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

1 **Two-colour fluorescence fluorimetric analysis for direct quantification of bacteria and**

2 **its application in monitoring bacteria growth in cellulose degradation systems**

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15 **Highlights**

16 • An assay for quantification of bacteria in mixtures containing insoluble substrates

17 • The assay uses SYBR Green I and propidium iodide dual staining with fluorimetry

18 • DNA standards were used to define an equivalent fluorescence DNA (EFD) unit

19 • Inter-lab/instrument comparisons are possible when the DNA standards are used

20

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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.

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

3

78 discrimination between living and dead bacteria. This transfer is due to the extremely high

79 quantum yield of DNA bound SYBR-I complex (~0.8, Molecular Probes Inc., USA) and the

80 overlapping of its emission spectrum (Fig. 1) with the absorption spectrum of PI. As a result,

81 the fluorescence of SYBR-I is 'quenched' by PI when stained with both. 'Dead cells' are

82 regarded as cells with compromised membranes. The compromised membrane integrity

83 allows both PI and SYBR-I to permeate the cells at which point such cells will fluoresce red

84 (PI) when excited. 'Live cells' on the other hand allows only SYBR-I to permeate and when

85 excited, fluoresce green.

86

87 Figure 1. Excitation and emission spectra of SYBR green I and propidium iodide from

88 Fluorescence SpectraViewer, Life Technologies

89

90 Fluorescence has been used in quantitation for a long time. Although the technique has

91 improved over time, a major challenge it faces is standardization and references for

92 fluorescent measurements. A special issue on "Quantitative Fluorescence Cytometry: An

93 Emerging Consensus" published by the journal *Cytometry* identified some of these challenges

94 (Lenkei et al., 1998). Among them were **(1)** inter-laboratory comparisons (Purvis and Stelzer,

95 1998, Waxdal et al., 1998, Zenger et al., 1998), **(2)** instrumentation (Purvis and Stelzer, 1998,

96 Wood, 1998, Wood and Hoffman, 1998), and **(3)** reagent and calibration standards (Gratama

97 et al., 1998, Lenkei et al., 1998, Purvis and Stelzer, 1998, Schwartz et al., 1998, Shapiro et

98 al., 1998, Wood and Hoffman, 1998, Zhang et al., 1998). A common response to these

99 challenges has been the development of a fluorescence intensity standard (FIS), the MESF

100 (molecules of equivalent soluble fluorochrome) for use in flow cytometry (Gaigalas et al., 2001,

101 Schwartz et al., 2004, Schwartz et al., 2002, Wang et al., 2002). The MESF is based on a

102 comparison between the number of fluorophores in two solutions, where one solution is a

103 standard with known values. The standard is often a suspension of labelled microbeads.

104 Although all these standardizations contribute to making the use of flow cytometry a better

105 platform for enumerating live bacteria than OD600 and colony counting, there are some

4

106 challenges that do not favour its wide use. First, the flow cytometers cost relatively high and

107 not available in many laboratories monitoring and enumerating bacteria growth. Secondly, the

108 use and interpretation of flow cytometric data require special training and expertise.

109 Furthermore, flow cytometers require regular servicing which is not available particularly in

110 most developing regions such as sub-Saharan Africa. There is therefore a need to adapt the

111 SYBR-I/PI principle of differentiating and enumerating bacteria for a simple and cheaper

112 platform such as fluorimetry. As mentioned above, a major challenge for fluorescence

113 measurement is the inability to make comparable fluorescence intensity measurements

114 across laboratories and between different instruments. In response to this, a FIS based on

115 DNA stained with SYBR-I and PI was also developed. This standard, like the MESF used in

116 flow cytometry, is based on equivalency between the intensity of fluorophores in two solutions,

117 one standard (known concentration(s) of DNA) and the other the unknown sample. An

118 alternative method for semi-quantification of bacteria using the total protein content of the

119 sample was also evaluated and compared with the two-colour fluorescence method.

120

121 As the world's fossil fuel reserves deplete, there is a growing need to develop sustainable

122 fuels (Creutzig et al., 2015, Fulton et al., 2015, Nuffield Council on Bioethics, 2011). Solar

123 electric, hydropower (hydroelectric, tidal and ocean thermal power) and geothermal appear

124 are safe sustainable sources of energy. However, these sources are not practically useful in

125 the long-distance transport sector, thus emphasizing the need for liquid fuels. The only

126 sustainable source of liquid fuels appears to be plant derived biofuels. The United States (US)

127 and the European Union (EU) have individually set targets to expand the production and use

128 of biofuels. The Energy Independence and Security Act (EISA) of 2007 establishes that

129 blending of renewable fuels (Biomass diesel and cellulosic biofuels) into transportation fuels

130 in the US should increase from 9 billion gallons in 2008 to 36 billion gallons by 2022 (Van Dyk

131 and Pletschke, 2012). Similarly, the EU has also projected biofuel supply in transportation

132 fuels to reach 25% by 2030 (Himmel *et al.*, 2007; US EPA, 2013). Despite the potential of

133 plant derived biofuels to meet these targets, large scale applications are currently problematic

134 due to the difficulties in converting plant biomass (mostly made of cellulose, hemicellulose,

135 lignin, pectin, and protein) into bioalcoholic derivatives and biodiesel. Naturally occurring

136 cellulose degrading microbes use a battery of multiple catalytic enzymes to hydrolyse

137 cellulose. We have described applications of synthetic biology that expand the technical

138 capabilities of engineering efficient cellulose degrading enzyme systems (Duedu and French,

139 2016, French et al., 2015, Lakhundi et al., 2016) making it potentially easier to develop an

140 ideal biofuel producing microorganism (IBPM) (French, 2009). Characterization and fine

141 tuning of microbial cellulose degrading systems requires reliable methods for monitoring

142 growth of cells on cellulose as a main carbon source. Substrates used for such experiments

143 (eg. avicel, paper and pre-treated plant materials) are generally insoluble. This makes the use

144 of turbidimetric methods (eg. OD600) which are fast and suitable for screening large numbers

145 of samples unreliable. Colony counting on the other hand is an arduous process. Dual staining

146 of samples with SYBR-I and PI has been demonstrated as a useful method for quantifying

147 bacteria in environmental and food samples using flow cytometry. The application of this

148 method for fluorimetry has not been reported. Here, SYBR-I/PI dual staining was used to

149 evaluate the cell content of cultures containing microcrystalline cellulose (avicel).

150

151 **2.   Materials and Methods**

152 *2.1   Reagents, equipment and sample preparation*

153 The SYBR-I/PI protocol was evaluated using DNA and cell suspensions of *Escherichia coli*

154 strains JM109 and DH5α and *Citrobacter freundii* strains ATCC8090 and SBS197. The

155 SBS197 strain was obtained from the Biology Teaching Laboratory of the School of Biological

156 Sciences (SBS), University of Edinburgh. *E. coli* and *C. freundii* were chosen because they

157 are the hosts we use in our biomass degradation experiments (Lakhundi, Duedu, Cain, Nagy,

158 Krakowiak and French, 2016). *E. coli* and *C. freundii* were grown in 100 ml Luria Broth (LB)

159 overnight at 37°C, 200 RPM. The cells were centrifuged at 5000 x g for 10 minutes, the

160 supernatant discarded and the pellet resuspended in 5 ml 1 x PBS. Human genomic DNA

161   (200 ng µl^{-1}) was obtained from Bioline, London, UK and used as a calibration standard.

162   Additionally, plasmid DNA was extracted from *E. coli* JM109 harbouring the BioBrick plasmid,

163   pSB1A2-BBa_K523025 using the QIAGEN plasmid midi kit (QIAGEN GmbH, Hilden,

164   Germany) according to the manufacturer's instructions. Dilutions of the DNA were made in

165   nuclease free water and stained with the fluorochromes. Propidium iodide (1mg ml^{-1} stock

166   solution) and SYBR-I (10,000 X concentrate) were obtained from ThermoScientific, Eugene,

167   OR, USA. SYBR-I dilutions were made using Pierce™ Dimethylsulfoxide (DMSO)

168   (ThermoScientific, Rockford, IL, USA). Fluorescence measurements were taken using the

169   Modulus™ Single Tube multimode reader (P/N 998-9203, Turner BioSystems, Sunnyvale,

170   CA, USA).

171

2.2   *Fluorescence staining of cells and DNA*

173   One ml of diluted DNA or cell suspension was stained with 10 µl each of SYBR-I (1:30 dilution

174   of commercial stock) and PI in a cuvette (FisherBrand FB55147, Fisher Scientific,

175   Loughborough, UK). The samples were covered with parafilm, mixed by inverting about 6

176   times and incubated in the dark for 15 minutes at room temperature.

177

2.3   *Fluorescence measurement and energy transfer*

179   Fluorescence was measured using the blue (P/N 9200-040, λ_{ex} = 460 nm, λ_{em} = 515 - 570 nm)

180   or green (P/N 9200-042, λ_{ex} = 525 nm, λ_{em} = 580 - 640) Modulus™ fluorescence kits (Turner

181   BioSystems, Sunnyvale, CA, USA). Measurements obtained from the instrument were given

182   in fluorescence units (FSU). To convert the FSU to a FIS which can be compared between

183   instruments and across labs, a standard was generated using dilutions of DNA stained with

184   either SYBR-I, PI or both. For each standard 10 ml was prepared. Aliquots of 1 ml were

185   transferred to labelled cuvettes in triplicates for SYBR-I, PI and SYBR-I/PI staining. All

186   samples were measured individually in both the red and green fluorescence channels. Energy

187   transfer from SYBR-I to PI was evaluated.

188

189 ***2.4   Discrimination and semi-quantification of cells***

190 To assess the discriminatory capacity of the protocol, various degrees of damage were

191 induced in the *E. coli* and *C. freundii* cells. To induce damage, 500 µl of cell suspension was

192 ultrasonicated at 10 µm (amplitude) for various intervals. The MSE Soniprep 150 Plus

193 Ultrasonic Disintegrator (MSE (UK) Ltd, London, UK) with the exponential probe was used to

194 sonicate cells. Cell damage was assessed by performing total protein quantification on 100 µl

195 of each sample using the Pierce Coomassie plus (Bradford) assay kit (ThermoScientific,

196 Rockford, IL, USA). The ultrasonicated samples were then stained with SYBR-I, PI or both.

197 Measurements were taken for all samples in both the green and red fluorescence channels.

198

199 ***2.5   Determination of how presence of cellulose particles affects fluorescence***

200 To assess the effects of addition of cellulose on fluorescence, 20 mg of cellulose powder (20

201 µm microcrystalline powder, Sigma Aldrich, Irvine, UK) was added to cell suspensions. Two

202 sets of cell suspensions were prepared at different cell densities. The turbidity of cells was

203 determined by measuring the absorbance at 600 nm using the absorbance module (Model

204 E6076, GLOMAX MultiJR, Promega, Southampton, UK) with the Modulus™ reader. To one

205 set of cells, cellulose powder was added and the turbidity re-measured. Both sets of samples

206 were stained in parallel with SYBR-I/PI and the fluorescence determined as described above.

207

208 ***2.6   Total protein assay***

209 The Pierce Coomassie plus (Bradford) assay was used to determine the total protein

210 concentration in cells. Reactions were set up in 1 ml volumes containing 100 µl of sample and

211 900 µl of Bradford reagent and mixed. To determine release of soluble protein from sonicated

212 cell suspensions, cells were removed by centrifugation (10,000 g, 10 min) and 100 microliters

213 of supernatant was assayed. The reaction mixture was incubated at room temperature for 10

214 minutes according to the manufacturer's instructions after which absorbance was measured

215 at 600 nm using the Modulus™ reader. To measure total protein content of intact cells, 100 µl

8

216   of the cell suspension was added to 900 μl of Bradford reagent, mixed and incubated at 65°C

217   for one hour to lyse the cells. The samples were allowed to cool for one hour at room

218   temperature during which time cellulose particles settle at the bottom that so they do not

219   interfere with the absorbance measurements. Absorbance values were converted to protein

220   concentration by comparing with a standard curve prepared with dilutions of the 2 mg ml^{-1}

221   bovine serum albumin (BSA) included in the kit.

222

223   *2.7   Statistical analysis*

224   Microsoft Excel was used for data entry, organization and generation of graph plots. Statistical

225   tests were conducted in IMB SPSS version 21. Correlations and tests for linearity between

226   fluorescence and DNA concentration or cell density were analysed using Pearson's product-

227   moment correlation tests. To determine whether fluorescence depended on cell density or

228   degree of damage, Kendall's rank correlation (tau b) was used. All experiments were

229   performed in at least three replicates and the means plotted with standard error values.

230

231   **3.   Results**

232   *3.1   Staining characteristics and energy transfer*

233   Both stains fluoresced in either channel due to the overlap between their absorption and

234   emission spectra. Furthermore, fluorescence values recorded for SYBR-I were higher than

235   those of PI for the same sample. SYBR-I fluorescence in the green channel was 10.06 % (SD

236   = ± 2.16) that of the fluorescence recorded from the red channel. Fluorescence of propidium

237   iodide in the green channel was 0.34 % (SD = ±0.25) of that in the red channel (supplementary

238   information figure 1). Background fluorescence of PI is not likely to affect fluorescence of

239   SYBR-I in the green channel but that of SYBR-I will potentially affect the fluorescence of PI in

240   the red channel.

241

242   Energy transfer was assessed after compensation for SYBR-I and PI emission in the red and

243   green channels respectively as described by Barbesti et al (2000). The energy transfer from

244   SYBR-I to PI was evaluated as a decrease in green SYBR-I fluorescence and increase in PI

245   fluorescence of DNA samples stained with both fluorochromes as compared to the same

246   samples stained with only one of the two fluorochromes. Reduction of SYBR-I fluorescence

247   was 98.6 % (SD = ±0.18) whereas increase in PI was 120.2 % (SD = ±15.5) indicating a strong

248   discriminatory capability (table 1 and figure 2).

249

250   *Table 1: Energy transfer from SYBR-I to PI*

251   *Figure 2: Differences between single and dual staining of DNA*

252

253   ### 3.2   Definition of an equivalent fluorescent DNA (EFD) unit

254   A fluorescence intensity standard was developed based on the fluorescence of DNA stained

255   with either of the two fluorochromes to enable comparison of fluorescence across laboratories

256   and instruments. An equivalent fluorescent DNA (EFD) unit was defined as the amount of

257   fluorescence obtained from staining 1 ng μl^{-1} of DNA for 15 minutes at room temperature

258   (25°C). A best fit line obtained for each stain was used to determine the EFD unit for that

259   particular stain after compensating for fluorescence of SYBR-I and PI in the red and green

260   channels (figure 3a).

261

262   ### 3.3   Assessment of single fluorescent staining of bacteria cultures and their

263   discriminatory capability

264   Quantification of bacterial cells was assessed first by staining dilutions of *E. coli* suspensions

265   with single fluorochromes. There was no correlation between cell density (OD600) and the

266   fluorescence obtained when PI alone was used to stain cells (figure 3b). On the other hand, a

267   strong correlation (R^2 = 0.988) between cell density and fluorescence was obtained with

268   SYBR-I alone. Although SYBR green I is a green fluorescent stain, red fluorescence was

269   detected from cultures stained with SYBR green I. This fluorescence was however 3 to 6 fold

270   lower than the green fluorescence detected.

271

272   To investigate discrimination of live and dead cells, we needed a system where specific

273   degrees of cell damage could be attained. Ultrasonication of cell suspensions for increasing

274   duration of time gave increasing extracellular protein concentrations indicating release of

275   protein from cells whereas the cell density measured by absorbance at 600 nm slightly

276   decreased (figure 3c). Complete lysis of all cells by incubating at 65 °C for one hour post-

277   sonication resulted in protein concentrations levels that were similar to each other irrespective

278   of the duration of sonication. (supporting information figure 2). To confirm that this damage

279   also resulted in release of varying amounts of DNA, suspensions were stained with both

280   SYBR-I and PI. Fluorescence increased with staining by both stains confirming that varying

281   degrees of cell damaged had been achieved (Figure 3d).

282

283   *Figure 3: DNA standard preparation and estimation of the EFD of damage-induced and non-*

284   *damaged cells stained with single fluorochromes*

285

286   **3.4   Discriminating between live and dead cells by dual staining**

287   To demonstrate that simultaneous staining of cells with both SYBR-I and PI can allow

288   quantification of live and dead cells using fluorimetry, cell suspensions of *E. coli* JM109 and

289   *C. freundii* NCIMB11409 were sonicated to induce various degrees of cell damage and stained

290   with both SYBR-I and PI. The population of live cells as determined by dual staining decreased

291   with increasing sonication duration whilst the dead cells increased (Figure 4a and 4b). There

292   was a strong direct correlation between duration of sonication and the dead cell population for

293   both *E. coli* (R^2 = 0.99; figure 5-8c) and *C. freundii* (R^2 = 0.99 for both *E. coli* and *C. freundii*).

294   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

12

322 correlation was used to test whether there was any correlation between OD600 before addition

323 of avicel and the live cells (green fluorescence) before and after addition of avicel. Strong

324 correlations were obtained for all cells whether avicel was present or not (Table 2). To

325 determine whether the fluorescence measured depended on the OD600, Kendall tau

326 correlation test was performed (Table 2). The results showed that irrespective of the type of

327 bacteria used or whether there was avicel or not, fluorescence depended on the OD600 before

328 addition of avicel.

329 *Table 2: Significance of correlations between OD600 and fluorescence in the presence or*

330 *absence of cellulose*

331

332 ### 3.7 Assessing growth in the presence of cellulose by total protein estimation

333 Total protein was determined using the coomassie assay on samples following incubation at

334 65°C for one hour as described above. There was no significant difference between total

335 protein for cells with or without avicel (p = 0.117 (*E. coli*) and 0.600 (*C. freundii*); figure 5 (b)).

336 Significant correlations were observed between OD600 and the total protein of *E. coli* without

337 cellulose (p = 0.001, R^2 = 0.960) and with cellulose (p = 0.003, R^2 = 0.923) as well as *C.*

338 *freundii* without cellulose (p < 0.001, R^2 = 0.990) and with cellulose (p < 0.001, R^2 = 0.984).

339 Total protein was statistically dependent on OD600 of cells (tau_b = 1, p ≤ 0.011).

340

341 **4.   Discussion**

342 There have been tremendous advances in fluorescence cytometry (instruments, fluorophores

343 and methods) over the years which enable direct analysis and quantification of bacteria and

344 other cells in different environments (Foladori, Bruni, Tamburini and Ziglio, 2010, Gregori,

345 Citterio, Ghiani, Labra, Sgorbati, Brown and Denis, 2001, Lebaron et al., 1998, Lenkei, Mandy,

346 Marti and Vogt, 1998, Melamed et al., 1972, Nunez et al., 2004, Tamburini, Foladori,

347 Ferrentino, Spilimbergo and Jousson, 2014). In this study, it has been demonstrated that a

348 simple two colour fluorescence fluorimetric technique can effectively be used to monitor

349 growth of bacteria in turbid growth cultures including those containing insoluble (eg. cellulosic)

350 substrates. This is essential for assessing *in vivo* activity of cellulose degrading enzyme

351 systems for biofuel production (Duedu and French, 2016, Lakhundi, Duedu, Cain, Nagy,

352 Krakowiak and French, 2016). The total protein assay evaluated in this study was also found

353 to be suitable for this purpose. The fluorescence technique however has some advantages

354 over the total protein technique. Whilst the fluorescence method can be completed in about

355 20 minutes, the total protein will be completed in not less than two hours. Additionally, the

356 stains used in the fluorescence technique are not regarded as harmful by European Union

357 regulations whereas the protein reagent is harmful. Another advantage of using the SYBR-I/PI

358 method over the total protein is that it allows direct estimation of live and dead cells making it

359 suitable for experiments where cell lysis is suspected. With an appropriate standard curve (eg.

360 plate count or flow cytometry), the green fluorescence values can be converted directly to cell

361 numbers.

362

363 Barbesti et al (2000) demonstrated that there is energy transfer from SYBR-I to PI in bacterial

364 cells stained with both fluorochromes. This study has demonstrated that the energy transfer

365 also occurs with DNA in solution but at a slightly higher percentage. This is expected as the

366 fluorochromes readily bind DNA and do not need to cross barriers (eg. membranes) as occurs

367 for cells (Lebaron, Parthuisot and Catala, 1998, Melamed, Adams, Zimring, Murnick and

368 Mayer, 1972). The strong energy transfer facilitates discrimination of live and dead bacteria

369 and it has been shown that it is not affected by the metabolic state (eg. stationary or

370 exponential) or the type of bacteria (gram positive or negative) (Barbesti, Citterio, Labra,

371 Baroni, Neri and Sgorbati, 2000). The discriminatory properties of the SYBR-I/PI dual staining

372 have been shown to be very effective, with strong correlations demonstrated between dead

373 cells (red fluorescence) and the degree of induced damage (sonication). This also means that

374 the fluorimetric application of the SYBR-I/PI method can account not only for cells with

375 compromised cell membranes as happens in flow cytometry but also for completely lysed

376 cells. Flow cytometry will count cells meaning completely lysed cells will not be accounted for.

377 This makes the application of two colour fluorescence staining with fluorimetry applicable in a

378 wider context.

379

380 Obtaining conditions necessary for the ideal measurement of fluorescence intensity is in

381 practice very difficult (Gaigalas, Li, Marti, Henderson, Vogt and Barr, 2001). The development

382 of the MESF unit is a practical approach that seeks to take the application of fluorescence

383 cytometry from just enumeration to actual quantitation. With this unit, quantitative fluorescence

384 data are no longer dependent on the instrument or the environment within which cells are

385 present (eg. media) but are standardized and comparable with others (Schwartz, Gaigalas,

386 Wang, Marti, Vogt and Fernandez-Repollet, 2004). Although the unit has not been evaluated

387 on other fluorescence platforms such as fluorimeters, there are some foreseeable challenges.

388 Fluorimeters do not count cells but rather give a value for the total fluorescence obtained. The

389 fluorescence obtained is also dependent on the amount of DNA present which these stains

390 (i.e. SYBR-I and PI) bind to. Thus, a recombinant organism harbouring a large piece of foreign

391 DNA will likely produce higher fluorescence than the wild type. The equivalent fluorescent

392 DNA (EFD) unit developed in this study leverages the principles of the MESF and the

393 foreseeable challenges of its application in fluorimetry to best serve its purpose. The EFD

394 value obtained for a sample can be directly compared to another sample of the same

395 constitution provided the DNA standard and samples were measured on the same instrument.

396 Rather than using a known quantity of standard beads, the EFD can be converted to a

397 quantitation standard when cell quantities are standardized with plate counts or another

398 appropriate measure.

399

400 Despite the advantages highlighted, the method does have some limitations. On its own, dual

401 staining followed by fluorescence measurement does not give absolute quantification of cells.

402 A standard curve must be generated, for example from plate counts or other suitable method,

403 and used to estimate the number of cells from the fluorescence reading. Furthermore, the

404 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

420 **6.   References**

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522

523 **Figures**

524 *Figure 1. Excitation and emission spectra of SYBR green I and propidium iodide from*

525 *Fluorescence SpectraViewer, Life Technologies*

526

527 *Figure 2: Differences between single and dual staining of DNA*

528 DNA was serially diluted and stained with either SYBR-I or PI alone and fluorescence

529 determined. Fluorescence determination was performed in the red fluorescence channel for

530 PI alone (a) and in the green fluorescence channel for SYBR-I alone (b). Dual stained DNA

531 was measured in both the red and green fluorescence channels. Six replicates were

532 performed.

533

534 *Figure 3: DNA standard preparation and estimation of the EFD of damage-induced and*

535 *non-damaged cells stained with single fluorochromes*

536 A. Standard curves for DNA stained with each of the fluorochromes were made to develop

537 the equivalent fluorescent DNA unit. DNA was serially diluted and the concentrations

538 determined using the nanodrop 2000 (ThermoScientific, Wilmington, DE, USA).

539 Diluted DNA was stained with either SYBR-I or PI and the fluorescence measured in

540 the green or red fluorescence channels respectively. Plots represent six replicates of

541 each dilution that was prepared and measured.

542 B. Cell suspensions were tested and compared with the DNA standard curves to

543 determine the EFD. Cell suspensions were prepared by resuspending pellets from

544 overnight cell cultures. The OD600 was predetermined and the pellets were serially

545 diluted and stained with either SYBR-I or PI. Two biological replicates (each made up

546 of three technical repeats) were performed.

547 C. Cell damage was confirmed by determining the total protein concentration of the cell

548 suspensions. Cell suspensions were ultrasonicated to induce various degrees of cell

549 damage. Suspensions were centrifuged to remove debris and non-lysed cells and the

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

551   assay.

552   D. Fluorescence and total protein were determined for cell suspensions sonicated to

553   induce various degrees of membrane damage. Fluorescence of PI as a result of cell

554   damage determined by total protein was compared.

555

556 *Figure 4: Simultaneous staining with SYBR-I and PI to discriminate live and dead E. coli*

557 *and C. freundii cells*

558 Discrimination of live and dead cells was achieved by simultaneously staining cell suspensions

559 of *E. coli* and *C. freundii* that have been damaged to various degrees. Live cells were obtained

560 as the fluorescence in the green channel whereas dead cells were obtained as the

561 fluorescence in the red channel. Three biological repeats were performed.

562

563

564 *Figure 5:*

565 *(a) Simultaneous staining is sufficient to discriminate and quantify live and dead cells*

566 *in the presence of insoluble cellulose*

567 Cell suspensions of different densities were used. Fluorescence measurements were obtained

568 for cells prior to and after addition of avicel. Cell suspensions of *E. coli* (a and b) and *C. freundii*

569 (c and d) were used. Three biological repeats were performed.

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

571 Total protein were obtained for cell suspensions prior to and after addition of avicel. The plots

572 show the original OD600 (i.e. before addition of avicel) versus the total protein with and without

573 avicel. Three biological replicates were performed.

Tables

575 *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

576 Values are means of three measurements. SYBR-I fluorescence was almost quenched when DNA was

577 stained with both stains.

578

579 *Table 2: Significance of correlations between OD600 and fluorescence in the presence or*

580 *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

581

582 Correlations were tested for linearity (Pearson) and dependence (Kendall's tau) between the

583 OD600 values and the fluorescence measurements. **Correlation is significant at the 0.01

584 level (2-tailed).

A. Red fluorescence channel

$y = 1862x + 89.031$
$R^2 = 0.9964$

$y = 877.26x + 401.53$
$R^2 = 0.9893$

Red fluorescence (FSU)

DNA concentration (ng μl^{-1})

▲ PI alone ◆ Both Red

B. Green fluorescence channel

$y = 3313.3x$
$R^2 = 0.9908$

$y = 53.04x - 109.49$
$R^2 = 0.9505$

Green fluorescence (FSU)

DNA concentration (ng μl^{-1})

● SYBR-I alone ■ Both Green

A. DNA standards

$y = 869.96x$
$R^2 = 0.9943$

$y = 32355x$
$R^2 = 0.9908$

● SYBR-I   ▲ PI

B. Discrimination of cells

$y = 0.4308x - 0.2225$
$R^2 = 0.9884$

● SYBR-I   ▲ PI

C. Total protein concentration following sonication

■ Total Protein   ▲ OD600

D. Fluorescence of supernatants following sonication

■ PI   ● SYBR-I