Video Article

A Platform of Anti-biofilm Assays Suited to the Exploration of Natural Compound Libraries

Malena E. Skogman[1], Pia M. Vuorela[1], Adyary Fallarero[1]

[1]Pharmaceutical Design and Discovery (PharmDD), Faculty of Pharmacy, University of Helsinki

Correspondence to: Adyary Fallarero at adyary.fallarero@helsinki.fi

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Abstract

Biofilms are regarded as one of the most challenging topics of modern biomedicine, and they are potentially responsible for over 80% of antibiotic-tolerant infections. Biofilms have displayed an exceptionally high tolerance for chemotherapy, which is thought to be multifactorial. For instance, the matrix provides a physical barrier that decreases the penetration of antibiotics into the biofilm. Also, cells within the biofilms are phenotypically diverse. Likely, biofilm resilience arises from a combination of these and other, yet unknown, mechanisms. All of the currently existing antibiotics have been developed against single-cells (planktonic) bacteria. Therefore, so far, a very limited repertoire of molecules exists that can selectively act on mature biofilms. This situation has driven a progressive paradigm shift in drug discovery, in which searching for anti-biofilms has been urged to occupy a more prominent place. An additional challenge is that there are a very limited number of standardized methods for biofilm research, especially those that can be used for large-throughput screening of chemical libraries. Here, an experimental anti-biofilm platform for chemical screening is presented. It uses three assays to measure biofilm viability (with resazurin staining), total biomass (with crystal violet staining), and biofilm matrix (using a wheat germ agglutinin, WGA-fluorescence-based staining of the poly-*N*-acetyl-glucosamine, PNAG, fraction). All the assays were developed using *Staphylococcus aureus* as the model bacteria. Examples of how the platform can be used for primary screening as well as for functional characterization of identified anti-biofilm hits are presented. This experimental sequence further allows for the classification of the hits based upon the measured end-points. It also provides information on their mode of action, especially on long-term versus short-term chemotherapeutic effects. Thus, it is very advantageous for the quick identification of high-quality hit compounds that can serve as starting points for various biomedical applications.

Video Link

The video component of this article can be found at http://www.jove.com/video/54829/

Introduction

Bacteria can switch between two very different lifestyles, planktonic and sessile, of which a biofilm is the most common example. In biofilms, bacteria form structured communities embedded in a self-produced matrix[1]. This self-produced matrix is a barrier between the bacteria and their external environment, and it protects the microbial cells, keeping them in close proximity. The composition of the biofilm matrix varies between and even within species, but it mostly consists of a tight network of lipopolysaccharides, extracellular DNA, and proteins. The matrix serves as a physical barrier inhibiting the entrance of harmful agents, but it also protects the biofilm from dehydration and prevents nutrients from escaping the cell[2].

Biofilms are regarded as one of the most challenging topics of modern biomedicine, and they are purportedly responsible for over 80% of antibiotic-tolerant infections[3]. They display an inherently high tolerance against external threats: humidity, osmotic pressure, mechanical stress[4], heat, UV radiation[5], disinfectants, antimicrobial agents, and the host immune system[1]. For example, the required antimicrobial agent concentration required to kill a biofilm has been shown to be up to 1,000 times higher in comparison to that required to kill planktonic bacteria. The explanation for this higher tolerance seems to be multifactorial. The matrix provides a physical barrier that decreases the penetration of antibiotics into the biofilm. Also, cells within the biofilms are phenotypically diverse; they transition between different metabolic states due to an existing gradient of oxygen, nutrients, and metabolites between the inner and outer parts of the biofilm[6]. Hence, in some biofilm regions, such as the core, bacteria are deprived of oxygen and nutrients and live in a metabolically less active or a completely dormant state[7]. The completely dormant cells are referred to as persister cells, and they are not susceptible to conventional antimicrobial treatments[8]. Thus, it is likely that biofilm resilience arises from a combination of the presently suggested and other, yet unknown, mechanisms. *Staphylococcus* spp. are still among the most problematic gram-positive bacteria, causing severe, often biofilm-related, infections[4]. It is suggested that up to 99% of all bacteria are associated within biofilms, making it the predominant bacterial lifestyle[3]. However, all of the currently existing antibiotics have been developed against single-cell (planktonic) bacteria. So far, a very limited repertoire of molecules exists that can selectively act on mature biofilms. This situation has driven a progressive paradigm shift in drug discovery, in which searching for anti-biofilms has been urged to occupy a more prominent place.

From a methodological perspective, additional challenges exist, as only a limited number of biofilm methods have been developed by standard-setting organizations, especially those applicable to the high-throughput screening of chemical libraries. All standardized assays (with only one exception) are based upon biofilm reactors, and these methods require large working volumes and large amounts of compounds to be tested, which are usually unavailable during the early investigational stage[9-12]. The only existing standardized screening-applicable assay is the so-called Calgary Biofilm Device, from which the commercially available minimum biofilm eliminating concentration (MBEC) system was developed[13-15]. However, the limitation of this assay is that the biofilms are grown on pegs, and not all bacterial species or even strains within the same species are able to form biofilms on this device. Moreover, methods that can be particularly applied to the exploration of natural compounds are needed. Natural products have been the major source for innovation in antimicrobial drug discovery over the past century[16]. They can provide novel anti-biofilm compounds with unique mechanisms of action that can also be effective against persister cells. Thus, the exploration of natural and naturally inspired libraries has high chances of producing promising and unique anti-biofilm leads.

Here, we present the experimental details of a platform of assays that was developed for the chemical screening of anti-biofilm compounds using three assays to measure the effects on the viability, total biomass, and matrix of *Staphylococcus aureus* biofilms. The first assay measures biofilm viability, and it is based on resazurin staining. Resazurin is a redox stain that is blue and non-fluorescent in its oxidized state and turns into pink, highly fluorescent resorufin when reduced by the metabolic activity of the bacteria. It is a very simple and fast method suitable for primary screenings[17-20]. The second assay, based on crystal violet staining, measures total biofilm mass. Crystal violet is a widely used stain for studying bacteria and bacteria in biofilms[19,21-23]. The assay is based on inexpensive reagents and has a simple absorbance endpoint reading. Finally, the third assay targets the extracellular polymeric substance (EPS)-matrix of the biofilm via wheat germ agglutinin (WGA), which binds specifically to poly-*N*-acetyl-glucosamine residues (PNAG) present in the matrix of staphylococcal biofilms[24]. The WGA is conjugated with a fluorophore that can be detected using fluorescence intensity readers[25]. We present here the rationale and details of the platform we developed, including examples of applications.

Protocol

1. Growing the Bacteria

1. Pre-culture the bacteria overnight in a tryptic soy broth (TSB) at 37 °C with 220 rpm shaking (16-18 hr).
2. Dilute the pre-culture 100-1,000 times (depending on the growth rate of the bacteria; here, 1,000 times is used for *S. aureus*) in fresh TSB and let it grow at 37 °C and 200 rpm to reach exponential growth (optical density at 595 nm (OD_{595}) between 0.2 and 0.6).
   NOTE: This step requires strain-specific optimization.

2. Biofilm Formation: Pre- and Post-exposure

1. Dilute the exponentially grown culture 100 times (this equals approximately 10^6 colony-forming units per milliliter (CFU/ml)).
2. **Pre-exposure protocol**
   1. For untreated control samples, add 200 µl of the diluted bacterial culture per well of a sterile 96-microwell plate.
   2. Add 4 µl of a test compound or a control antibiotic (50x stock solution) and 196 µl of the diluted bacterial culture per well.
   3. Incubate it at 37 °C on a plate shaker at 200 rpm for 18 hr.
      Note: Here we use a shaker with a 2 mm orbit.
3. **Post-exposure protocol**
   1. For all samples, add 200 µl of the diluted bacterial culture per well of a sterile 96-microwell plate.
   2. Incubate it at 37 °C on a plate shaker at 200 rpm for 18 hr.
      Note: Here we use a shaker with a 2 mm orbit.
   3. Remove the entire planktonic solution carefully, without touching the biofilm, using a multichannel pipette.
   4. Add 4 µl of a test compound or a control antibiotic (50x stock solution) and 196 µl of TSB per well.
   5. Incubate it at 37 °C on a plate shaker at 200 rpm for an additional 24 hr.
      Note: Here we use a shaker with a 2 mm orbit.

3. Resazurin Staining Protocol for the Viability Assessment of Biofilms

1. Prepare a stock solution of 0.1 mg/ml resazurin (0.4 mM) in sterile PBS. Keep this stock sterile, protected from light exposure, and at 4 °C.
2. Dilute the resazurin stock 1:50 in sterile phosphate-buffered saline (PBS) to achieve a final concentration of 20 µM.
3. Transfer the entire planktonic solution (leaving the biofilms in the wells) carefully, without touching the biofilms or creating air bubbles, to a separate, clean 96-well plate using a multichannel pipette.
4. Wash the biofilms once with sterile PBS by adding 200 µl per well, and remove it carefully using a multichannel pipette.
5. Add 200 µl of the diluted resazurin per well of the biofilm plate using a multichannel pipette.
6. Incubate it in darkness, at room temperature (RT), and 200 rpm, shaking for approximately 20 min until the untreated biofilm controls are evenly pink.
7. Measure the fluorescence at λ_{exc} = 560 nm and λ_{em} = 590 nm with a plate reader (top reading).

4. Crystal Violet Staining Protocol for Biomass Quantification of Biofilms Using the Same Plate as in Step 3

1. Remove the resazurin stain carefully from the wells using a multichannel pipette.

2. Fix the biofilms with 200 µl of methanol for 15 min.
3. Let the plate air dry for 10 min.
4. Add 190 µl of 0.02 % (vol/vol, diluted in deionized water) crystal violet solution, carefully using a multichannel pipette. Avoid touching the sides of the wells with the stain while pipetting and prevent the formation of air bubbles by not pressing the pipette to complete blow-out.
5. Incubate it for 5 min at RT.
6. Remove the stain carefully, using a multichannel pipette.
7. Wash it twice with deionized water (200 µl each time).
8. Let the wells dry for 5 min at RT and dissolve the remaining stain in 96% ethanol or 33% acetic acid.
9. Incubate it for 1 hr at RT and read the absorbance at 595 nm.

5. Evaluating the Bactericidal Effect on Planktonic Bacteria Using the Same Sample Plate as for the Biofilms

1. Measure the turbidity at 595 nm of the plate with the planktonic solution from 3.3.
2. Stain the planktonic bacteria with resazurin by adding 10 µl of the resazurin stock per well. Mix well by pipetting.
3. Incubate it in darkness at RT for approximately 5 min, until the untreated controls are evenly pink.
4. Measure the fluorescence at λ_{exc} = 560 nm and λ_{em} = 590 nm.

6. Wheat Germ Agglutinin Staining for Matrix Quantification and Fluorescence Microscopy Imaging of Biofilms

1. **Matrix quantification**
    1. Prepare a 5 µg/ml solution of the WGA probe in sterile PBS.
    2. Use a parallel sample plate for this staining. Remove the planktonic solution from the wells and wash once with sterile PBS (200 µl per well), carefully using a multichannel pipette without touching the biofilms.
    3. Add 200 µl of WGA solution per well to be stained.
    4. Incubate it in darkness at 4 °C for 2 hr.
    5. Remove the unbound stain by washing the wells with 200 µl of PBS three times.
    6. Let the plate dry for 15 min at RT.
    7. Dissolve the bound stain in 33% acetic acid, using 200 µl per well.
    8. Seal the wells with strip caps and sonicate them using a water bath sonicator for 30 sec at RT and 40 kHz.
    9. Incubate the plate for 1 hr at RT.
    10. Repeat the sonication step. The wells can remain sealed between the sonication steps.
    11. Measure the fluorescence at λ_{ex} = 495 nm and λ_{em} = 520 nm with a plate reader (top reading).

2. **Visualizing the matrix with fluorescence microscopy**
    1. Use the same protocol as above until step 6.1.6.
    2. After the drying step, visualize the samples with a fluorescence microscope, using a FITC filter (or another suitable green excitation filter).

7. Staining Viable and Dead Cells within the Biofilms for Imaging with Fluorescence Microscopy and Quantification of the Signal for the Green-to-red-ratio (G/R)

1. **Imaging with fluorescence microscopy**
    1. Prepare a solution of each probe (one staining viable cells and the other one staining dead cells), according to the manufacturer's guidelines.
    2. Remove the planktonic solution from the wells and wash once with sterile PBS (200 µl per well) using a multichannel pipette.
    3. Add 6 µl of the staining solution per well.
    4. Incubate the plate in darkness for 15 min.
    5. Before the microscopy, remove the excess liquid manually using a multichannel pipette.
    6. Capture the images using a fluorescence microscope, using for instance a FITC filter (for green fluorescence, viable cells) or a TRITC filter (red fluorescence, dead cells).

2. **Quantification of the signal as a green-to-red-fluorescence ratio (G/R)**
    1. Follow the same protocol as in steps 7.1.1 and 7.1.2.
    2. Add 200 µl of the staining solution per well and incubate them for 15 min in darkness.
    3. Measure the fluorescence with a plate reader at excitation/emission wavelengths 485/535 nm and 485/635 for green and red fluorescence, respectively (top reading).

Representative Results

In the proposed platform, the effects on the viability, biomass, and biofilm matrix are quantified. In the working sequence (**Figure 1**), one sample plate is stained with resazurin and subsequently with crystal violet to simultaneously evaluate the effects on bacterial biofilm viability and on total biofilm biomass. Both assays can be performed consecutively in the same plate because it was demonstrated earlier that a first staining with resazurin had no statistically significant impact on the crystal violet staining result (p = 0.4149 for comparison of maximal signal absorbance units between crystal violet plates stained separately or after resazurin-staining, n = 20)[26]. The effect on planktonic bacteria can be simultaneously evaluated.

When hits are identified from either the viability or the biomass-based assay, a second plate is stained with the WGA probe to quantify the effect of the compounds on the polysaccharide component (PNAG) of the biofilm matrix. This workflow can be applied to the screening of chemical libraries, as well as to functional follow-up studies for potency measurements of hit compounds, as exemplified below.

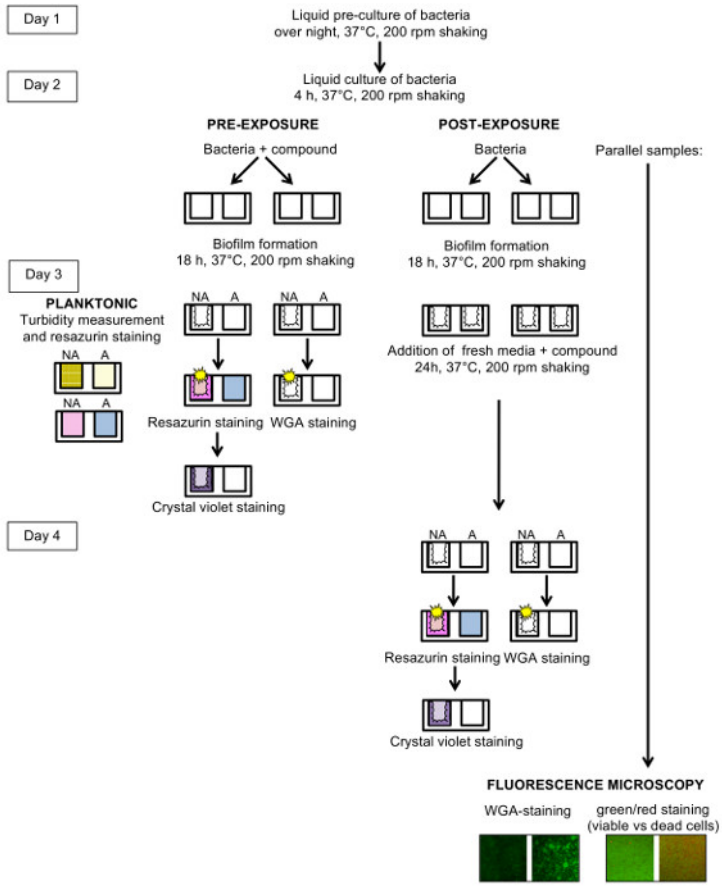

Figure 1: Workflow of the three-assay platform. Schematic representation of the workflow combining the three assays on biofilm samples. The platform includes staining for an effect on viability, biomass, and EPS layer; imaging using fluorescence microscopy; and evaluating the effect on the planktonic phase. "A" and "NA" stand for "Active" and "Not Active," respectively. Modified from Skogman *et al.*[26] Please click here to view a larger version of this figure.

Performance of the Three-assay Platform for Screening Purposes

Here, two screening runs are shown as representative results of the performance of the platform. In the first campaign (**Figure 2A**), a small library of cinchona alkaloid derivatives was screened for anti-biofilm activity against *S. aureus* biofilms[27], while in the second example (**Figure 2B**), a natural and naturally inspired library of abietane-type diterpenoids and derivatives was explored[28,29]. The active hits were determined using the calculated hit limits (thresholds; Equation 6, **Table 1**). In each screening study, the results of the first two assays correlated very well; the hits were all able to reduce the viability and the biofilm biomass, while the inactive compounds showed no effect on either assay. All the identified hits were then tested on the third (WGA) assay, but they had no matrix-disassembly (or matrix-degrading) effects (results not shown).

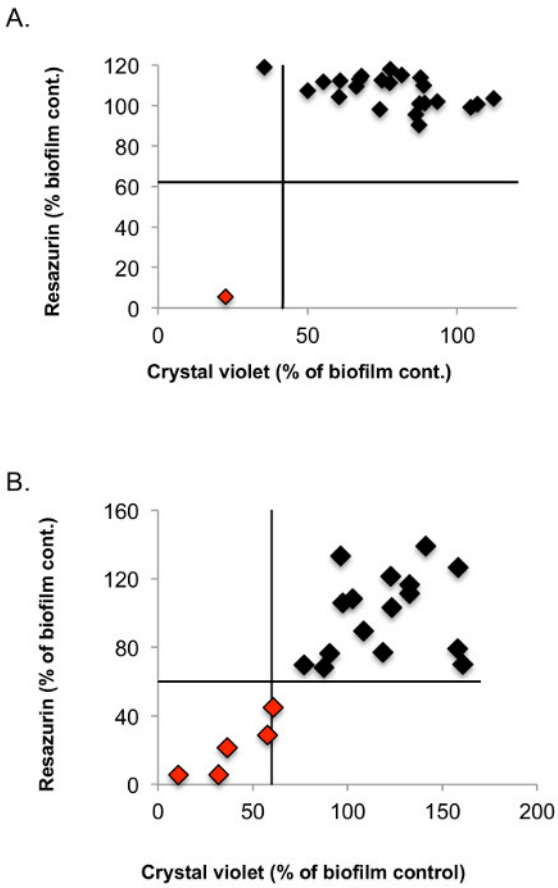

Figure 2: Representative results of screening campaigns using the platform with *S. aureus* biofilms. Two examples of proof-of-concept validation screens performed using the assay platform. The results are presented as a percent of the untreated biofilm control, the hit compounds are indicated in red, and the lines represent the calculated hit limits. (**A**) The screening of a cinchona-alkaloid derivative library identified one active compound. (**B**) The screening of a library of abietane-type diterpenoids and derivatives identified five active compounds. Please click here to view a larger version of this figure.

Performance of the Three-assay Platform for Follow-up Studies

Representative results can be seen in **Figure 3**, where two known antibiotics were tested at a very broad range of concentrations (0.5 nM-5 mM) against *S. aureus* biofilms. Minimum inhibitory concentration (MIC) values of reference compounds against planktonic bacteria (either from the literature or performed in the lab) serve as guidelines for choosing the concentrations to test against biofilms of the same species. In addition, knowledge of the concentration values in which no cytotoxicity is detected in mammalian cells can also help in the selection of the concentration to test for anti-biofilm follow-ups. Ideally, if both antimicrobial and cytotoxicity data for a certain compound are available, a parameter known as the "Biocompatibility Index" (BI) can be calculated, as defined by Müller and Kramer[30]. Here, MIC values against planktonic *S. aureus* for penicillin G and ciprofloxacin were determined to be 0.04 µM and 6 µM, respectively (results not shown). Thus, the concentration interval ranged from approximately 10^5 x MIC to 10^{-2} x MIC and 10^3 x MIC to 10^{-4} x MIC for penicillin and ciprofloxacin, respectively. Both antibiotics could significantly decrease the viability, biomass, and biofilm matrix PNAG content when they were exposed to single-cell bacteria prior to the initiation of the biofilm formation process (**Figure 3a**). However, despite the very high concentration of antibiotics tested on preformed (18 hr) biofilms, the viability and the biomass were only reduced to approximately 50% of those of untreated biofilms (**Figure 3b**). The most prominent result here was the increase of the biofilm matrix (to over 200%) when preformed biofilms were treated with high concentrations of penicillin G. No changes in the content of the biofilm matrix were detected when preformed biofilms were treated with ciprofloxacin.

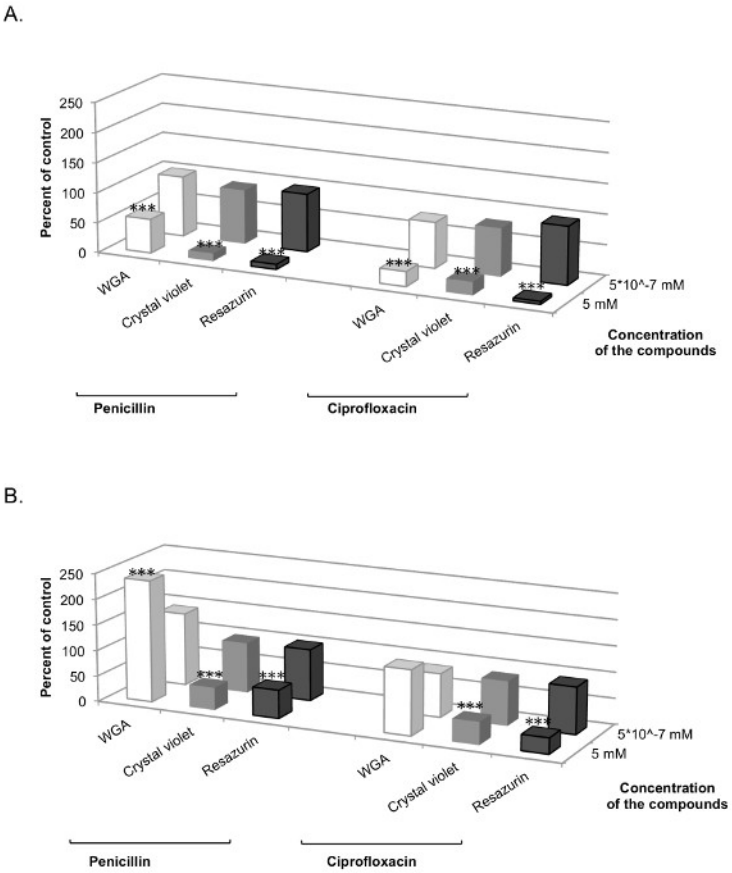

Figure 3: Example of a follow-up study of anti-biofilm effects using two model antibiotics against *S. aureus* biofilms. The effects of penicillin G and ciprofloxacin are presented here: (**A**) prior to biofilm formation (pre-exposure) and (**B**) post-biofilm formation (post-exposure). For figure clarity, only the lowest and the highest concentrations tested are shown. The standard deviations do not exceed 20%. *** equals $p < 0.01$. Modified from Skogman *et al.*[26] Please click here to view a larger version of this figure.

Fluorescence Microscopy-based Imaging

The result that penicillin G at 400 µM (as well as higher concentrations, as shown in **Figure 3b**) kills about 50% of the preformed *S. aureus* biofilm bacterial population was confirmed with another viability staining assay. The average of the green-to-red (G/R) fluorescence ratios for the untreated biofilm wells was 2.75, indicating a predominance of live (green-stained) cells, over dead (red-stained) cells. However, in penicillin-treated cells the G/R ratio decreased to 1.54, corresponding to 56% of the control wells. The cells remaining alive after penicillin treatment produced a significantly higher amount of EPS, as judged from the detected increase in green (WGA) fluorescence when compared with untreated biofilms (**Figure 4b**). We recommend, whenever possible, to perform these imaging-based experiments to further confirm the results of the platform, especially during the stage of characterization of hit candidates.

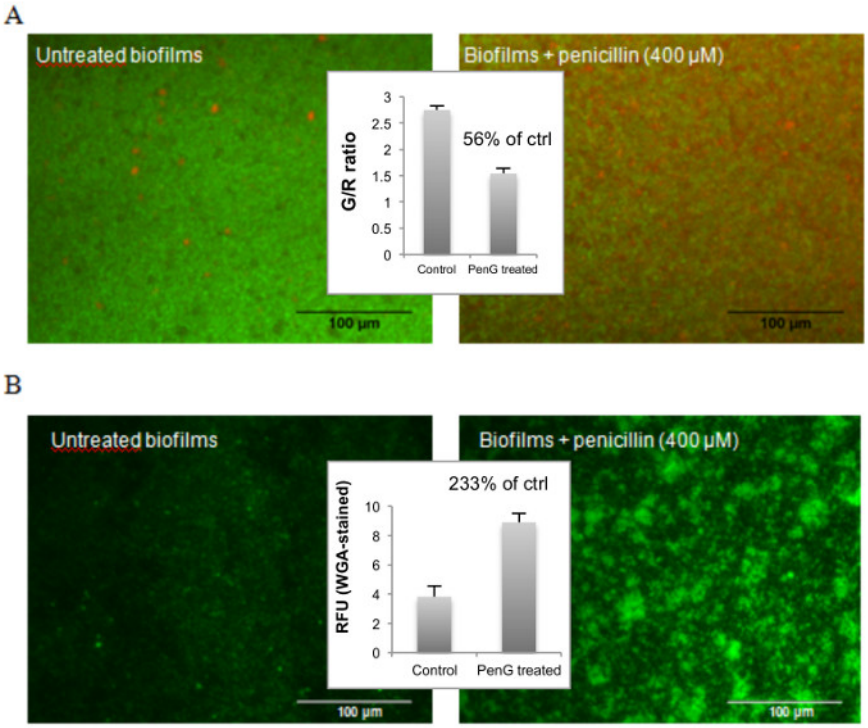

Figure 4: Fluorescence microscopy. The effect of penicillin addition (400 µM) on viability and EPS production are shown here. The top images (**A**) correspond to untreated biofilms and penicillin-treated biofilms, where viable cells are stained green and dead cells are stained red. The inserted graph illustrates the calculations of G/R fluorescence ratios. The bottom images (**B**) correspond to untreated biofilms and penicillin-treated cells stained with the WGA probe. The inserted graph presents the quantification of the WGA signals for treated and untreated biofilm samples, and error bars represent the standard deviations. Modified from Skogman *et al.*[26] Please click here to view a larger version of this figure.

Data Processing and Statistical Analysis

Statistical parameters are calculated to characterize the quality of the assays and to follow their performance during screening runs. The equations of the most important parameters used, as well as the obtained and target values, are listed in **Table 1**. In all equations, SD_{min}, μ_{min}, SD_{max}, and μ_{max} represent the standard deviations and means of the minimal (min) and maximal (max) signals, respectively. In the results shown here, paired comparisons of the original values were done with an unpaired t-test with Welch's correction, where $p < 0.05$ was considered to be statistically significant.

Parameter	Equation	Target value	Actually reached values[§]
1. Screening window coefficient	$Z' = 1 - \frac{(3 * SD_{max} + 3 * SD_{min})}{(\mu_{max} - \mu_{min})}$	0.5 – 1.0 (> 0.3 acceptable for cell based assays)	RES: > 0.6 CrV: > 0.4 WGA: > 0.3
2. Signal to noise	$S/N = \frac{(\mu_{max} - \mu_{min})}{\sqrt{(SD_{max}^2 + SD_{min}^2)}}$	The higher the better	RES: > 20 CrV: > 6 WGA: > 6
3. Signal to background	$S/B = \frac{\mu_{max}}{\mu_{min}}$	> 2	RES: > 20 CrV: > 5 WGA: > 10
4. Separation band	$Sb = (\mu_{max} - \mu_{min}) - (3 * SD_{max} + 3 * SD_{min})$	Depends on the measurement method	RES: > 1000 RFU CrV: > 1.0 RAU WGA: > 7 RFU
5. Coefficient of variation of the assay	$CVA\% = \frac{100 * SD_{max}}{(\mu_{max} - \mu_{min})}$	< 20%	RES: < 10% CrV: < 10% WGA: < 13%
6. Hit limit	$HL = \mu_{max} - 3 * SD_{max}$	Can also be empirically set, depending on assays	RES: > 80% of the ctrl CrV: > 50% of the ctrl WGA: > 50% of the ctrl

Table 1: Statistical parameters used to evaluate the performance of the assays. In all equations, SD_{min}, μ_{min}, SD_{max}, and μ_{max} represent the standard deviations and means of the minimal (min) and maximal (max) signals, respectively. The staining methods, resazurin, crystal violet, and wheat germ agglutinin, are abbreviated RES, CrV, and WGA, respectively. RFU: relative fluorescence units; RAU: relative absorbance units. Please click here to download this file.

Discussion

There is no single method that can simultaneously measure the effect of a compound on the viability, biomass, and biofilm matrix. Therefore, there is a need for combining assays in order to detect an effect on the three endpoints, preferably at a primary screening stage.

Resazurin is a very simple staining protocol consisting only of the addition of the redox probe. However, establishing the optimal incubation time of the biofilms with the resazurin is crucial to the success of this assay. In some bacterial strains, the reduction of the resazurin probe to the pink, fluorescent resorufin occurs very quickly, while in others it can last several hours[19]. Moreover, within the same bacterial strain, there may also be significant differences between planktonic bacteria and biofilms. The planktonic bacteria are typically more easily reached, mainly because the bacterial density is lower in planktonic populations than in biofilm populations, which promotes faster turnover of the reaction. In a published comparison of biofilm viability staining probes, resazurin was concluded to be one of the most accurate, simple to use, and least expensive assays[19]. Moreover, it has been shown to be applicable to a range of bacterial and fungal organisms[19]. On the other hand, the crystal violet assay is the most widely used for biofilm mass quantification[19,32,33]. The most critical steps of this protocol are the addition and removal of the crystal violet stain solution. These steps have to be conducted very carefully to avoid unspecific staining (*e.g.,* due to drops of stain on the walls of the wells). One of the main benefits of using resazurin as the initial staining assay is the fact that it is non-toxic to the cells, which enables the use of the same plate for crystal violet staining. The combination of both assays significantly simplifies the workflow, lowers the costs, saves consumables, and minimizes the use of the test compounds, which is particularly valuable in a screening environment.

As indicated earlier, the self-produced extracellular polysaccharide matrix is an essential component of biofilms. To measure the matrix content (particularly essential polysaccharides), a third assay was included here, based on fluorescence-labeled WGA. Originally the WGA quantification was described as an enzyme-linked lectin-sorbent assay (ELLA)[24]. A similar assay based on staining glycosaminoglycans (GAGs) in the matrix of *S. aureus* biofilms with dimethylmethylene blue (DMMB) has also been used[31,34]. GAGs are similar to the polysaccharide intercellular adhesins (PIAs) found in the matrix of *Staphylococcus* spp. biofilms[35]. The WGA-assay similarly targets PIAs, but WGA more specifically binds to poly-*N*-acetyl-glucosamine residues, which play an essential role in the biofilm matrix[36]. Targeting the matrix is of high importance due to the fact that biofilms can easily regrow if the matrix is left after a chemical or antibiotic treatment. Bacteria can also more easily attach to a surface that is matrix pre-coated[35]. It has been shown that some antibiotics can effectively lower the viability and biofilm biomass, but without any effect on the matrix. In our experiments, this is exemplified by the case of ciprofloxacin[31]. Some antibiotics can even increase matrix production, as demonstrated here with penicillin G[26]. Based on these changes, antibiotics can be classified into the categories listed in **Table 2**. All the hits in our screening studies (**Figure 2**) can be classified, together with ciprofloxacin, as compounds that kill the bacteria in the biofilm and reduce the biomass but do not disassemble or disrupt the biofilm matrix.

Category	Effect on bacteria	Effect on matrix	Example antibiotic (target biofilm)	Reference
1	none	none	ampicillin (SA)	(Toté *et al.* 2009)
2	none	**–**	cefalotin (SA)	(Toté *et al.* 2009)
3	**–**	none	polymyxin (SA)	(Toté *et al.* 2009)
4	**–**	**–**	kanamycin (PA)	(Toté *et al.* 2009)
			ciprofloxacin (SA)	(Skogman *et al.* 2012)
5	**–** (**+**)	**–**	doxycycline (PA)	(Toté *et al.* 2009)
6	**–**	**+**	penicillin G (SA, EC)	(Skogman *et al.* 2012)

Table 2: Classification of antibiotics. The antibiotics are divided into categories based on their effect on the viability of the biofilm bacteria (using resazurin staining) and the matrix (using WGA or dimethylmethylene blue (DMMB) staining). SA: *Staphylococcus aureus*; PA: *Pseudomonas aeruginosa*; EC: *Escherichia coli*. Modified from Toté *et al.*[31]

Furthermore, this platform is ideal for performing primary screening campaigns. The most cost- and time-effective strategy is to apply resazurin and crystal violet-based assays as a first-tier strategy and to move on to the WGA-matrix assay in second-tier (follow-up) studies. This is due to the fact that the WGA probe is rather expensive and that this assay consists of several steps, which makes it more laborious and time-consuming.

A relevant feature of this platform is the possibility to evaluate the long-term effects of the identified antimicrobials. Long-term chemotherapeutic effects can only be achieved if the biofilm matrix is disassembled. It has been shown that the risk of resuscitation of the biofilm infection is much higher if the matrix is left behind. With this platform, it is possible to first assess if the overall biofilm biomass is affected using crystal violet staining. A more detailed assessment of the biofilm matrix polysaccharides, using the WGA assay, can follow. Of note, the likelihood of identifying a single molecule that can both disassemble the matrix and exert antibacterial (biocidal) effects may be regarded as low. Indeed, thus far, the only compounds of this type that we have identified are antimicrobial peptides[37]. To address this, follow-ups are performed in this platform with either resazurin- or crystal violet-active hits, since this strategy permits the identification of compounds with separate biocidal or matrix-degrading activity. Such leads can be potentially interesting for multi-component strategies. A combination of matrix-degrading molecules with biocidal or antibiotic-type compounds is expected to provide more efficient biofilm eradication.

In summary, the platform presented here provides a good basis for anti-biofilm screening using viability and biomass measurements together with matrix quantification and visualization. Both the resazurin and crystal violet staining assays can also be used for other kinds of biofilm-forming microorganisms. However, these assays require separate optimization for both growing and staining conditions. The applicability of the WGA staining assay is limited to those bacteria in which the poly-*N*-acetyl-glucosamine is a main component of the biofilm matrix. Other matrix quantification assays (such as the one based on dimethyl-methylene blue staining) can be applied in other cases. Altogether, the assays in

this platform are fairly easy to perform, and all reagents are easily available as well as generally affordable. This platform is suitable for low- to medium-throughput anti-biofilm screening without the need for expensive equipment investments.

Disclosures

The authors have no conflicts of interest to disclose.

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