from the fluorescence reading. Furthermore, the method is not suitable for directly comparing quantitation obtained from different bacteria, due

405 to its dependence on the amount of DNA present and inability to separate brightly and dimly
406 fluorescing cells.
407
408 **5. Conclusions**
409 Analysis of bacterial cells stained with SYBR-I and PI using a fluorimeter has been evaluated.
410 The application of this dual fluorescence staining technique in fluorimetry is simple, fast and
411 can be easily adopted for automation or large screening applications such as with 96-well
412 plates. With fluorimetry, dual staining of samples with SYBR-I and PI will detect not only cells
413 with compromised membranes but completely lysed cells as well. This property can be an
414 advantage or disadvantage depending on the application. The use of the EFD as a unit for
415 comparing fluorescence intensity in fluorimetry across laboratories and instrument platforms
416 has also been demonstrated. This standard is cheap and can easily be made, yet is suitable
417 for comparison with other samples or applications.
418
419
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522

Figures

Figure 1. Excitation and emission spectra of SYBR green I and propidium iodide from Fluorescence SpectraViewer, Life Technologies

Figure 2: Differences between single and dual staining of DNA

DNA was serially diluted and stained with either SYBR-I or PI alone and fluorescence determined. Fluorescence determination was performed in the red fluorescence channel for PI alone (a) and in the green fluorescence channel for SYBR-I alone (b). Dual stained DNA was measured in both the red and green fluorescence channels. Six replicates were performed.

Figure 3: DNA standard preparation and estimation of the EFD of damage-induced and non-damaged cells stained with single fluorochromes

A. Standard curves for DNA stained with each of the fluorochromes were made to develop the equivalent fluorescent DNA unit. DNA was serially diluted and the concentrations determined using the nanodrop 2000 (ThermoScientific, Wilmington, DE, USA). Diluted DNA was stained with either SYBR-I or PI and the fluorescence measured in the green or red fluorescence channels respectively. Plots represent six replicates of each dilution that was prepared and measured.
B. Cell suspensions were tested and compared with the DNA standard curves to determine the EFD. Cell suspensions were prepared by resuspending pellets from overnight cell cultures. The OD600 was predetermined and the pellets were serially diluted and stained with either SYBR-I or PI. Two biological replicates (each made up of three technical repeats) were performed.
C. Cell damage was confirmed by determining the total protein concentration of the cell suspensions. Cell suspensions were ultrasonicated to induce various degrees of cell damage. Suspensions were centrifuged to remove debris and non-lysed cells and the

supernatant collected and tested. The total protein was determined using the Bradford assay.

D. Fluorescence and total protein were determined for cell suspensions sonicated to induce various degrees of membrane damage. Fluorescence of PI as a result of cell damage determined by total protein was compared.

Figure 4: Simultaneous staining with SYBR-I and PI to discriminate live and dead E. coli and C. freundii cells

Discrimination of live and dead cells was achieved by simultaneously staining cell suspensions of *E. coli* and *C. freundii* that have been damaged to various degrees. Live cells were obtained as the fluorescence in the green channel whereas dead cells were obtained as the fluorescence in the red channel. Three biological repeats were performed.

Figure 5:

(a) Simultaneous staining is sufficient to discriminate and quantify live and dead cells in the presence of insoluble cellulose

Cell suspensions of different densities were used. Fluorescence measurements were obtained for cells prior to and after addition of avicel. Cell suspensions of *E. coli* (a and b) and *C. freundii* (c and d) were used. Three biological repeats were performed.

(b) Total protein estimation to estimate growth in the presence of cellulose

Total protein were obtained for cell suspensions prior to and after addition of avicel. The plots show the original OD600 (i.e. before addition of avicel) versus the total protein with and without avicel. Three biological replicates were performed.

Tables

Table 1: Energy transfer from SYBR-I to PI

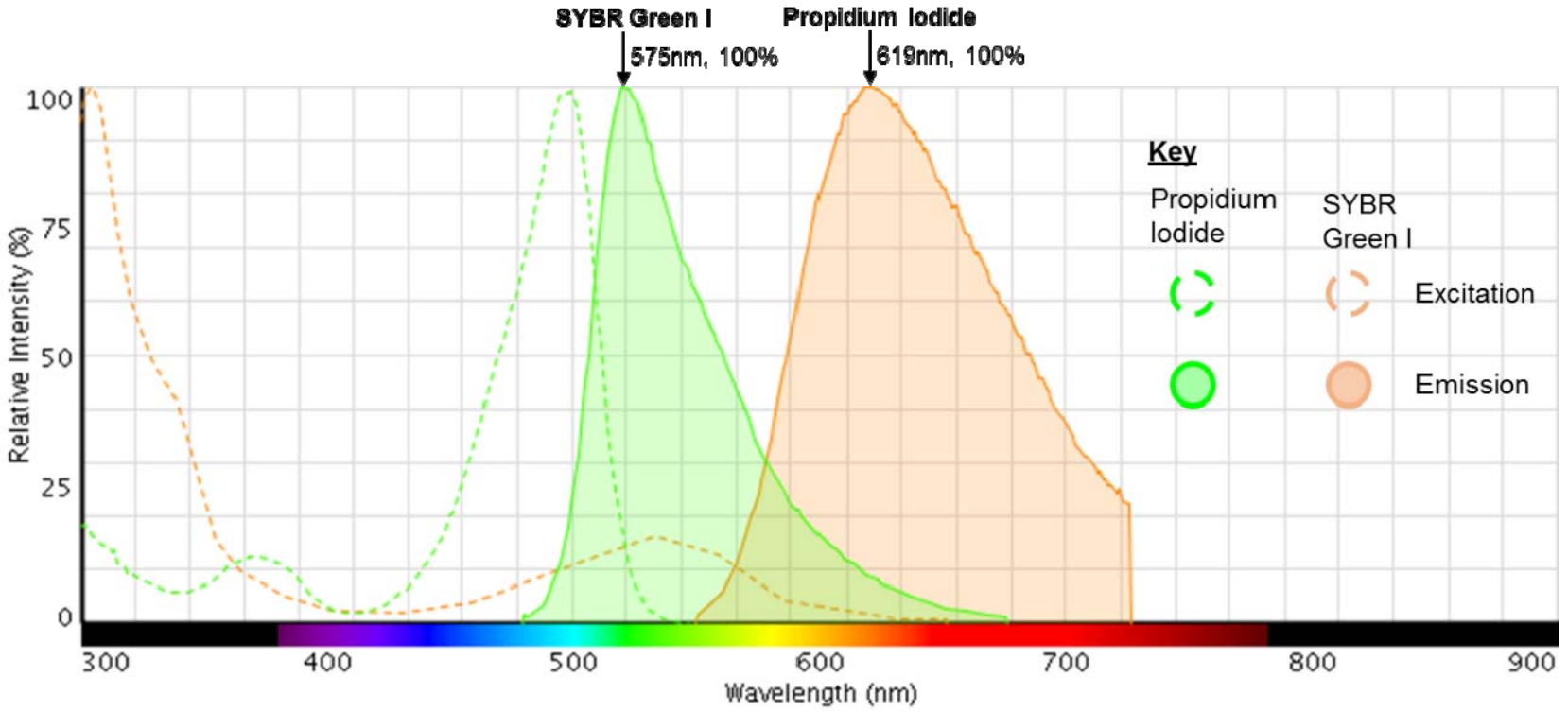
Amount of DNA (ng µl^{-1})	SYBR-I decrease (%)	Standard Deviation (±)	PI increase (%)	Standard Deviation (±)
10	-99.0	0.27	146.7	23.31
15	-99.8	0.07	121.9	17.84
20	-99.4	0.14	98.4	31.13
25	-99.7	0.11	115.6	31.60
30	-99.4	0.13	118.6	37.61

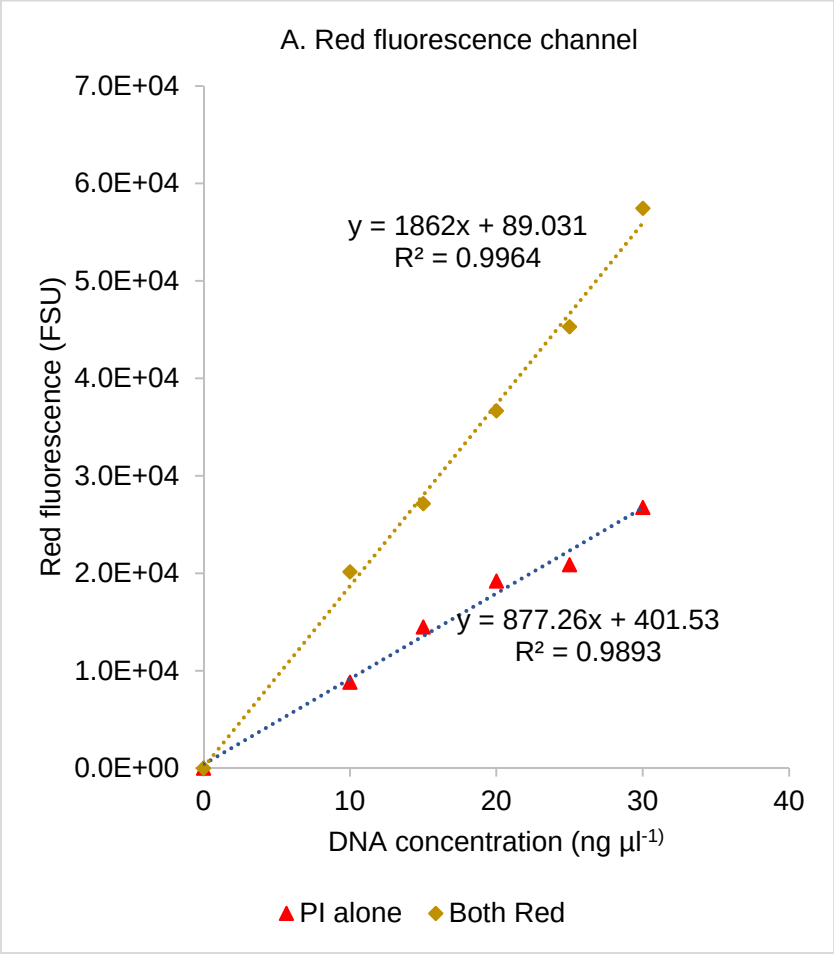
Values are means of three measurements. SYBR-I fluorescence was almost quenched when DNA was stained with both stains.

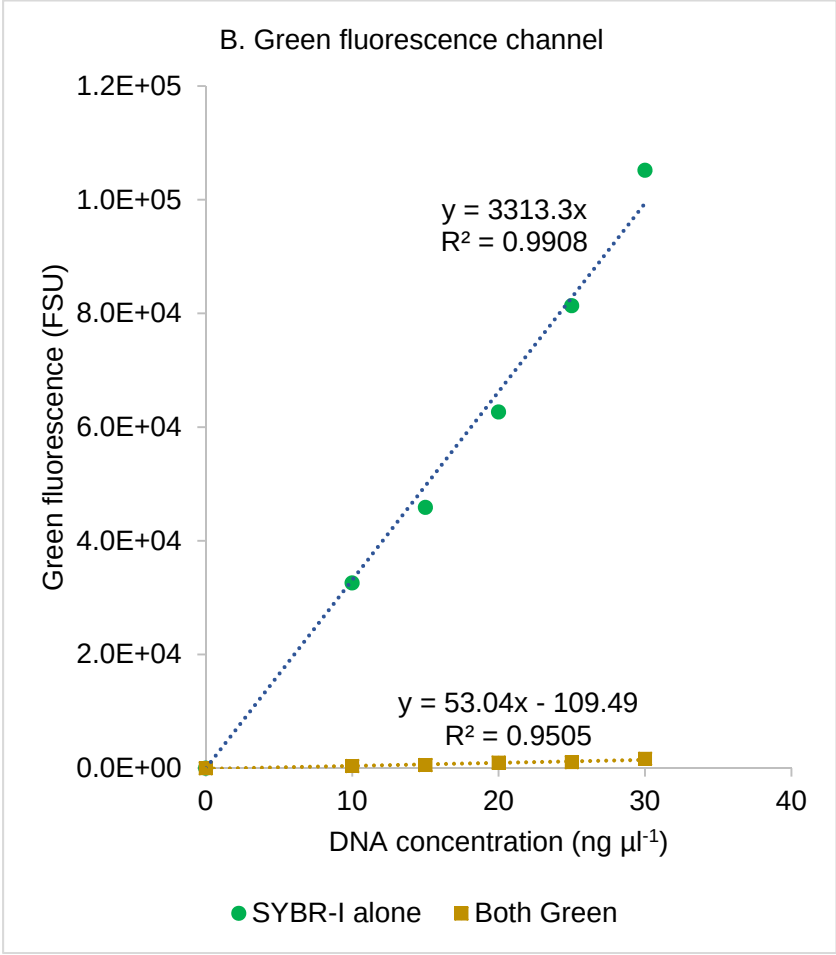
Table 2: Significance of correlations between OD600 and fluorescence in the presence or absence of cellulose

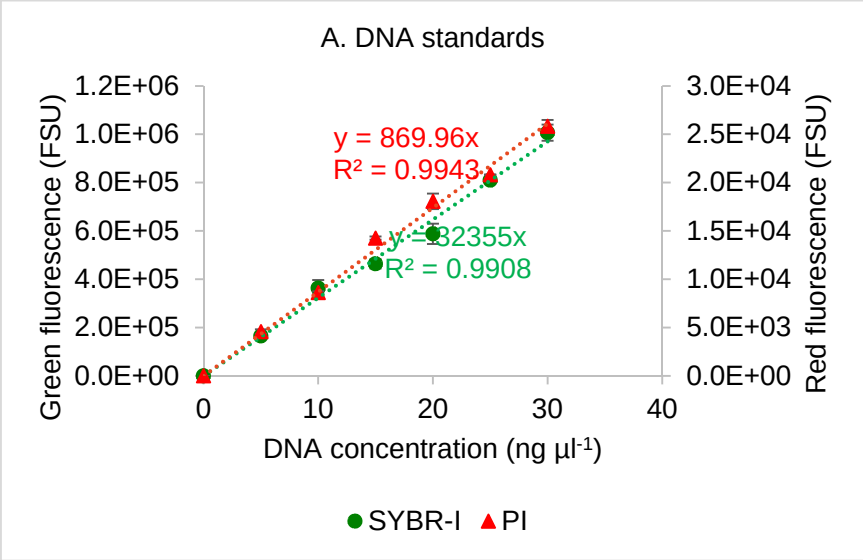
		Pearson Correlation	Kendall's tau_b Correlation
JM109	Correlation Co-efficient	0.995**	1.000**
	Sig. (2-tailed)	0.000	0.000
JM109+avicel	Correlation Co-efficient	0.990**	1.000**
	Sig. (2-tailed)	0.000	0.000
NCIMB	Correlation Co-efficient	0.978**	1.000**
	Sig. (2-tailed)	0.000	0.000
NCIMB+avicel	Correlation Co-efficient	0.942**	1.000**
	Sig. (2-tailed)	0.002	0.000

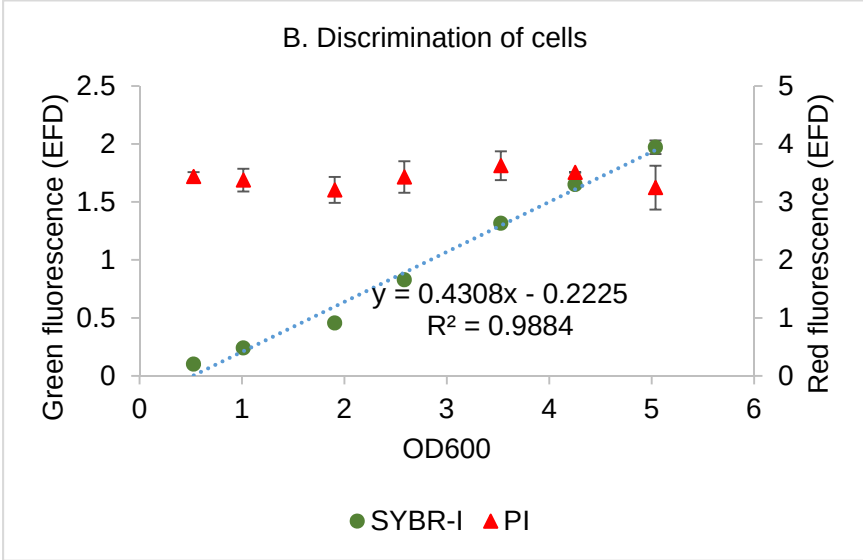
Correlations were tested for linearity (Pearson) and dependence (Kendall's tau) between the OD600 values and the fluorescence measurements. **Correlation is significant at the 0.01 level (2-tailed).

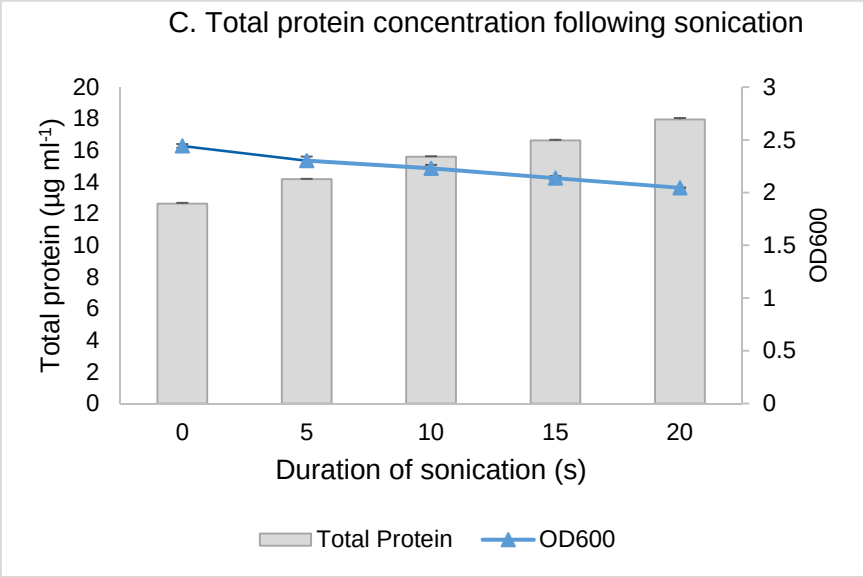

SYBR Green I
575nm, 100%
Propidium Iodide
619nm, 100%
Key
Propidium Iodide
SYBR Green I
Excitation
Emission
Relative Intensity (%)
100
75
50
25
0
300
400
500
600
700
800
900
Wavelength (nm)

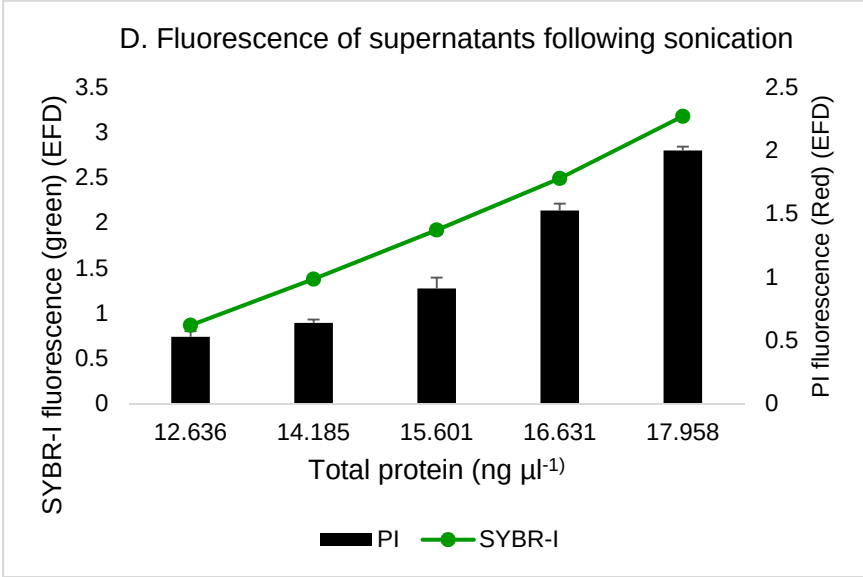

A. Red fluorescence channel
7.0E+04
6.0E+04
5.0E+04
4.0E+04
3.0E+04
2.0E+04
1.0E+04
0.0E+00
Red fluorescence (FSU)
y = 1862x + 89.031
R² = 0.9964
y = 877.26x + 401.53
R² = 0.9893
0
10
20
30
40
DNA concentration (ng µl-1)
PI alone
Both Red


B. Green fluorescence channel
1.2E+05
1.0E+05
8.0E+04
6.0E+04
4.0E+04
2.0E+04
0.0E+00
Green fluorescence (FSU)
y = 3313.3x
R² = 0.9908
y = 53.04x - 109.49
R² = 0.9505
0
10
20
30
40
DNA concentration (ng µl-1)
SYBR-I alone
Both Green


A. DNA standards
Green fluorescence (FSU)
Red fluorescence (FSU)
y = 869.96x
R² = 0.9943
y = 32355x
R² = 0.9908
DNA concentration (ng µl-1)
SYBR-I
PI


B. Discrimination of cells
Green fluorescence (EFD)
Red fluorescence (EFD)
y = 0.4308x - 0.2225
R² = 0.9884
OD600
SYBR-I
PI


C. Total protein concentration following sonication
Total protein (µg ml-1)
OD600
Duration of sonication (s)
Total Protein
OD600


D. Fluorescence of supernatants following sonication
SYBR-I fluorescence (green) (EFD)
PI fluorescence (Red) (EFD)
Total protein (ng µl-1)
PI
SYBR-I