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ABSTRACT

Since biofilms are important in many clinical, industrial and environmental settings, reliable methods to quantify these sessile microbial populations are crucial. Most of the currently available techniques do not allow the enumeration of the viable cell fraction within the biofilm and are often time-consuming. This paper proposes flow cytometry (FCM) using the single-stain viability dye TO-PRO®-3 iodide as a fast and precise alternative. Mature biofilms of *Candida albicans* and *Escherichia coli* were used to optimize biofilm removal and dissociation, as a single-cell suspension is needed for accurate FCM enumeration. To assess the feasibility of FCM quantification of biofilms, *E. coli* and *C. albicans* biofilms were analyzed using FCM and crystal violet staining at different time points. A combination of scraping and rinsing proved to be the most efficient technique for biofilm removal. Sonicating for 10 min eliminated the remaining aggregates, resulting in a single-cell suspension. Repeated FCM measurements of biofilm samples revealed a good intraday precision of approximately 5 %. FCM quantification and the crystal violet assay yielded similar biofilm growth curves for both microorganisms, confirming the applicability of our technique. These results show that FCM using TO-PRO®-3 iodide as a single-stain viability dye is a valid fast alternative for the quantification of viable cells in a biofilm.

Keywords: flow cytometry, TO-PRO®-3 iodide, biofilm, viability, single-cell suspension, enumeration

ABBREVIATIONS

A = absorbance

C. albicans = *Candida albicans*

CFU/mL = colony forming units per ml

CV = crystal violet

E. coli = *Escherichia coli*

FCM = flow cytometry

PBS = Phosphate Buffered Saline

TP3 = TO-PRO®-3 iodide

VBNC = viable but nonculturable cell

VPC = viable plate count

INTRODUCTION

Biofilms are surface-attached microbial communities embedded in an extracellular polymeric matrix. The cells within these biofilms differ phenotypically from their planktonic counterparts (Cos *et al*. 2010). This widespread mode of sessile growth promotes microbial survival since the matrix protects against external influences such as antimicrobial compounds, while different species within the biofilm can show synergistic behavior (Cos *et al*. 2010; Dunne 2002; Flemming 2002).

Biofilms are important in clinical, industrial and environmental settings (Nickzad and Deziel 2014). The majority of all microbial infections involve biofilm formation, while biofouling, *i.e.* the unwanted growth of a biofilm on a surface, presents a costly nuisance for the industry (Coenye and Nelis 2010; Flemming 2002). In contrast, biofilms can also be beneficial, *e.g.* for the biodegradation of pollutants (Cos *et al*. 2010).

To study biofilms, reliable quantification methods are essential (Cos *et al*. 2010; Morikawa 2006; Toté *et al*. 2009). Current techniques allow quantification of the entire biofilm, the biofilm matrix or biofilm cells (An and Friedman 1997; Cos *et al*. 2010; Denkhaus *et al*. 2006). Microscopy and image analysis enable quantitative assessment of the biofilm architecture, but this time-consuming approach can only address a limited number of cellular parameters (An and Friedman 1997; Behnam *et al*. 2012; Coenye and Nelis 2010). High-throughput quantification can be achieved through staining of biofilms grown in a microtiter plate and subsequent spectrophotometric measurement of the amount of desorbed dye. Depending on the stain, this method enables quantification of the total biofilm biomass (*e.g.* crystal violet (CV)), the biofilm matrix (*e.g.* DMMB) or the metabolic activity of the biofilm cells (*e.g.* resazurin) (Peeters *et al*. 2008; Toté *et al*. 2008). However, these spectroscopic assays are not only species-dependent, requiring individual optimization of staining protocols, but also non-specific since most dyes cannot discriminate between the different biofilm components (Cos *et al*. 2010; Hannig *et al*. 2010). To enumerate viable cells within a biofilm, viable plate counting (VPC) is often employed, although this technique is time-consuming, labor-intensive and does not allow detection of viable but nonculturable cells (VBNCs) (An and Friedman 1997; Coenye and Nelis 2010).

Flow cytometry (FCM) could present a fast and precise alternative to count cellular subpopulations in a biofilm. Moreover, it enables detailed investigation of the heterogeneous biofilm population due to its ability to perform multiparametric single-cell analysis. This powerful technique is also capable of observing ten thousands of cells in a matter of minutes, providing statistically relevant data for the analysis of biofilm populations (Muller and Nebe-von-Caron 2010).

Previously, we reported an FCM approach using the membrane impermeable dye TO-PRO®-3 iodide (TP3) to discriminate and quantify viable and dead cells with a single stain only (Kerstens *et al*. 2013). In the present paper, we describe how this technique, which requires a single-cell suspension as starting material, could be applied to quantify biofilms. The first part focused on the careful optimization of a protocol to remove sessile cells from the surface of a well plate and to disintegrate biofilm aggregates without affecting cell viability. Next, the precision of the FCM assay was determined and its feasibility was evaluated by comparing FCM with CV staining for biofilm growth assessment.

MATERIAL AND METHODS

Bacterial strains and growth conditions. *Escherichia coli* ATCC 10536 and *Candida albicans* SC5314, both known as biofilm-forming strains, were maintained at 37 °C on Tryptic Soy Agar (Lab M, Lancashire, UK) and Sabouraud Dextrose Agar (LAB M, Lancashire, UK) respectively (Jaglic *et al*. 2012; Krom *et al*. 2007). Strains were subcultured every 24 h. A fresh culture was prepared weekly from a cryostock.

Biofilm growth. The protocol for biofilm growth was adapted from Jaglic *et al.* for *E. coli* and from LaFleur *et al.* and Krom *et al.* for *C. albicans* (Jaglic *et al*. 2012; Krom *et al*. 2007; LaFleur *et al*. 2006). The inoculum for biofilm growth was prepared by scraping off an overnight microbial culture and suspending the cells in the appropriate medium. The *E. coli* suspension was diluted in RPMI 1640 (GIBCO, NY, USA) to achieve a final concentration of 1×10^6 Colony Forming Units per mL (CFU/mL), while Yeast Nitrogen Base medium (Difco, MI, USA) was used to dilute the *C. albicans* suspension to 4×10^5 CFU/mL. The microbial suspensions (1 mL) were then added to the wells of a 24-well plate (Greiner Bio-One, NC, USA). Uninoculated medium was included as a blank. The plates were placed in an Innova 4300 shaking incubator (25 rpm; New Brunswick Scientific, NJ, USA) at 37 °C for 48 h. Every 24 h, the culture medium was replenished with fresh medium.

Biofilm quantification: crystal violet assay. Prior to the biofilm analysis, wells were washed twice with 1 mL Phosphate Buffered Saline (PBS; GIBCO, NY, USA) to remove residual planktonic cells. The staining procedure was based on the protocol described by Toté *et al.*, with minor modifications (2009). Fixation of the biofilm was achieved by adding 1.5 mL 99 % (vol/vol) methanol (Merck Millipore, Darmstadt, Germany) to each well. After 15 min, the methanol was removed and the plates were allowed to dry. The biofilm was then stained with 2 mL of a CV solution (Merck Millipore, Darmstadt, Germany) for 5 min, followed by another washing step. The CV concentrations used were 0.02 % (g/vol) for *E. coli* and 0.1 % (g/vol) for *C. albicans*, based on previous optimization experiments (data not shown). After air-drying the plates, 2.5 mL 33 % acetic

acid (vol/vol; Sigma-Aldrich, MI, USA) was added to each well to release the bound dye. Then, 250 µL of each CV solution was transferred to a 96-well plate (Greiner Bio-One, NC, USA). The absorbance (A) was measured at 570 nm after 45 min (Labsystems Multiskan MCC/340 microplate reader, Fisher Scientific, PA, USA).

Biofilm quantification: flow cytometric assay.

From biofilm to single-cell suspension.

Influence of sonication on viability (i). Sonication is often used to remove biofilms from its substrate. To exclude any detrimental influences of sonication on the viability of the microorganisms, planktonic suspensions of both species were subjected to sonication for periods of increasing duration (0 min, 10 min, 30 min, 1 h, 2 h). Planktonic samples were obtained by suspending a colony of the species in 10 mL of buffered sodium chloride solution (1 g of peptone, 3.6 g KH_2PO_4, 7.2 g $Na_2HPO_4.H_2O$ and 4.3 g NaCl dissolved in 1 L of ultrapure water with a pH of 7.0 ± 0.2). Sonication was performed on a sonicator bath (B2510; Branson, CT, USA) at a frequency of 40 kHz. During sonication, ice was added to the sonicator bath to minimize the effect of temperature on the cell viability. After each interval, the total amount of cells and viable cells was determined by FCM. The amount of viable cells was expressed relatively to the total amount of cells to avoid sampling errors. The means of nine counts were compared statistically (Kruskal–Wallis test with Dunn's multiple comparison post-hoc test; GraphPad 4 Prism software, CA, USA).

Detachment of sessile cells from a surface (ii). Before removal of the biofilm, 1 mL of buffered sodium chloride solution was placed in each well of the plate. Several mechanical techniques were explored to detach the mature biofilm from the wells. The first approach was sonication of the well plate, based on the procedure described by Van den Driessche *et al*. (2014). The plate was sonicated on the sonicator bath for 10 min. For the second approach, the biofilm was dislodged by scraping the wells with a cell scraper (TPP, Trasadingen, Switzerland). A third technique involved the use of a 2 mL syringe with a blunt 18G fill needle (BD biosciences, NJ, USA) to thoroughly rinse the wells five times. The following combinations were tested as well: scraping and rinsing; scraping and sonication; rinsing and sonication; scraping, rinsing and sonication. "Untreated" wells containing intact biofilm were included in the assay as a negative control value.

After dislodging the biofilm, the obtained cell suspension was collected by aspiration using a pipet. The residual biofilm in the well was assessed by CV staining after application of the different removal strategies. Wells were inspected microscopically (Axio observer Z1; Zeiss, Oberkochen, Germany) before the addition of acetic acid. The means of nine replicates of each technique were compared statistically using a Kruskal-Wallis test (Dunn's post-hoc test).

Disruption of biofilm aggregates (iii). Biofilms were grown as described above and removed by scraping and rinsing. The biofilm suspensions thus obtained were pooled and sonicated for 10 min, 30 min or 60 min using a sonicator bath. A non-sonicated sample was included as a control. On these samples, a total cell count was performed nine times by FCM for both species. The resulting counts were analyzed statistically (Kruskal-Wallis test; Dunn's post-hoc test).

Flow cytometric analysis. Analyses were performed as previously described (Kerstens *et al*. 2013). Briefly, 25 µL CountBright absolute counting beads (Molecular Probes®, OR, USA) were added to 470 µL of the sample to determine the volume analyzed. Next, the sample was stained with 5 µL TP3 (20 µmol/L) for 10 min in the dark. A FACScalibur flow cytometer (BD Biosciences, NJ, USA) equipped with a red diode laser (λ_{ex}=635 nm) and a band pass filter measuring red fluorescence (653–669 nm) was used to analyze the suspension. The sample flow rate was set at "low" (12 ± 3 µL/min). Table 1 gives an overview of the software settings. The amount of viable cells per mL was calculated from the events recorded within a preset gate defining the viable cell population, and from the volume aspirated by the flow cytometer.

Precision of FCM quantification of the biofilm. The repeatability (intraday precision) was evaluated by performing FCM on nine technical replicates of a mature biofilm (48 h of growth). From these results, the coefficient of variation was determined. To assess the interday precision, mature biofilms were grown in two separate 24-well plates, representing biological replicates. From nine wells of each plate, biofilm was harvested and analyzed separately by FCM. The resulting means were compared between plates using a Mann-Whitney U test.

Biofilm growth curve. Biofilm growth was initiated as described above. After 1 h, 6 h, 24 h, 30 h, 48 h and 72 h, plates were analyzed by FCM and CV staining (nine replicates). Wells stained with CV were checked microscopically before resolubilization of the dye. The resulting biofilm growth curves for both methods were compared.

RESULTS

Biofilm quantification: flow cytometric assay.

From biofilm to single-cell suspension.

Influence of sonication on viability (i). Sonication for 2 h significantly reduced the amount of viable cells in the sample and is unsuitable for the disaggregation of biofilm lumps (fig. 1). The other sonication times (10 min, 30 min, 1 h) showed no significant impact on cell viability.

Detachment of sessile cells from a surface (ii). Different approaches to remove the biofilms from the wells were compared (fig. 2). Combined scraping and rinsing was the most effective method to remove the biofilm, as reflected by the lowest residual amount of biofilm (lowest A) in comparison with the control wells (no deliberate biofilm removal).

For *C. albicans*, there was no significant difference between the A of the control wells and the A obtained after sonication, scraping or a combination of the two methods. These techniques left residual biofilm in the well, while scraping followed by sonicating and rinsing, scraping and rinsing, rinsing and sonicating or rinsing alone removed most of the biofilm in comparison with the control. The four latter techniques performed equally well. In the case of *E. coli*, scraping and sonicating, sonicating or rinsing removed less biofilm than the other methods, as was indicated by the statistically higher A value. All the other removal techniques performed equally well. Microscopic analysis of the CV stained plates confirmed these findings. Since the combination of scraping and rinsing allowed the most efficient collection of biofilm for both microorganisms, this approach was applied for biofilm removal in all further experiments.

Disruption of biofilm aggregates (iii). For *E. coli*, sonication had no influence on the total cell count per mL (fig. 3). For *C. albicans*, sonication for 10 min yielded a significantly higher total cell count per mL in comparison with the control sample. Microscopic analysis revealed that, though large aggregates were not abundantly present before sonication, 10 min did reduce the number of cell clumps. Hence, a sonication step of 10 min was included in the protocol.

Precision of FCM quantification of the biofilm. The repeatability was characterized by a coefficient of variation of 5.51 % for nine replicate measurements of *C. albicans* and 4.76 % for *E. coli* (table 2). The interday precision of the FCM quantification of the biofilm was confirmed for both *E. coli* and *C. albicans*. No significant differences were found between the means of the well plates analyzed on two different days ($p > 0.05$). However, the interday coefficient of variation was about five times larger than the intraday coefficient of variation for *C. albicans*.

Biofilm growth curve. The CV assay and the FCM assay indicated a similar growth pattern for *C. albicans* biofilms (fig 4.). After an initial delay of 24 h, both curves showed a similar increase in viable cells per mL or in A_{CV}. A plateau phase was reached at t = 48 h for both techniques, which lasted for at least an additional 24 h.

For *E. coli*, the A_{CV} also seemed to correlate well with the FCM quantification of the biofilm. Both techniques showed an increased signal after a shorter delay of about 6 h. The signals continued to rise swiftly until approximately 30 h, after which the increase was more gradual.

DISCUSSION

Since biofilms are omnipresent and important in clinical, industrial and environmental settings, the development and validation of fast and reliable quantification techniques is a microbiological research priority (Cos *et al*. 2010). From a fundamental research perspective, the analysis of the cellular components is important to gain more insight into the biofilm lifestyle. In addition, efficient assessment of the viable fraction could facilitate screening for new antibiofilm therapeutics (Almeida *et al*. 2007; Ammann *et al*. 2013; Coenye and Nelis 2010).

Both direct and indirect methods exist to quantify this aspect of the biofilm. Among the indirect methods, the microtiter plate assays are widely employed due to their ease of use and high-throughput possibilities (Van den Driessche *et al*. 2014). The viable cell fraction is often determined by the addition of dyes, such as resazurin, XTT and FDA, which are metabolically converted to a measurable end product (Cos *et al*. 2010). However, since the metabolism of viable cells is often diminished in biofilms, these assays may underestimate the viable fraction (Welch *et al*. 2012). PCR is another widespread indirect method to quantify the cellular fraction in biofilms (Klosterman 2012). This technique can analyze the entire cellular fraction or identify viable and dead subpopulations after addition of propidium monoazide or by performing Reverse Transcriptase PCR (Costerton *et al*. 2011; Dolan *et al*. 2010; Girones *et al*. 2010). However, molecular techniques require knowledge of the target organisms, which may pose a problem in non-research settings (Smith and Osborn 2009; Wolcott *et al*. 2013). Additionally, inhibitory substances in the sample may interfere with efficient PCR amplification (Suzuki *et al*. 2005).

VPC allows direct quantification of viable cells, though this labor-intensive and time-consuming technique fails to quantify VBNCs, which may constitute a large fraction of the biofilm (Cerca *et al*. 2011a; Cos *et al*. 2010). VBNCs are suspected to be the main reason why culture-dependent methods exhibit a low biofilm detection rate (Fux *et al*. 2005).

Microtiter plate assays, VPCs and PCR fail to take into account the heterogeneous nature of the biofilm in terms of composition (presence of different species and matrix components) and/or cell status (*e.g.* metabolic activity) as they offer limited information on these parameters (Almeida *et al*. 2011; Fux *et al*. 2005). Though microscopy, particularly CLSM, is appropriate to address these needs, image acquisition and analysis is time-consuming and complex (Cos *et al*. 2010; Mueller *et al*. 2006). Additionally, the thickness of the biofilm presents a challenge as it limits the penetration depth of both the (fluorescent) dyes and the laser into the biofilm (Paramonova *et al*. 2007; Vroom *et al*. 1999).

Our research proposes FCM as a fast and precise technique for biofilm quantification. For accurate FCM quantification, the biofilm needs to be transformed into a single-cell suspension (Nebe-von-Caron *et al*. 2000). Our results indicate that the best approach for the preparation of a single-cell suspension for FCM analysis consists of the following steps: removal of the biofilm cells from the wells of a 24-well plate using a combination of scraping and rinsing, sonication of the biofilm suspension for 10 min and vortexing of the suspension.

For biofilm removal, literature mentions several mechanical methods as well as chemical approaches, often entailing the use of surfactants such as Tween 80 (An and Friedman 1997). The use of chemical substances to disrupt the biofilm was unsuitable for our FCM application, since Tween influenced TP3 staining. Sonication, scraping or rinsing are most commonly described and were selected for further investigation (Cerca *et al*. 2011b; El-Azizi *et al*. 2004; Harriott and Noverr 2010; Koo *et al*. 2003). In our experiments, a combination of scraping and rinsing most efficiently removed the biofilm. Scraping mainly detached the biofilm in the middle of the well, whilst rinsing with a syringe was more adequate to remove the biofilm near the edges. Sonication did not contribute to the removal of the cells, but was important for the dissociation of biofilm aggregates of *C. albicans*. It did not significantly contribute to the disaggregation of *E. coli* biofilms clumps, demonstrating that repeated extrusion through a syringe needle was sufficient for disruption.

Our protocol gives rise to a single-cell suspension that can be investigated by FCM. The intraday coefficient of variation was approximately 5 %, demonstrating the high precision of our technique (Higgs *et al*. 2014). The analysis of two plates on two different days yielded statistically equivalent results, indicating a good interday precision. The interday coefficient of variation depended on technical as well as biological variation, since different plates and thus different biofilms were analyzed. This caused a larger interday coefficient of variation for *C. albicans* compared with the intraday coefficient of variation. To ensure a high repeatability and to minimize variation, care must be taken when rinsing the biofilm twice with PBS prior to analysis. If performed too roughly, biofilm can unintentionally be washed away (Coenye and Nelis 2010). Only two washing steps are included in our sample preparation, presenting an additional advantage in comparison with other techniques that involve the addition and subsequent removal of fixatives and staining solutions.

To demonstrate the ability of the FCM method to monitor biofilm maturation over time, biofilm growth curves were made using CV staining and our optimized FCM technique. FCM was able to detect an increase of the viable fraction over time and yielded a similar growth curve as the CV stain, which supports the use of FCM for the quantification of viable biofilm cells.

Up to now, few studies have described the application of FCM for the analysis of mature biofilms, although recently the technique has gained more attention. Kim *et al.* used green fluorescent protein strains of *Pseudomonas aeruginosa* to identify and sort active or dormant biofilm cells (2009). To analyze different species within the biofilm, FCM has been combined with fluorescence *in situ* hybridization (Flow-FISH) (Nettmann *et al*. 2013). Though selective oligonucleotide probes enable the specific detection of certain bacteria, discrimination between viable and dead cells is not feasible (Almeida *et al*. 2007). The studies on FCM analysis of biofilms that discriminate between viable and dead cells use dual-stain procedures to analyze both subpopulations. The described techniques mostly involve the application of propidium iodide in combination with Syto9, 5(6)-carboxyfluorescein diacetate or thiazole orange (Dashper *et al*. 2013; Ksontini *et al*. 2013; Torres *et al*. 2012). Using a combination of dyes to distinguish live and dead cells implies the occupation of two fluorescent channels which might limit the analysis of other fluorescent parameters.

The FCM strategy described in this paper is based on TP3 as a single-stain viability dye. The unique spectral characteristics of TP3 enable the addition of other fluorochromes with minimal spectral overlap, creating interesting possibilities for multiparametric measurements. Since only one fluorescent channel is occupied for viability assessment, other channels remain available. In the context of biofilm research, multiparametric experiments allow the qualitative and quantitative assessment of different species within the biofilm and the study of different physiological states of the biofilms cells.

CONCLUSION

This paper concludes that biofilms can be transformed into single-cell suspensions for FCM analysis. Furthermore, we demonstrated that TP3-based FCM allows precise, repeatable and accurate quantification of viable biofilm cells. Since FCM permits rapid and direct analysis of many cells in a short period of time, even rare events can be detected. All these features put FCM forward as a promising technique to study the heterogeneous nature of biofilms and to evaluate the efficacy of new therapeutic compounds and/or approaches.

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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Fig. 1 The influence of sonication on the viability of cells in a planktonic culture

The amount of viable cells per mL relative to the total amount of cells per mL (% VC/TC) was compared for different sonication intervals. The columns in the graph display the average of nine replicates. The error bars represent the standard error of the mean of nine technical replicates. Statistical differences (Kruskal-Wallis test; Dunn's post-hoc) are indicated with * ($p < 0.05$), ** ($p < 0.01$) or *** ($p < 0.001$).

Fig. 2 Detachment of sessile cells from a surface

Overview of the average absorbance at 570 nm ± standard error of the mean of nine biological replicates, shown per biofilm removal technique and per species. Microscopic images of the remaining biofilm are displayed within the small circle accompanying each technique (magnification is indicated in the panels). The solid lines (———) denote a statistical difference ($p < 0.05$) between the techniques connected as determined by a Kruskal-Wallis test (Dunn's post-hoc), while the dashed lines (- - - -) indicate that there is no statistical significant difference ($p > 0.05$).

Fig. 3 Disruption of biofilm aggregates

The average total cell count per mL (TC/mL) determined by flow cytometry in function of the sonication time of the biofilm sample was plotted. Statistically significant differences (Kruskal-Wallis, Dunn's post-hoc test) are marked with an asterisk. The standard error of mean of nine technical replicates is indicated by the error bars.

Fig. 4 Biofilm growth curves

The average flow cytometric (FCM) count of the viable cells per mL (VC/mL) and the average crystal violet absorbance (A_{CV}) at 570 nm were determined at different time points during biofilm formation. Biofilm formation was initiated by adding 1 mL of a suspension containing respectively 4×10^5 VC/mL *C. albicans* or 1×10^6 VC/mL *E. coli* to the wells of the plate. Measurements were made at t = 1 h; t = 6 h; t = 24 h; t = 30 h; t = 48 h and t = 72 h. The points in the graphs show the average of nine biological replicates per plate ± the standard error of the mean. A. *C. albicans* SC5314; B. *E. coli* 10536; ▲= A_{CV} at 570 nm, ■ = VC/mL (FCM)