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High-throughput screening alternative to crystal violet biofilm assay combining fluorescence quantification and imaging

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\textbf{A R T I C L E  I N F O}

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\textbf{A B S T R A C T}

Biofilms are microbial communities commonly composed of mixed bacterial species where frequent inter- and intraspecies interactions occur (Costerton et al., 2003; Røder et al., 2016; Tan et al., 2017). The use of biofilms as model systems for investigating such interactions creates a need for suitable tools that enable high-throughput screening of the adhesive capabilities of the contributing species and their synergistic effects. Even though multiple methods have been developed for studies of such interactions there are still many limitations regarding reproducibility and resolution (Azeredo et al., 2017).

Among these methods, crystal violet (CV) staining of biofilms in microplate wells and pegs (Christensen et al., 1985; Ceri et al., 1999; Štepanović et al., 2000) is one of the most extensively used platforms for high-throughput quantification of biofilm biomass (Djordjević et al., 2002; Extremia et al., 2011; Merritt et al., 2011; Røder et al., 2015; Doll et al., 2016). Crystal violet binds negatively charged molecules and thus stains both bacteria and the surrounding biofilm matrix. However, the use of CV as a quantitative method has many limitations, including i) toxicity (Merek, 2017); ii) unspecific binding to negatively charged molecules and iii) low reproducibility (Peeters et al., 2008; Krägli et al., 2019) due to uneven dye extraction or differential removal of biofilm biomass throughout the washing steps.

Staining with safranin has been proposed as a non-toxic alternative for quantifying biofilm biomass (Christensen et al., 1982; Ommen et al., 2017); however, the need for dye extraction can lead to limitations similar to CV. Other studies have focused on assessing metabolic activity in biofilms using redox indicators such as triphenyltetrazolium chloride (TTC), 2,3-bis (2-methoxy-4-nitro-5-sulphophenyl)-5-[(phenylamino) carbonyl]-2H-tetrazolium hydroxide (XTT) or resazurin (Pettit et al., 2005; Koban et al., 2012; Sabaeifard et al., 2014). Despite the advantages of measuring biofilm metabolically active cells, the main drawback is that different microorganisms metabolize the dyes at different rates, requiring prior optimization and making it difficult to apply to microbial communities (Peeters et al., 2008; Sandberg et al., 2009; Van den Driessche et al., 2014).

Other methods involve dyes staining specific biofilm components such as nucleic acids or chromosomal tagging with fluorescent proteins for strains compatible with genetic manipulation. These approaches have previously been used for imaging and quantifying microbial biofilms (Lawrence et al., 1998; Klausen et al., 2003; Peeters et al., 2008; Larrosa et al., 2012; Tolker-Nielsen and Sternberg, 2014; Sanchez-Vizute et al., 2015; Stiefel et al., 2015), but are so far not applicable for high-throughput screenings.

In this study, we combine quantitative and imaging features in a single method to facilitate high-throughput biofilm screening for i.e., genetic mutants, growth conditions or species combinations in microbial communities. We assessed biofilm adhesion and variation by the conventional CV assay compared to i) fluorescent staining with SYTO 9 and ii) gfp-tagged strains using the Cytation 5 instrument, a multi-mode plate

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We were interested in comparing CV staining and fluorescence (FL) for biofilm biomass quantification of isogenic strains (mutant screening) but also more complex biofilms such as multispecies biofilms. For the former, we chose *Pseudomonas putida* KT2442 (wt), that undergoes rapid biofilm dispersal in response to nutritional stress, and its derivative mutant MRB1 which is resistant to dispersal due to a mutation in the lapG protease gene (Gjermansen et al., 2005; López-Sánchez et al., 2013). Additionally, we tested a four-species community (SPMX) composed of *Stenotrophomonas rhizophila*, *Paenibacillus amylolyticus*, *Microbacterium oxydans* and *Xanthomonas retroflexus*, where only the latter is capable of forming abundant biofilm in monoculture in microplate wells. These strains have shown a strong synergy in mixed vs. single cultures and have been extensively used as a model for investigating interactions in multispecies biofilms (Liu et al., 2021; Ren et al., 2014, 2015; Hansen et al., 2016; Herschend et al., 2017).

Biofilm formation in microtiter plate wells was quantified using a modified method (O’Toole and Kolter, 1998). Three replicate cultures of each strain were grown overnight (16 to 20 h) in 5 ml LB at 30 °C and 250 rpm. Cultures were adjusted to optical density (OD$_{600}$) 1, diluted 100 $\times$ in 20% LB (w/v in 1 $\times$ PBS) and 150 μl was inoculated into 96-well microtiter plates (Cellstar, Greiner) in replicate plates (for CV and SYTO 9 staining) and incubated at 30 °C for 24 h. Planktonic cells were removed by inversion of the plate and washed 3 times with 150 μl 1 $\times$ PBS. For CV, biofilms were stained with 160 μl 1% CV (w/v, in water) for 20 min and followingly washed 3 times with 160 μl 1 $\times$ PBS. Bound CV was finally extracted with 200 μl 100% Ethanol (AnalaR NORMAPUR®, VWR) for 20 min at 450 rpm in a Heidolph Vibramax100 mixer (López-Sánchez et al., 2013). Biofilm was quantified as a measurement of absorbance at 590 nm using the Cytation 5 Cell Imaging Multi-Mode Reader, BioTek (Cytation 5). For SYTO 9 staining, the planktonic cells were removed and the attached cells were washed as indicated above before adding 160 μl 5 μM SYTO 9 solution in 20% LB. SYTO 9-stained biofilms were measured with the Cytation 5, (Ex. 479/20, Em. 520/20 nm).

### Table 1

<table>
<thead>
<tr>
<th></th>
<th>Fluorescence (RFU)</th>
<th>Crystal violet (A$_{590}$)</th>
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<td></td>
<td>Average</td>
<td>StDev</td>
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<tr>
<td><strong>X</strong></td>
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<td>607</td>
</tr>
<tr>
<td>SPMX</td>
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</tr>
<tr>
<td>KT2442</td>
<td>878</td>
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</tr>
<tr>
<td>KT2442 MRB1</td>
<td>1661</td>
<td>215</td>
</tr>
</tbody>
</table>

Fig. 1. Biofilm formation of *Xanthomonas retroflexus* (X) mono- and mixed cultures (SPMX) after 24 h. Biomass quantitation of 24-h biofilms stained with SYTO9 (A, fluorescence area scan) or crystal violet (B, 590 nm). Black dots outside the boxes denote outliers. C, Images of *X. retroflexus* (X) and multispecies (SPMX) biofilms stained with SYTO 9, 20× PL FL objective. Scale bar corresponds to 72 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 1

Data summary for biomass quantification by fluorescence and crystal violet. RFU: relative fluorescence units; StDev: standard deviation; CoV: coefficient of variation (%); ratio: fold change relative to *Xanthomonas. retroflexus* monospecies (X) or *Pseudomonas putida* wild type (KT2442). $n = 21$, 3 biological replicates each including 7 technical replicates.
using both endpoint and well area scan measurements (Fig. 3A, 5 × 5 points; well diameter 6604 μm; probe diameter 2000 μm) expressed in relative fluorescence units (RFU). For biofilm biomass calculations, average values, standard deviations, and coefficient of variation were used. For statistical analysis, Welch’s t-test was generally applied, unless otherwise stated, with P-values < 0.05 considered as significant. Sidák’s correction was applied in multiple comparisons (MC) and Pearson’s correlation coefficients (r) were calculated for comparing endpoint vs. area scanning methods, and corresponding P-values (supplementary). All statistical analyses were done using GraphPad Prism 6.01 (GraphPad Software, Inc.).

Fluorescence images were acquired with the Cytation 5 Gen5 software Image Prime 3.10 using manual mode with LED intensity 10, integration time 5 ms and camera gain 22.9 with a 20 × PL FL objective (Olympus) and GFP 469,525 filter cube (P/N 1225101). Three replicate wells were recorded, two images per well and random pictures shown.

We compared quantification of biofilm biomass between SYTO 9 staining and CV for X. retroflexus monospecies (X) vs. mixed-species biofilms (SPMX). When using fluorescent staining, we observed a 3.3-fold significant induction in biofilm biomass in the bacterial community compared to single species (P < 0.0001, two-tailed Welch corrected t-test) (Fig. 1A, Table 1), while CV staining showed non-significant difference (P = 0.0819, Welch corrected t-test) (Fig. 1B). Variation was similar for both methods, reflecting that more complex communities or types of biofilm produced may have an impact of biomass variability. However, the discrimination level differed depending on the staining method used. Mono- and multispecies biofilms had significantly higher biomass than the blank when stained with SYTO 9 but not with CV (Sidák’s MC test, P < 0.05, Table S1). Imaging of the wells also provided evidence of a distinct biofilm for the multispecies combination regarding surface coverage and topology (Fig. 1C) consistent with the fluorescent quantitative data (Fig. 1A). Additionally, differences in estimated biomass in X. retroflexus monospecies biofilms may be explained by the nature of both stains since CV stains cells and the extracellular matrix (CV) while SYTO 9 stains DNA, and, to a lower extent, eDNA (Li et al., 2003).

Next, we examined biofilm biomass and structure in a chromosomally gfp-tagged P. putida KT2442 strain and its isogenic mutant MRB1 (Fig. 2). Comparison of P. putida fluorescently tagged strains resulted in significant difference between wild-type and mutant strains (P < 0.0001, Welch corrected t-test) irrespective of the quantification method, likewise mutant vs. wt biomass ratios (fluorescence, 1.9-fold; CV, 1.8-fold; Table 1). CV yielded higher variation regardless of the strain tested (FL 4–13%; CV 20–33%). The sensitivity of both assessment methods was also evaluated by comparison with blank values (Table S1). MRB1 biofilm was significantly different from the baseline when measured by fluorescence (Sidák’s MC test, P < 0.001), unlike KT2442 biomass regardless of the quantification method (P = 0.1077, FL; 0.9997, CV). Besides the increased biomass in the MRB1 mutant, imaging of the wells also evidenced differential biofilm structure in both strains, showing scattered, adhered wild-type cells in comparison with more complex microcolony structure covering a larger surface area for the for the MRB1 mutant (Fig. 2C).

We further tested whether the fluorescence scanning method would
influence the results and variation. The Cytation 5 enables two types of readings: endpoint measurement acquires a single measurement in the centre of each well, resulting in faster readings and available in all plate readers. In contrast, area scan acquires multiple measurements in each well (Fig. 3A), and thus a more integrated reading of the entire well. We measured fluorescence of KT2442-gfp, MRB1-gfp and non-inoculated wells to assess the variability but also the discrimination level of both scanning methods. We observed significant positive correlation between endpoint and area scan fluorescence measurements irrespective of the type of biofilm tested ($r = 0.8634$, KT2442-gfp; $r = 0.4412$, MRB1-gfp; $r = 0.6111$, blank) although the strongest correlation was found in the MRB1 mutant (Fig. 3B). Fluorescence of KT2442-gfp was significantly different from that of MRB1-gfp regardless of the method used ($P < 0.0001$) but not from the blank ($0.4580$, endpoint; $0.3112$, area scan). This suggests that sensitivity of the method could be a limitation for strains producing low amounts of biofilm. Biofilm type accounted for most of the variation (88.27%), unlike the scanning method (1.36%), even though both factors were found significant (Two-way ANOVA, $P$-value $< 0.0001$). Variability was generally higher using endpoint mode than area scan but only MRB1-gfp showed significant difference (Fig. S1, Table S2). Thus, we can conclude that biofilm complexity rather than measurement mode was responsible for the variation found and endpoint reading may therefore safely be used when area scan is not available, even though it may not be suitable for all types of biofilm topology.

This study presents an alternative to CV assay for monospecies but also more complex biofilms, in terms of robustness, simplicity and information recorded, such as biofilm morphology. Unlike the CV assay, biofilm quantification using the Cytation 5 does not involve additional dye incubation, extraction or washing steps, making biofilm processing milder and faster for high-throughput screenings. Viability of stained cells should nonetheless be tested in case that subsequent physiological assays of continuous monitoring are wanted. Other studies have reported downsides of SYTO 9 staining associated with different binding affinity to live and dead cells or permeability in Gram-positive and Gram-negative cells (Stiefel et al., 2015; McGoverin et al., 2020). However, there is a wide array of nucleic acid and biomass stains that can circumvent this problem (Thermo-Scientific, 2014). Even though we used the Cytation 5 imaging reader, comparison of endpoint and area scan measurements proves that this workflow is also compatible with conventional plate readers and microscopy. We envision our approach as a streamlined alternative to CV quantification that could facilitate high-throughput biofilm screenings for i.e. genetic mutants, growth conditions or species combinations in microbial communities, but also acquisition of topological information of such biofilms.

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Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.mimet.2021.106343.

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