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

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Abstract

The crystal violet assay is widely used for biofilm quantitation despite its toxicity and variability. Here, we instead combine fluorescence labelling with the Cytation 5 multi-mode plate reader, to enable simultaneous acquisition of both quantitative and imaging biofilm data. This high-throughput method produces more robust data and provides information about morphology and spatial species organization within the biofilm.

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; Stepanović et al. 2000) is one of the most extensively used platforms for high through-put quantification of biofilm biomass (Djordjevic et al. 2002; Extremina 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 (Merck 2017); ii) unspecific binding to negatively charged

molecules and iii) low reproducibility (Peeters et al. 2008; Kragh 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 2011; Sanchez-Vizueté 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 multimode plate reader integrating imaging and quantitative capabilities.

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 *P. 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.; 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 (OD₆₀₀) 1, diluted 100x in 20 % LB (w/v in 1x 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 1x PBS. For CV, biofilms were stained with 160 µl 1 % CV (w/v, in water) for 20 minutes and followingly washed 3 times with 160 µl 1x PBS. Bound CV was finally extracted with 200 µl 100 % Ethanol (AnalaR NORMAPUR®, VWR) for 20 minutes 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 using both endpoint and well area scan measurements (Figure 3A, 5x5 points; well diameter 6604 microns; probe diameter 2000 microns) 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. Šidák'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.).

Fluorescent images were taken 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 20x 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 (Šidák's MC test, P < 0.05, Table S1). Imaging of the

wells also provided evidence of a distinct biofilm for multispecies 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* KT2242 strain and its isogenic mutant MRB1 (Figure 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 ± 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 (Šidák's MC test, $P < 0.001$), unlike KT2242 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 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 (Figure 3A), and thus a more integrated reading of the entire well. We measured fluorescence of KT2242-*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 fluorescent measurements irrespective of the type of biofilm tested ($r = 0.8634$, KT2242-*gfp*; $r = 0.4412$, MRB1-*gfp*; $r = 0.6111$, blank) although the strongest correlation was found in the MRB1 mutant (Fig. 3B). Fluorescence of KT2242-*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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Figures and tables

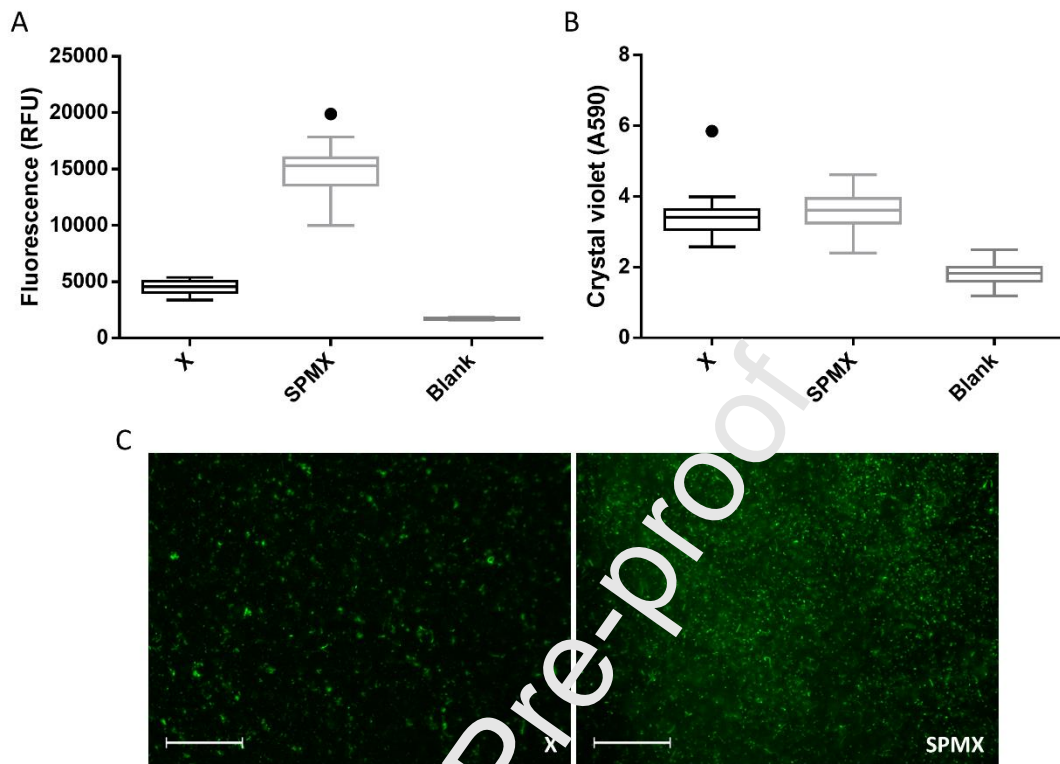


Figure 1. Biofilm formation of *X. retroflexus* (X) mono- and mixed cultures (SPMX) after 24 hours. Biomass quantitation of 24-hour biofilms stained with SYTO9 (A, fluorescence area scan) or crystal violet (B, 590 nm). Black dots outside the boxes denote outliers. C, Images of *X. retroflexus* (X) and multispecies (SPMX) biofilms stained with SYTO 9, 20X PL FL objective. Scale bar corresponds to 72 μm .

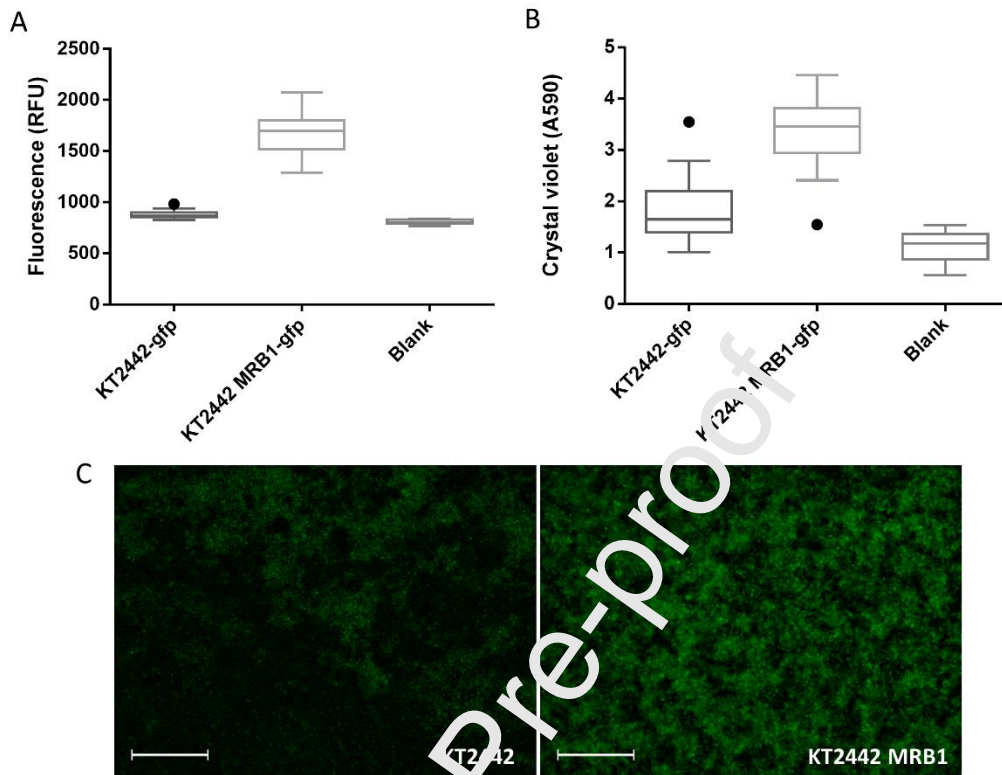


Figure 2. Biofilm formation of *gfp*-tagged *P. putida* KT2442 and MRB1 after 24 hours. Biomass quantification of 24-hour biofilms (A, fluorescence area scan) or crystal violet (B, 590 nm). Black dots outside the boxes denote outliers. C, Images of KT2442-*gfp* and KT2442 MRB1-*gfp* biofilms, 20X PL FL objective. Scale bar corresponds to 72 μm .

	Fluorescence (RFU)				Crystal violet (A_{590})			
	Average	StDev	CoV	Ratio	Average	StDev	CoV	Ratio
X	4492	607	14%	1	3,40	0,51	15%	1
SPMX	14931	2255	15%	3,3	3,60	0,51	14%	1,1
KT2442	878	37	4%	1	1,85	0,62	33%	1
KT2442 MRB1	1661	215	13%	1,9	3,35	0,67	20%	1,8

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 *X. retroflexus* monospecies (X) or *P. putida* wild type (KT2442). n = 21, 3 biological replicates each including 7 technical replicates.

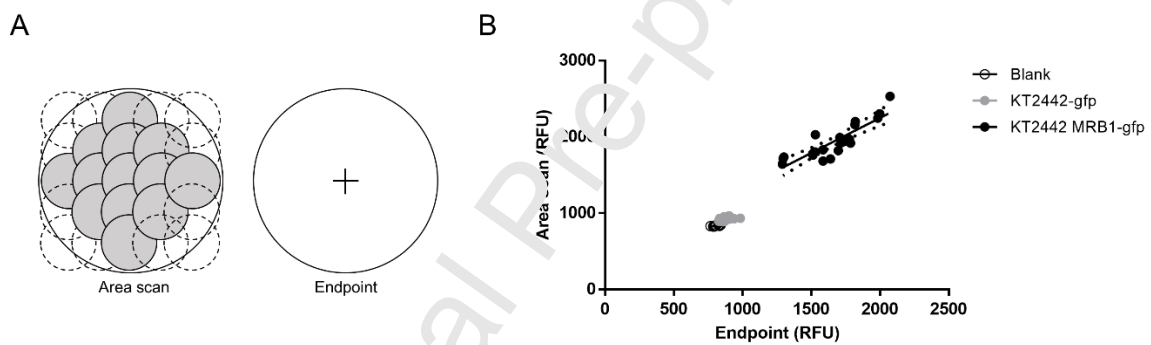


Figure 3. Biofilm biomass comparison of fluorescence by measuring method. A. Representation of area scan and endpoint measurement. B. Correlation of fluorescent readings using endpoint vs. area scan methods for *P. putida* KT2442-*gfp*, *P. putida* KT2442 MRB1-*gfp* and blanks. Individual points represent 21 replicates corresponding to 3 biological replicates each including 7 technical replicates. Lines represent linear regressions of each data series with the 95 % confidence interval (dotted lines).

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.

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Highlights

- Fluorescent staining was evaluated as an alternative to crystal violet for biofilm quantitation.
- Fluorescent quantitation proved higher sensitivity in complex communities.
- Fluorescent staining of monospecies biofilms produced similar results as crystal violet but with lower variation.
- Cytation 5 further provided topological information of simple or complex biofilms.
- We recommend fluorescent imaging and quantitation for high-throughput biofilm screenings.

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