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Robust biofilm assay for quantification and high throughput screening applications

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ABSTRACT

Bacterial biofilms are populations of bacteria within a self-produced adherent extracellular matrix that are notoriously resistant to treatment. Existing methods for biofilm quantification are often limited in their dynamic range of detection (signal-to-background), throughput, and require modifications to the protocol depending on the bacterial species. To address these limitations, a broad utility, high-throughput (HTP) method was required. Using a fluorescent dye, FM1-43, we stained the biofilm, followed by solvent extraction and quantitation of biofilm employing a fluorescent plate reader. Utilizing eight different bacterial pathogens, we demonstrate that this method is widely applicable for biofilm quantification. Depending on the species, this biofilm assay offered a large dynamic range of 8–146 fold change compared to 2–22 fold for crystal violet staining under similar conditions. In addition to routine biofilm quantification using this new assay, as a proof-of-concept, 1200 compounds were screened against two different bacterial species to identify biofilm inhibitors. In our HTP screens we successfully identified compounds rifabutin and ethavarine as potential biofilm inhibitors of *Burkholderia pseudomallei* Bp82 and *Acinetobacter baumannii* biofilm production respectively. This newly validated biofilm assay is robust and can be readily adapted for antibiofilm screening campaigns and can supplant other less sensitive and low throughput methods.

1. Introduction

Bacterial biofilm are groups of bacteria which coalesce to form a protective barrier against the external environment. The primary feature of a biofilm is its extracellular matrix, which offers a layer of protection against hostile environmental conditions, including environmental stressors, host defenses and antibiotic treatment (Wei and Ma, 2013). Establishment of biofilms by infectious human pathogens is particularly threatening as the bacteria in the biofilm can resist very high concentrations of known antibiotics that would effectively target their planktonic cell counterparts (Ceri et al., 1999). Well-documented examples include patients with cystic fibrosis, where *Pseudomonas aeruginosa* establishes a chronic infection and exists in a biofilm in the dense sputum of the lung milieu. This results in an infection that cannot be eliminated even with the most aggressive antibiotic regimens and often resulting in chronic infection and death in patients with this genetic disorder (Hoiby et al., 2010).

Similarly, a number of other pathogenic bacteria, including biothreat agents are documented to form persistent biofilms (Stewart and Costerton, 2001). Medical devices and implants are also prone to house biofilms that result in chronic infections in the host (Marrie et al., 1982). In a nosocomial environment, *Acinetobacter baumannii, Staphylococcus aureus* and *Pseudomonas aeruginosa* are amongst the most commonly isolated organisms (Lu et al., 2009). *Acinetobacter baumannii* has garnered significance as a common pathogen recovered from various wound infections of US military personnel deployed to areas such as Iraq (Howard et al., 2012).This opportunistic pathogen is intrinsically resistant to several classes of antibiotics and has also been recovered from endotracheal biofilms (Lorente et al., 2002). *Staphylococcus aureus* is commensal in the anterior nares of about 20% of the

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population and can produce chronic infections in almost every niche of the body (Kluytmans et al., 1997). Its ability to form biofilms in these niches is also wide-ranging, with biofilms present in bone, indwelling medical devices, periodontal implants and wounds (Archer et al., 2011). Owing to their innate resistance to common antibiotics, ease of transmission, high morbidity and mortality, *Burkholderia mallei* and *Burkholderia pseudomallei* were reclassified as potential biothreat agents (Gilad et al., 2007). *B. pseudomallei* is the causative agent of the debilitating disease melioidosis, while *B. mallei,* a zoonotic pathogen, produces the disease glanders in horses and related animals (Gilad et al., 2007; Stewart and Costerton, 2001). The formation of biofilms by both *B. mallei* and *B. pseudomallei* would potentiate their virulence and render disease more intractable (Kunyanee et al., 2016). Other commensal enterics like *Proteus mirabilis* and *Enterococccus faecium* become pathogenic when introduced to the blood or wounds (Kotnis-Gaska et al., 2018). They are also common in urinary tract infections and often exacerbate clinical prognoses by forming biofilms on indwelling catheters (Kwiecinska-Pirog et al., 2014). Previously considered a contaminant of clinical specimens, *Bacillus cereus,* has been isolated as the primary pathogen in infections of the blood, intestines and urinary tract (Bottone, 2010). *B. cereus,* like other *Bacillus* species, can form biofilms on fomites and urinary catheters (Auger et al., 2009).

There are a number of different technologies available for the investigation of bacterial biofilms (staining, engineered bacteria, etc.) in different biofilm states (air-liquid interface, surface attached, etc.) (Christensen et al., 1985; Franklin et al., 2015). The most commonly used static biofilm quantification methods employ microtiter dishes (e.g.: 96 well-plate), which rely primarily on colorimetric dyes (e.g.: crystal violet, safranin, etc.) that are extracted from stained biofilms and absorbance measured using a spectrophotometer. Though easy to execute, they offer limited high throughput (HTP); additionally, the assay execution is constrained by the need for at least two separate assay plates: Plate 1 for biofilm culturing, staining and dye extraction and plate 2 for extracted dye transfer and absorbance recording in flat clear bottom well plates. These quantification protocols are in general customized specifically for each targeted bacterial species and employs choice matrices for biofilm growth (PVC, polypropylene, polystyrene, slides, etc.), biofilm bacterial fixing methods (chemical fixing, air drying, no fixing etc.,) and alternate dye extraction methods (solvents, acids, detergents, etc.) (Merritt et al., 2005; O'Toole, 2011; Ommen et al., 2017; Pakharukova et al., 2018). Most often the colorimetric readouts are marred by relatively low sensitivities and limited dynamic range when compared to no biofilm controls. To address these limitations and to develop a reliable and possibly universally adaptable HTP biofilm quantification method that also offers imaging potential, we explored the establishment of a fluorescence based single plate (e.g.: 96 well) biofilm imaging and quantification assay.

Fluorescent dyes such as FM1-43 (SynaptoGreen C4) and its derivatives were developed as optical imaging tools for studying synaptic activity by staining synaptic vesicles at the nerve terminal or at the neuromuscular junctions (Betz and Bewick, 1992). Virtually non-fluorescent in aqueous media, FM1-43, when bound to the plasma membrane of cells, becomes increasingly fluorescent. This dye has also been shown to be a useful agent for staining and imaging bacterial biofilms (Franklin et al., 2015). However, to the best of our knowledge, there is no recorded evidence of FM1-43 being adapted for microtiter plate based biofilm quantification applications. Using a range of opportunistic and biothreat pathogens (*A. baumannii, B. mallei, B. pseudomallei, P. mirabilis, P. aeruginosa, S. aureus, E. faecium and B. cereus*), we demonstrate that this dye can be used for staining followed by solvent extraction for fluorescence based biofilm quantitation. When compared to the existing crystal violet based method, the FM1-43 assay provides a higher signal-to-background ratio than the bacteria free media only controls. Further, using *A. baumannii* and *B. pseudomallei* as representative pathogens, we show that this method is readily adaptable for HTP assays and offers great promise in identifying small molecules that can both inhibit and/or alter biofilm growth properties.

2. Materials and methods

2.1. Strains, media and culture conditions

The bacterial strains used in this investigation, *Acinetobacter baumannii* ATCC 19606, *Burkholderia mallei* CLH001 (tonB-, hcp1-; BSL-2 non-BSAT derivative), *Burkholderia pseudomallei* Bp82 (purM-; BSL-2 non-BSAT derivative), *Proteus mirabilis* (CDC 0159), *Pseudomonas aeruginosa* PAO1 (ATCC 15692), *Staphylococcus aureus* ATCC 43300, *Enterococcus faecium* JMI10032 and *Bacillus cereus* (environmental isolate) were cultured as follows: *Acinetobacter baumannii*, *Pseudomonas aeruginosa* and *Proteus mirabilis* were grown in Mueller-Hinton II (MH) broth, *Burkholderia pseudomallei* Bp82 and *Burkholderia mallei* CLH001 were grown in Modified Vogel Bonner Media (MVBM), except that *B. pseudomallei* Bp82 was supplemented with a final concentration of 100 μg/ml Adenine and 5 μg/ml Thiamine, while *B. mallei* CLH001 was supplemented with 4% glycerol and 200 μM Iron (II) Sulfate. *Staphylococcus aureus, Enterococcus faecium* JMI10032 and *Bacillus cereus* were grown in Tryptic Soy Broth (TSB), *Bacillus cereus* was supplemented with 3% sodium chloride and 0.5% dextrose.

2.2. Reagents for biofilm assay

FM1-43 biofilm staining kit (Thermofisher) was used in the assessment of the initial method. Larger quantities of the FM1-43 dye (or SynaptoGreen C4, CAS # 149838-22-2) were purchased from Proactive Molecular Research (Florida, USA). Primary stock of FM1-43 was made in 100% Dimethyl sulfoxide (DMSO) at 100 mg/ml and diluted as needed. Working stocks of FM1-43 dye were prepared by diluting 10 μl of the primary stock into 990 μl of DMSO (1 to 100 dilution) and diluting it further by adding 100 μl of this in to 900 μl of water (1 to 10 dilution) to make 1.0 ml of working stocks of FM1-43 dye solution. Crystal violet powder (Sigma) was used for making 1% (*w/v*) stock solution in water. A drug library containing about 1200 off-patent compounds (about 95% FDA approved) was obtained from Prestwick Chemical (Prestwick Chemical Library®).

2.3. Biofilm staining with FM1-43 and quantification

Saturated bacterial cultures grown overnight (37 °C, 225 rpm, for 16 h) in respective media were diluted 1:100 in respective fresh media and 100 μl aliquots of this culture was added to 96-well Costar black flat clear bottom non-tissue culture treated plates (Corning #3631) or tissue culture treated plates (Corning #3603) depending on the pathogen under investigation. Bacteria-free fresh media (100 μl) was added to select wells to serve as both biofilm background and plate sterility controls. All plates were covered with lids (Corning #3931) and incubated at 37 °C. It is important to note that though non-tissue culture treated plates (Corning #3631) were not certified to be sterile, their sterility was verified. This was achieved by adding fresh media to a random sampling of plates and incubating for an extended period of time and also by including wells with fresh media during each assay to confirm that there was no bacterial growth. After 24 h incubation, the plate was removed and absorbance at 600 nm (OD600) was recorded to determine cell density. To initiate biofilm staining, the non-adherent contents in the assay plate were removed by pipetting or decanting and wells were washed 3–4 times with double-distilled water. 100 μl of a working stock of FM1-43 was added to each well and the plate was incubated at room temperature in the dark for 30 min. The dye was then removed from the plate and the wells washed 3–4 times with double-distilled water to remove any excess dye before imaging and/or solvent extraction to quantify the biofilm. To quantify biofilm, 100 μl of 50% ethanol in water was added to the stained wells and incubated for 1 h at room temperature in the dark. The assay plate was then measured

using a Fluorimeter (Tecan Infinite M1000) set at an Excitation wavelength – 480 nm and Emission wavelength – 600 nm. All experiments were performed two or more independent times and each biological experiment included 3–5 technical replicates.

2.4. Crystal violet biofilm staining and quantification

For crystal violet (CV) staining, after 24 h incubation, the biofilm plates were washed 3–4 times in a similar way to the above procedure to remove planktonic cells. To each well 100 µl of 1% CV was added and incubated for 15 min. To remove unbound dye, the wells were washed 3–4 times with double-distilled water and the dye extracted with the appropriate extraction agent. 100 µl of each extraction agent was added to the relevant wells for 10 min. After 10 min the contents of the wells (100 µl) were transferred to a new 96 well clear bottom plate to measure absorbance at 595 nm (Tecan Infiniti M1000). The extraction agent for each organism is as follows: *A. baumannii* 0.2% Triton X-100 (Pakharukova et al., 2018), *B. mallei* and *B. pseudomallei* 30% acetic acid (Ramli et al., 2012), *P. aeruginosa*, *S. aureus* and *E. faecium* 30% acetic Acid (O'Toole, 2011), *P. mirabilis* and *B. cereus* 95% ethanol (Czerwonka et al., 2016; Ma et al., 2017). As needed all the assays were quantified using 3–4 independent biological replicates and the averages and standard deviations were enumerated from these observed results.

2.5. Biofilm imaging

Stained biofilms were imaged using a high content imager (Operetta, Perkin Elmer) with Emission / Excitation filters 460–490 nm and 560–630 nm respectively. Images were acquired at 20× magnification in a 96-well format with 20 image fields / well. Consistency of biofilm production was determined using multiple independent replicates (3 or more) for each pathogen under similar assay conditions.

2.6. Compound library screening for biofilm inhibitors

The Prestwick Chemical library (1200 compounds, 96 well format) was screened against *A. baumannii* and *B. pseudomallei* Bp82 to identify biofilm inhibitors. All assay wells had a final concentration of 50 µM of compound, 0.5% DMSO in a total volume of 100 µl. Briefly, using PerkinElmer JANUS, 10 µl of 500 µM solution of each compound in DMSO was transferred into wells of 96-well plates containing 70 µl of the appropriate media (Mueller Hinton for *A. baumannii* and MVBM for *B. pseudomallei* Bp82). Using the INTEGRA VIAFILL liquid dispensing system, 20 µl of a 24 h bacterial culture that was freshly diluted (1:20) in their respective media, was added to wells achieving a total volume of 100 µl and the final compound concentration of 50 µM and 0.5% DMSO. Wells containing the above volumes of media, DMSO (no compound) and bacteria served as positive controls and account for 100% biofilm production. Wells containing the media only with 0.5% DMSO and no bacteria served as no biofilm production controls. Additional controls included 0.2% TWEEN 20 for *A. baumannii*, which prevented biofilm production without affecting bacterial growth and viability. For *B. pseudomallei* Bp82, 8 µg/ml of doxycycline (that kills bacteria) was used as a control.

2.7. Statistical analysis

The statistical reliability of the biofilm formation was evaluated by calculating the Z′ factor using the formula $1 - [(3\sigma p + 3\sigma n)/|\mu p - \mu n|)]$, where µp, σp, and µn, σn are the mean (µ) and standard deviations (σ) of both positive (p) and negative (n) controls. The bacteria free media wells and bacteria containing biofilm wells (no compound addition) were used as negative and positive controls, respectively. For primary screening, Z′ factor ≥ 0.5 was used to validate the results of the assay plates. To determine the compounds antibiofilm activity, the fluorescence values of bacteria + compound treated wells were normalized with bacteria containing biofilm wells, which were considered as 100% biofilm production, and the values obtained were subtracted from 100 to determine the percentage biofilm inhibition. The formula used to calculated biofilm inhibition or bacterial optical density inhibition is, 100 - {[OD or Fluo] / (AvgBacOnly)]*100}, where OD is optical density of the bacteria containing wells, Fluo is the fluorescence of the dye that is bound to the biofilm, AvgBactonly is the OD or Fluo of wells containing bacteria only. A compound is considered as a "Hit" if it prevented ≥80% of biofilm formation and has ≤20% antibacterial/bacteriostatic activity compared to media only controls. Selected hit compounds were verified using dose response studies as follows. The activity of the hits compounds were verified using dose response function. The 50% biofilm inhibitory concentration (EC_{50}) values for each hit compound was determined using Genedata Analyst Software Version 14.0.6 from Genedata (Basel, Switzerland).

3. Results

3.1. Biofilm establishment and FM1-43 staining

Surface untreated black clear flat bottom 96-well polystyrene plates were used to establish biofilms with *P. aeruginosa, A. baumannii, B. pseudomallei, B. mallei, P. mirabilis* and *S. aureus*. For *B. cereus* and *E. faecium* the biofilm establishment was conducted in tissue culture treated polystyrene black clear flat bottom 96-well plates. Production of static biofilm in each of the wells (selected fields) was assessed with FM1-43 staining and fluorescence imaging of the biofilm plates. Images obtained using a high-content imager showed the successful establishment of biofilms for all the tested species (Fig. 1), with some noticeable differences in the bacterial biofilm architecture. Control wells with media only showed no staining with FM1-43 and remained non-fluorescent in our images (data not shown).

3.2. Development of biofilm quantification using FM1-43 staining

To develop an easy to adapt and a robust plate based biofilm quantification assay, we optimized a dye extraction protocol post FM1-43 staining. For initial assessment, *A. baumannii* and *B. pseudomallei* biofilms stained with FM1-43 were extracted with various solvents and reagents including methanol and ethanol (ranging from 25 to 100% prepared in water), 95% isopropanol, acetone, acetonitrile and acetic acid. The fluorescence was measured using a plate reader (top read). We determined that solvents such as methanol and ethanol, preferably diluted in water, were better at extracting the FM1-43 stained biofilm (Fig. S1), as evident from the fluorescence measurements. Alternatively 30% acetic acid commonly used for extraction of crystal violet (CV) dye from biofilms resulted in no FM1-43 fluorescence, potentially from changing the chemical properties of the extracted dye. Each extraction solvent used gave varying quantities of fluorescent signal, possibly due to differences in their ability to interact with the biofilm and to extract FM1-43 bound to the biofilm. Amongst the reagents tested, it was established that 50% ethanol was better at both extracting the FM1-43 from stained biofilms (Fig. S1) and stably retaining the fluorescence over a long period of time (> 1 h) (Fig. S2). The fluorescence emission maxima for the extracted dye in 50% ethanol was also determined to be 600 nm (Fig. S1). Based on these observations, subsequent biofilm quantifications with FM1-43 were carried out using 50% ethanol under the following fluorescence parameters: excitation wavelength - 480 nm; emission wavelength - 600 nm. FM1-43 stained biofilms (Fig. 1) and subsequent FM-143 dye extraction produced a robust fluorescence signal for all eight representative pathogens compared to media control (Fig. 2). However, based on the differences in established biofilm mass, the observed signal intensity did vary between the different pathogens. In addition to the choice of 96 well plate composition (tissue culture treated versus untreated) (Fig. 1), media conditions and bacterial isolates also played a critical role in biofilm production (Fig. S3). This was

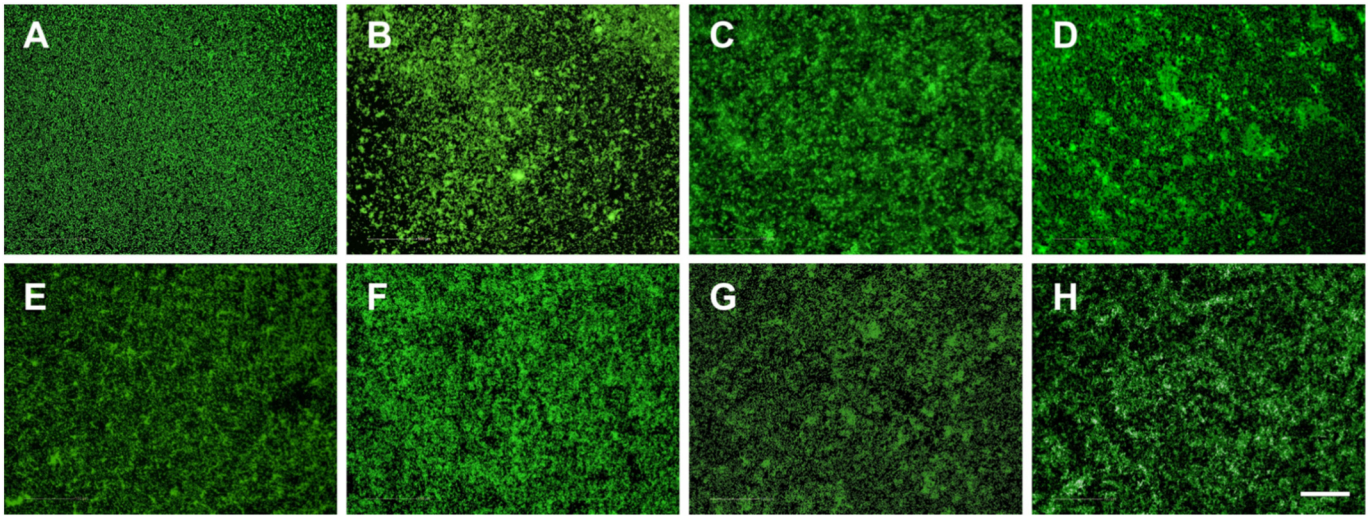

Fig. 1. Establishment of biofilms in 96 well plates. Representative images of biofilms grown in 96-well plates for 24 h, stained with FM1-43 and then images acquired using Operetta imaging system (20× magnification). Biofilm formation and associated observed morphology was consistent amongst multiple independent biological replicates. (A) *Acinetobacter baumannii,* (B) *Burkholderia mallei,* (C) *Burkholderia pseudomallei,* (D) *Proteus mirabilis,* (E) *Pseudomonas aeruginosa,* (F) *Staphylococcus aureus,* (G) *Enterococcus faecium* and (H) *Bacillus cereus*. The scale bar is equivalent to 100 μm.

underscored in our study with four different strains of *S. aureus,* where Tryptic soy broth (TSB) and TSB supplemented with glucose and sodium chloride had pronounced differences in biofilm production (Fig. S3).

3.3. Comparison of FM1-43 to crystal violet staining

Amongst biofilm quantification methods, crystal violet (CV) biofilm assays are most commonly used for biofilm estimation. In order to benchmark the FM1-43 assay to CV staining, we compared both biofilm staining methods simultaneously. Since CV staining protocols are varied and have gone through considerable modifications, for our study we decided to compare 1% CV to our FM1-43 staining. Extraction of FM1-43 stained biofilms was carried out with 50% ethanol, while CV extractions often varied depending on the strains (see materials and methods section). Based on multiple biological repeats of these assays with bacteria-free media wells as controls, it was demonstrated that FM1-43 offered substantially greater sensitivities in biofilm quantification compared to CV methods. As summarized in Table 1, depending on the pathogen, the fold changes observed for FM1-43 was at least 2–17 greater than the CV method. Amongst these pathogens tested, *P. aeruginosa* only had a 2 fold higher FM1-43 signal (24 ± 4.2) than the

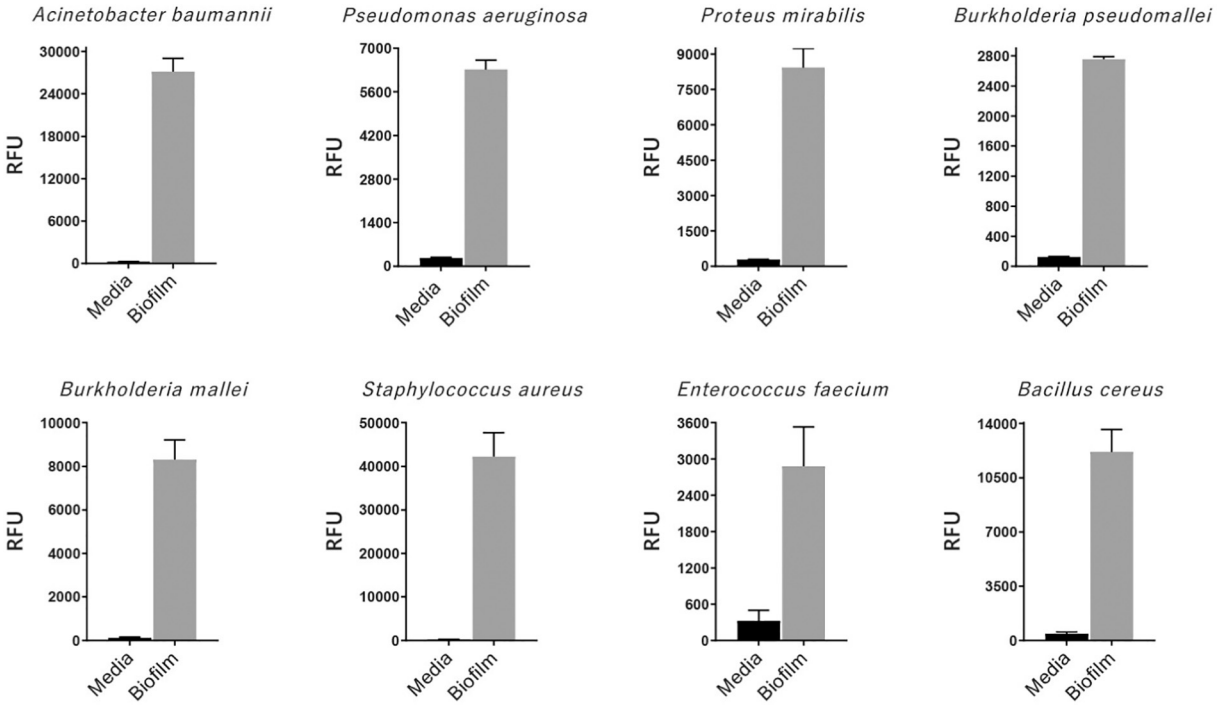

Fig. 2. Bacterial quantification of biofilms using FM1-43. Biofilms were established for eight representative pathogens in 96-well plates. At 24 h post biofilm establishment, the plates were washed, stained with FM1-43 and the dye was extracted using 50% ethanol (1 h) and the fluorescence intensity (RFU) was measured using a plate reader, at an excitation and emission wavelengths of 482 nm and 600 nm respectively. Averages and standard deviations were calculated using five independent well replicates for each pathogen.

Table 1
Bacterial strains and fold change established from biofilm quantification using FM1-43 and crystal violet (CV) dyes.

Bacteria strains	FM1-43 Fold change[a]	Crystal violet Fold change[a]	Ratio fold change FM 1–43/ CV
Acinetobacter baumannii	94 ± 7.3	11 ± 0.5	8.5
Burkholderia mallei CLH001	50 ± 2	3 ± 0.5	16.7
Burkholderia pseudomallei Bp82	24 ± 4.4	6 ± 1.6	4
Proteus mirabilis	18 ± 2.9	2 ± 0.4	9
Pseudomonas aeruginosa	24 ± 4.2	12 ± 3.4	2
Staphylococcus aureus	146 ± 16.9	22 ± 9.1	6.6
Enterococcus faecium	8 ± 1.2	2 ± 0.2	4
Bacillus cereus	37 ± 8.3	6 ± 1.5	6.2

Note: All crystal violet staining was performed using 1% solution of crystal violet (*w/v*).

[a] The fold change was estimated as a ratio of quantified biofilm versus media control wells. The averages and standard deviations were calculated from 3 to 4 independent biological replicates. Raw data values are shown in Table S1.

CV method (12 ± 3.4), whereas *B. mallei* has the highest difference of 17 fold (50 ± 2 for FM1-43 versus 3 ± 0.5 for CV). Amongst all these pathogens, *S. aureus* isolate (ATCC 43300) utilized here had the highest biofilm read out for both the methods employed (Fig. 2) with fold changes of 146 ± 16.9 and 22 ± 9.1 for FM1-43 and CV respectively. These results offer some indications as to how large the signal to background measurements could be when utilizing FM1-43 for biofilm quantification.

3.4. Chemical library screening for biofilm inhibitors

To demonstrate the utility of FM1-43 biofilm quantification method for HTP screening applications, the Prestwick library (containing 1200 off-patent compounds and about 95% FDA approved) was screened against *B. pseudomallei* and *A. baumannii*. The compounds were screened at a final concentration of 50 μM (0.5% DMSO), single replicate plates. Assay controls for both pathogens included medium alone and medium containing 0.5% DMSO and appropriate bacterium. Post 24 h incubation of biofilm assay plates with the compounds, the optical densities (OD600) were recorded first to identify compounds that caused potential growth inhibition. The plates were then washed and stained with FM1-43 as detailed before. The overall screening quality was determined using the Z' estimate with a cutoff score ≥ 0.5. Based on our measurements, compounds that showed ≥80% biofilm inhibition and ≤20% growth inhibition were identified as biofilm hits (Fig. 3). For *A. baumannii* we identified 5 compounds (Fig. 3a) and for *B. pseudomallei* two compounds (Fig. 3b) that are structurally unrelated to the *A. baumannii* biofilm inhibitory compounds. Upon validation of the down selected compounds, we determined that ethaverine and rifabutin (refer Fig. S4 for chemical structures) exhibited a dose-dependent inhibition of *A. baumannii* and *B. pseudomallei* biofilms respectively, without any significant alteration in their growth potential. The effective biofilm inhibitory concentration (EC_{50}) of ethaverine against *A. baumannii* was determined to be 160 μM, while rifabutin to *B. pseudomallei* was determined to be 29 μM. Interestingly, when we tested papaverine, an analog of ethaverine against *A. baumannii*, we did not observe any inhibition of biofilm production (data not shown), indicating that the observed biofilm inhibition is mediated specifically by ethaverine interacting with *A. baumannii*.

4. Discussion

Over the years several useful methods for growing and studying biofilms have emerged (Franklin et al., 2015). Amongst these methods, static biofilm employing microtiter dish has emerged as a choice method for assays consisting of multiple samples (e.g., screening transposon mutant libraries, biofilm disruptors, etc.). State-of-the-art static biofilm quantification assays predominantly utilized CV based colorimetric methods for biofilm quantification. Notably this method has undergone demonstrable alterations depending on the bacterial species under investigation, requiring a need for assay standardization for any new strains under investigation. Alternatively, our results indicate that the adaptation of FM1-43 fluorescent dye to quantify biofilm has broad utility, high sensitivity and a high signal to background ratio when compared to the more common CV staining method. We demonstrated that this assay can be applied to a range of organism including Gram negatives, Gram positives, cocci and bacilli; additionally, the assay functions well with biofilms formed in the presence of various types of media. (Figs. 1 and 2; Table 1). These improvements with FM1-43 could be due to a combination of biophysical properties of the dyes (fluorescence versus absorbance) or due to better FM1-43 staining of biofilm and/or better extraction of the dye from biofilms when using 50% ethanol. Further, an additional benefit of implementing this FM1-43 based assay is that both imaging and quantification of biofilm can be achieved with a single reagent. There appears to be no noticeable limitations to deploying this method for the quantification of all bacterial biofilms.

The goal for developing a new fluorescent assay is also to address the need for improved HTP assay tools that overcome the need for a two plate system as needed for CV biofilm quantification. Using our newly standardized assay we established the use of a single (96 well black clear bottom) plate for biofilm staining, dye extraction and subsequent fluorescence measurement (top read). Our successful execution demonstrated that the FM1-43 assays is readily adaptable for high throughput screening in a simple 96 well format. Starting with a library of 1200 compounds, which offered high chemical and pharmacological diversity and have documented data for their bioavailability and safety in humans, we were able to screen and obtain statistically robust (Z' > 0.5) data sets. Robustness of our assay helped with the down selection of one or more potential inhibitors of biofilm production against *A. baumannii* and *B. pseudomallei* that did not have marked effect on their cell densities. Representative inhibitors of these organisms further exhibited a dose dependent response to biofilm inhibition with relatively no significant impact on bacterial cell density (Fig. 3). Even though agents which kill bacteria within biofilms are highly useful, our goal for this screen was primarily to identify agents that disrupt the formation of biofilms. Interestingly, while standardizing FM1-43 biofilm assay conditions, we observed that 0.2% Tween-20 significantly impacts *A. baumannii* biofilm production without affecting bacterial cell density, and thus could potentially be used as control for biofilm inhibition assays. Identification of similar control compounds that inhibit biofilm without affecting growth for various bacterial pathogens would be highly useful as standards for biofilm assays.

Given FM1-43 fluorescence offers higher sensitivity than CV, it is most likely that even low biofilm biomass can be detected with FM1-43. This observation is highlighted in Table 1 where the fold differences are significantly higher for all the FM1-43 samples than CV samples. This offers the possibility of adapting this assay in a 384 well format, which could result in establishment of limited bacterial biofilm biomass in the wells. If successful, we will be able to increase the throughput and also decrease the reagents used from the chemical library, dye and the solvent for extraction. Importantly, few studies have successfully established biofilms in 384-well plates for fluorescence imaging based studies of the biofilm (Navarro et al., 2014; Peng et al., 2010). Though imaging can be useful, this requires sophisticated instrumentation and data processing capabilities. Further, the biofilm images captured are often representative of a small region in the well and prone to imaging biases. Whereas in our method that utilizes solvent extraction of a fluorescent dye, the plate-reader is relatively inexpensive and the reading is faster and does not require complicated data analysis. Our method further offers an ensemble measurement of the biofilm per well,

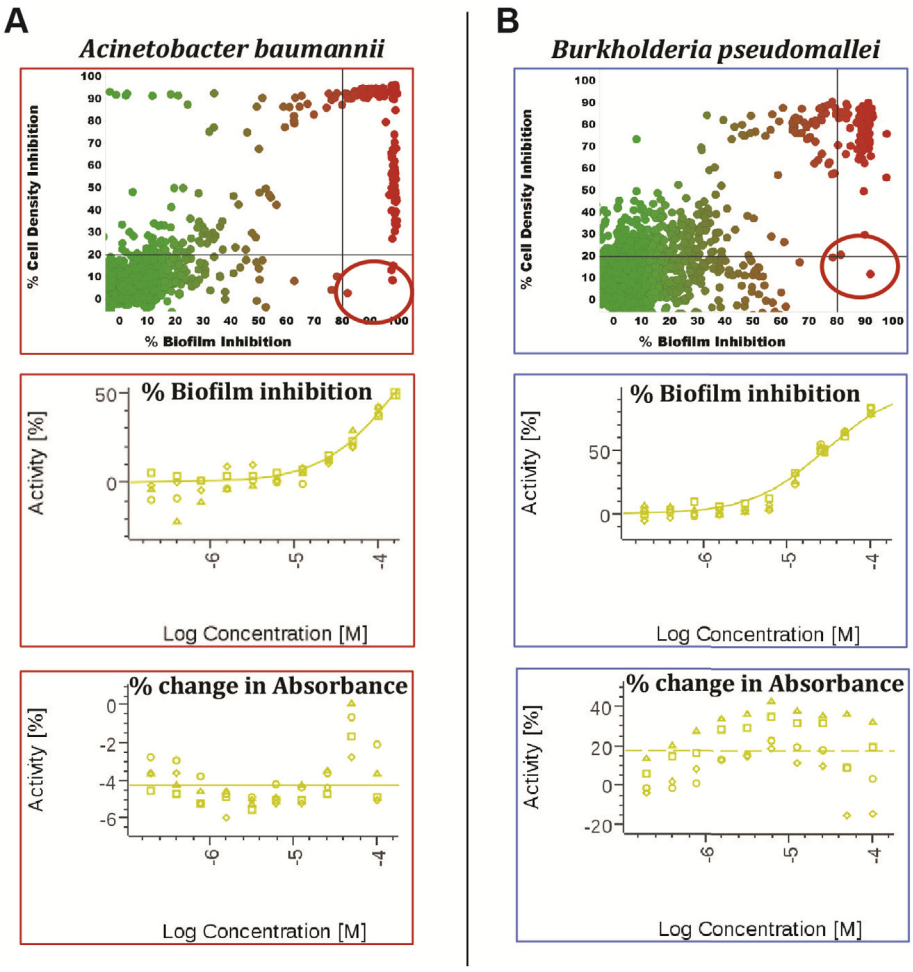

Fig. 3. Screening of Prestwick library to identify compounds that inhibit *A. baumannii* and *B. pseudomallei* biofilm formation. Fig. A (*A. baumannii*) and B (*B. pseudomallei* Bp82) top panels show scatter plots of all compounds screened against respective pathogens showing calculated % cell density inhibition versus % biofilm inhibition. The dots below horizontal and vertical lines marked in a red oval represent compounds of interest whose % cell density inhibition is ≤20 and % biofilm inhibition is ≥80%. Middle panels show dose-dependent biofilm inhibition of two compounds identified in our primary screen, ethaverine and rifabutin against *A. baumannii* and *B. pseudomallei* respectively. The bottom panels show % change in absorbance with compounds ethavarine and rifabutin against *A. baumannii* and *B. pseudomallei* respectively, showing no demonstrable bacterial growth inhibition at these tested concentrations. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

accounting for the entire biofilm produced. We are currently in the process of determining if our fluorescent biofilm assays can be executed in a 384-well format. If successful we will adapt the method for developing our subsequent HTP screening and other biofilm studies.

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Conflict of interest

No conflict of interest declared.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.mimet.2019.02.018.

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