Exploring photoinactivation of microbial biofilms using laser scanning microscopy and confined 2-photon excitation

Hanna Thomsen1,2* | Fabrice E. Graf2,3 | Anne Farewell2,3 | Marica B. Ericson1*

1Biomedical Photonics, Department of Chemistry and Molecular Biology, University of Gothenburg, Gothenburg, Sweden
2CARe, Center for Antibiotic Resistance Research, University of Gothenburg, Gothenburg, Sweden
3Microbiology, Department of Chemistry and Molecular Biology, University of Gothenburg, Gothenburg, Sweden

*Correspondence
Hanna Thomsen, Biomedical Photonics, Department of Chemistry and Molecular Biology, University of Gothenburg, Kemigården 4, 412 96 Gothenburg, Sweden.
Email: hanna.thomsen@chem.gu.se
Marica B. Ericson, Biomedical Photonics, Department of Chemistry and Molecular Biology, University of Gothenburg, Kemigården 4, 412 96 Gothenburg, Sweden.
Email: marica.ericson@chem.gu.se

Funding information
FP7 People: Marie-Curie Actions (FP7-PEOPLE-ITN-2013), Grant/Award Number: #608407;
Swedish Research Council, Grant/Award Number: 621-2011-5189

One pertinent complication in bacterial infection is the growth of biofilms, that is, communities of surface-adhered bacteria resilient to antibiotics. Photodynamic inactivation (PDI) has been proposed as an alternative to antibiotic treatment; however, novel techniques complementing standard efficacy measures are required. Herein, we present an approach employing multiphoton microscopy complemented with Airyscan super-resolution microscopy, to visualize the distribution of curcumin in Staphylococcus epidermidis biofilms. The effects of complexation of curcumin with hydroxypropyl-γ-cyclodextrin (HPγCD) were studied. It was shown that HPγCD curcumin demonstrated higher bioavailability in the biofilms compared to curcumin, without affecting the subcellular uptake. Spectral quantification following PDI demonstrates a method for monitoring elimination of biofilms in real time using noninvasive 3D imaging. Additionally, spatially confined 2-photon inactivation was demonstrated for the first time in biofilms. These results support the feasibility of advanced optical microscopy as a sensitive tool for evaluating treatment efficacy in biofilms toward improved mechanistic studies of PDI.

KEYWORDS
curcumin, hydroxypropyl-γ-cyclodextrin, microbial biofilms, multiphoton microscopy, super-resolution microscopy, Staphylococcus

INTRODUCTION

Growing resistance to antibiotics is made even more complicated by biofilm-caused infections; bacteria grow adhered to a surface and can grow into large microorganism communities including extensive defense mechanisms designed to protect the microbe community from surrounding environment, including potential antimicrobial actions [1–4]. These communities can be up to 1000-fold more resistant to antibiotics than planktonic counterparts [5]. The prevalence of biofilm cultures in chronic infections adds to the increasing challenge of treating, for example, nosocomial, dental, wound, cystic fibrosis, sinus and ear infections, naming just a few. Microbial biofilms are covered in an exopolysaccharide (EPS) matrix through which limited diffusion of common disinfectants and antimicrobials has been demonstrated [6, 7]. Thus, new treatment strategies are required, specifically, to bypass side effects associated with multdrug approaches, and to treat antimicrobial resistant infections.
Photodynamic inactivation (PDI) as a concept for elimination of bacteria has become increasingly popular stemming from success in photodynamic therapy for treatment of cancer. PDI has been presented in a variety of experimental studies focused on eradication of bacterial infections, development of novel photosensitizers for use in antimicrobial PDI and exploration of PDI in relation to microbial phenotypes [8–11]. A major expected advantage of PDI is that development of resistance is unlikely given that the mode of action of photosensitizers used in PDI is different from typical antibiotic drugs. Bacteria adopt mechanisms to increase antibiotic resistance such as encoding proteins that can prevent drug penetration, changing the structure of the outer cell wall (thus having an effect on drug efficacy), etc., whereas the use of light activates toxic reactive oxygen species (ROS) which do not have the same mode of action or biochemical target, thus does not lead to the same resistance mechanisms [12, 13]. PDI is a multitarget process, treatment is broad-spectrum (meaning that it can be effective against a variety of pathogens), and does not have the same specific target requirements as for many typical antibiotics, thus making it a potential treatment for multidrug resistant infections. For PDI to be effective for treatment of microbial biofilms, the photosensitizer must have successfully penetrated and distributed within the biofilm. It is particularly important to access all layers of biofilm where the adhered bacteria are protected by the EPS and thus thought to be more resistant to antibiotics [14, 15]. Furthermore, the light should have access to the biofilm. Although significant microbial death has been previously demonstrated in biofilms using PDI [16], the location of the photosensitizer and resultant toxicity in a biofilm should be of equal importance given the variation in susceptibility to antimicrobials that exists throughout a mature biofilm. Standard antimicrobial efficacy measurements lack in-depth insight into photosensitizer distribution, subcellular localization and effective treatment against the entire 3D bacterial structure [17]. Noninvasive optical techniques can be adapted as a complement, with further applications in spatially localized treatment of biofilms.

Curcumin has previously been explored as a proposed photosensitizer for PDI of biofilms [18–23]. Curcumin is a natural product present in turmeric, and is interesting also for its inherent anti-inflammatory properties and general potential as a natural therapeutic agent [24]. Despite this, curcumin remains pharmaceutically challenging due to being nearly insoluble in aqueous solutions, easily photodegradable and rapidly metabolized thus having low bioavailability, creating a challenge in clinical use of curcumin. Complexation of curcumin with cyclodextrin has been demonstrated to improve solubility and stabilize curcumin in solution [25]. Furthermore, curcumin-cyclodextrin complexed solutions have demonstrated elevated PDI activity against biofilms [9]. However, how complexation of curcumin affects bioavailability in the biofilm is poorly understood. Thus, new tools providing more in-depth studies are required.

Multiphoton microscopy (MPM) is used as a method for high-resolution visualization of optically dense biological tissue, for example, skin, etc. [26, 27]. Surprisingly, few
investigations have used MPM for exploring microbial biofilms so far [28, 29]. Two-photon excitation (2PE) takes advantage of 2-photon absorption, in which 2 photons of light are absorbed with lower energy than required to excite a molecule with 1 photon absorption. Excitation of fluorescence using lower energy near-infrared light enables microscopy at a less damaging and higher penetrating optical window [30]. MPM overcomes drawbacks of other optical microscopy techniques such as photobleaching and phototoxicity. In addition, 2PE allows for imaging of thick specimens; as near-infrared light is less absorbed. Since MPM is diffraction limited, additional tools are required to investigate subcellular localization within bacteria. Super-resolution optical techniques like stimulated emission depletion microscopy and stochastic optical reconstruction microscopy are of interest, but limited by the requirement for certain fluorophores. Instead, Airyscan confocal technology provides an interesting approach to acquire improved signal-to-noise ratio and higher resolution without putting constraints to choice of fluorophore [31].

Herein, we present a mechanistic study based on MPM and Airyscan microscopy to evaluate the efficacy of PDI using curcumin and curcumin complexed with hydroxypropyl-\(\gamma\)-cyclodextrin (HP\(\gamma\)CD), for treatment of Staphylococcus epidermidis biofilms. The 2-photon absorption cross section of curcumin has earlier been characterized [32], and thus chosen as a suitable photosensitizer in the study. The complexation of curcumin with HP\(\gamma\)CD was done according to Tønnesen et al. [25]. Figure 1 shows ultraviolet (UV)/Vis absorption and emission spectra of both solutions. Corresponding absorption and emission peaks were identified at 430 and 530 to 550 nm, respectively. Curcumin-CD was found to exhibit higher absorption (approximately 5\(\times\)) and emission intensity (approximately 2\(\times\)) compared to non-complexed curcumin at the same concentration. Furthermore, the results show that the tautomeric form of curcumin remains the predominant keto-enol form, known to have peak absorption near 420 nm [32], following complexation. This indicates that HP\(\gamma\)CD complexation offers enhanced solubility and photochemical properties necessary for using curcumin in a PDI setting.

In addition to 1PE spectroscopy, curcumin and curcumin-CD complex in aqueous solutions were investigated using 2PE. Figure 2 shows 3D z-stacks acquired from both curcumin solutions and corresponding 2PE fluorescence emission spectra. As shown by the figure, the fluorescence emission for both curcumin solutions using 2PE correspond to the 1PE emission spectra (Figure 1), and

2 RESULTS

2.1 Spectral characterization and visualization of curcumin

Spectral characteristics of curcumin and HP\(\gamma\)CD complexed curcumin (curcumin-CD) were evaluated using 1PE and 2PE. Curcumin is known to be poorly soluble in aqueous solvent (<200 \(\mu\)M). To solubilize curcumin in aqueous format, a stock solution of curcumin was first prepared in EtOH at a concentration of 1 mM. This stock solution was then diluted by a pH-adjusted phosphate buffered saline (PBS, pH 5.5) to a final concentration of 10 \(\mu\)M. Curcumin-CD at a concentration of 10 \(\mu\)M was prepared by dissolving curcumin in pH adjusted PBS (pH = 5.5, no EtOH) according to the Tønnesen method [25]. Figure 1 shows ultraviolet (UV)/Vis absorption and emission spectra of both solutions.

FIGURE 2 MPM z-stacks (\(\lambda_{ex} = 750\) nm) acquired from (A) curcumin-CD complex (10 \(\mu\)M, in PBS) and (B) curcumin (10 \(\mu\)M, in PBS with 1% EtOH); and (C) spectral 2PE emission data (10 nm resolution) from both solutions, where ROI1 and ROI2 correspond to regions with aggregates and solution in (B), respectively, while spectrum for the curcumin-CD complex is presented as a 3D volume average in (A). Z-stacks correspond to a volume of 425.10 \(\times\) 425.10 \(\mu\)m, and z-range of 134 \(\mu\)m for image (A) and 109 \(\mu\)m for image (B), using a z-step of 5 \(\mu\)m for both images.
match the spectral shape as earlier described for 2PE of curcumin in the literature [32]. Interestingly, the visualization of the 2 solutions demonstrates the differences in fluorescence distribution on the microscopic level. As can be observed, the fluorescence emission is more evenly distributed for the curcumin-CD solution (Figure 2A), whereas the z-stack obtained from the pure curcumin in PBS (Figure 2B) implies the presence of larger microscopic aggregates than curcumin-CD. This was confirmed by the spectral analysis (Figure 2C) by selecting regions of interest (ROIs) from the 3D data. The emission peak from curcumin in PBS solution was only observed from selected aggregates (ROI1), while the surrounding solution (ROI2) exhibited a flat baseline signal. Furthermore, the investigation confirms that 2PE can be applied to visualize curcumin in biologically appropriate aqueous medium.

2.2 | Visualizing photosensitizer distribution in fluorescent S. epidermidis biofilms

MPM was used to visualize the distribution of curcumin delivered to S. epidermidis biofilms. An S. epidermidis strain was constructed expressing a blue fluorescent protein (BFP) to allow visualization of the biofilm without the requirement of exogenous staining. Figure 3 shows MPM z-stacks as volumetric 3D images acquired from the biofilms following a 30-minute incubation with curcumin. The excitation wavelengths (760 nm for BFP; and 950 nm for curcumin) were chosen to minimize spectral cross talk between BFP and curcumin. Imaging settings were kept constant for all experiments. As can be seen from the figure, curcumin fluorescence was found distributed throughout the entire depth of the biofilms (up to 120 μm) for both administrations, although the fluorescence signal was found higher for the curcumin-CD complex (mid panel) compared to pure curcumin (lower panel). This result implies a more efficient uptake and biodistribution of curcumin-CD complex in the biofilm.

2.3 | Subcellular distribution of photosensitizer

To investigate the subcellular distribution of curcumin within bacteria having the size of about 1 μm, increased resolution was required. Thus, 1PE confocal laser scanning microscopy equipped with Airyscan detection was employed. Figure 4 shows fluorescence images of individual clusters of S. epidermidis bacteria using Airyscan microscopy. Here, the bacteria were incubated with curcumin or curcumin-CD complex for 30 minutes, as above, but in addition stained with FM4-64 membrane dye, to compare the distribution of curcumin in relation to the cell membrane. Fluorescence emission was collected in red, corresponding to membrane stain, and green for curcumin. As can be seen, curcumin was found within the bacteria, which was similar for both solutions. This result implicates that curcumin is entering the bacteria and localizes in the cytoplasm in both cases, and that complexation with HPγCD does not seem to alter the uptake by bacteria.
Biofilms were treated with curcumin-CD or noncomplexed curcumin and irradiated with 1PE-based PDI (λ = 360 nm, 5 J/cm²). To assess the treatment efficacy, the biofilms were stained with FilmTracer live/dead staining based on SYTO9 and propidium iodide and imaged using MPM. Figure 5 shows data from mature *S. epidermidis* biofilms following PDI treatment with curcumin solutions (Figure 5B,D) and controls (Figure 5A,C). As shown by the data, higher red (propidium iodide) signal, corresponding to membrane-compromised bacteria, is demonstrated for PDI in the presence of curcumin (Figure 5B,D) compared to control. This is further confirmed by emission spectra (Figure 5E). A minor increase in red emission spectra can be seen for biofilms treated with curcumin-CD compared to curcumin, although this difference is not significant. In addition to imaging, standard efficacy measures based on counting colony-forming units (CFUs) were performed. Based on the CFU counts, the entire biofilm culture appears to have been eliminated (Figure S3, Supporting Information) indicating a more drastic effect on cell viability than the propidium iodine staining indicated. It is known that various factors influence the reliability of CFU counting, particularly when it comes to biofilm cultures [17, 33]. For example, bacteria can remain adhered to the surface of the plates and are not counted within CFU, thus leading to misinterpretation of viability measurements. Furthermore, CFU only measures a subfraction of cells, which might explain the observed differences.
Thus, this supports that MPM imaging can be a valuable tool providing a 3D map of biofilm viability, complementary to traditional microbiology methods.

2.5 | Targeted 2PE PDI, 3D visualization of PDI with intrinsic control

To demonstrate localized PDI within a complex biofilm structure, 2PE was used to selectively irradiate a biofilm within a specific ROI and toxicity was visualized using live/dead staining using MPM as shown in Figure 6. As shown by the figure, spatially confined 2PE irradiated areas can be seen for both preparations of curcumin. 2PE emission spectra of the irradiated and nonirradiated regions were analyzed for spectral quantification of toxicity (Figure 6B,C). Biofilms treated with curcumin demonstrate significant localized cell death as a result of 2PE PDI. For the 2PE PDI region in the biofilms treated with curcumin-CD, the bacteria appear to have been eliminated in the irradiated region. This interpretation was confirmed by reduction in fluorescence emission (Figure 6B). These results suggest that curcumin complexed with cyclodextrin acts as a more potent photosensitizer in combination with 2PE, compared to noncomplexed curcumin. Furthermore, the experiments support the approach being a novel tool for spatially confined PDI within biofilms allowing for improved mechanistic studies.

3 | DISCUSSION

In this study, we demonstrate a combination of MPM and Airyscan microscopy as a novel approach complementing traditional microbiology methods for investigating bioavailability and efficacy of 1PE and 2PE PDI of *S. epidermidis*
biofilms using curcumin as a photosensitizer. Curcumin is well-known for its reported pharmacological effects, and has earlier been proposed as a potent photosensitizer for PDI of bacteria [9, 34, 35]. However, curcumin suffers from poor solubility in aqueous solutions making its pharmaceutical preparation challenging [36]. The results in this study demonstrate that curcumin-CD improves bioavailability of curcumin in the *S. epidermidis* biofilms, without modifying the subcellular uptake. In addition, the potency of 1PE and 2PE PDI was confirmed, supporting the feasibility of CD-complexation for improving pharmaceutical delivery of curcumin.

To visualize the biofilm structure simultaneously with curcumin, a fluorescent *S. epidermidis* strain was developed. The ability of compounds to penetrate throughout a microbial community is a complex problem and is dependent on dosage, transfer properties, biofilm thickness and chemical actions of the compound itself [37]. Thus, methods for non-invasive assessment of drug delivery uptake in biofilms are of interest from a more general perspective. The combination of fluorescent bacterial strains, together with MPM serves as an interesting approach for improved mechanistic understanding of novel antimicrobial treatments.

To study subcellular distribution of curcumin, Airyscan microscopy was implemented. Subcellular localization is of importance given the potential of understanding the photodynamic mode of action. Mechanisms involving ROS [38, 39] and/or intermediate hydrogen peroxide (H$_2$O$_2$) have been proposed [18]. Due to limited diffusion of these species, intracellular localization is preferable to ensure efficient PDI. It was here found that both curcumin and curcumin-CD are intracellularly localized, supporting that both preparations can act as efficient photosensitizers for PDI.

Biofilms are difficult to treat and a common cause of chronic infection. PDI has previously been proposed as a strategy for treatment of biofilms circumventing resistance development. Treatment efficacy is typically measured using microbiological assay such as CFUs. Although this method is standard for assessing viability of planktonic bacterial cells, it is insufficient for monitoring biofilm viability accurately [17]. Using MPM combined with live/dead staining, it was here shown that after 1PE PDI, intact cells remained adhered to the surface, despite CFU counts indicating all viable bacterial cells were killed. This discrepancy could possibly be explained by the growth of small colony variants (SCVs), a phenotypically different subpopulation of bacteria associated with biofilms, also reported previously in *S. epidermidis* [40, 41]. These colonies may not have been captured by overnight CFU due to their slow growth rate. This suggests that the approach proposed in this study can be implemented as a complementary and more sensitive tool for evaluating efficacy of PDI. Future experiments could be performed to elucidate the growth of SCVs following 2PE PDI, or 1PE and 2PE PDI in comparison. Furthermore, the method should be refined to include quantitative measures, for example, by calculating the percentage of live and dead cells found in the MPM images to compare with the number of live cells detected in CFU counting.

Surprisingly, the difference in efficacy of 1PE PDI between curcumin and curcumin-CD was found to be negligible, despite higher bioavailability for curcumin-CD. It could be argued that this effect might be due to limited light penetration; such as experienced through an inner-filter effect, meaning that excitation light is absorbed superficially. In contrast, the effect of 2PE PDI was found to substantially differ between the biofilms treated with curcumin-CD complex and curcumin. Since 2PE using NIR is only able to excite the photosensitizer in the focal plane, the confounding factors such as inner filtering effect are eliminated. Thus, it is likely that 2PE PDI serves as a more sensitive tool for mechanistic studies of PDI.

The potential clinical translation of curcumin-based PDI, as explored in this work, is most likely focused toward treatment of wound, oral or other surface-level infections that can easily be accessed by light sources. While several studies cite dose-dependent cytotoxic effects of curcumin to specific human cell lines [42, 43], future investigations should be undertaken to investigate the potential cytotoxic effects of these compounds specific to oral or topical delivery, in addition to cytotoxicity resulting from the light sources described in this work. With the combination of tools and novel approaches used in this study, there is great promise toward novel treatment strategies to fight antimicrobial resistance and biofilm infections.

4 | CONCLUSION

Taken together, this study explores MPM and Airyscan microscopy as novel approach to complement standard microbiology efficacy measures for assessment and improved mechanistic studies of PDI targeting biofilms. Herein, a cyclodextrin-complexed curcumin solution was compared to a standard saturated curcumin solution with respect to biodistribution within *S. epidermidis* biofilms. Complexed curcumin demonstrated higher bioavailability within the biofilms, while subcellular uptake was not altered. Similar efficacy of 1PE PDI was obtained using both curcumin preparations; however, spatially confined 2PE PDI was found most efficient for curcumin-CD. The approach allows for improved mechanistic studies for antimicrobial biofilm treatments, and the concept should be further exploited as a potential antimicrobial treatment against resistant infections.

5 | MATERIALS AND METHODS

5.1 | Bacteria culturing

*Staphylococcus epidermidis* (ATCC 35984) was kindly provided by the M. Andersson group (Department of Chemistry
and Chemical Engineering, Chalmers Technical University, Gothenburg, Sweden). Cultures were obtained from maintained subcultures in tryptic soy agar (TSA) plates and grown overnight in aerobic atmosphere at 37°C. Second day overnight cultures were diluted in TSB to optical density OD₆₀₀ = 0.25; corresponding to 10⁸ CFU/mL.

5.2 | Plasmid construction and transformation of *S. epidermidis*

The plasmids pCM29 [44] and pHC48 [45] expressing sGFP and dsRed, respectively, were kind gifts of Prof. Horswill (University of Iowa). The BFP gene mKalama1 [46] was codon-optimized for expression in *S. epidermidis* using Optimizer (http://genomes.urv.es/OPTIMIZER) [47] and the gene was synthesized at GeneArt (Thermo Fisher, Schwerte, Germany). The sequence is available at Genbank (MH256562). Sod ribosomal binding sites were identical to those in pCM29 and pHC48. The codon-optimized BFP was subcloned into the expression vector pCM29 replacing sGFP using the restriction enzymes KpnI and EcoRI to yield pFGbfp and amplified in *Escherichia coli* DC10B, a DNA cytosine methyltransferase deficient mutant which allows later direct transformation of plasmids into *S. aureus* and *S. epidermidis* [48].

Transformation by electroporation of *S. epidermidis* was performed as described by Monk et al. [48] with some minor changes. A single colony was inoculated in 10 mL tryptic soy broth (TSB) and grown overnight at 37°C. Cells were adjusted to OD₆₀₀ = 0.5 in 100 mL prewarmed TSB and further incubated for 30 minutes at 37°C. Thereafter, the cells were cooled immediately in ice/water slurry for 10 minutes (all subsequent steps were performed on ice and centrifugation steps at 4°C).

Cells were transferred to prechilled 50 mL tubes and centrifuged for 10 minutes at 4000 rpm, with subsequent washing steps in: (1) 35 mL ddH₂O, (2) 5 mL 10% glycerol and (3) 2 mL 10% glycerol and finally resuspended in 250 μL 10% glycerol. Aliquots of 50 μL were placed in prechilled microcentrifuge tubes and directly used for electroporation or stored at −80°C. In addition, 4 to 6 μg plasmid DNA (in a volume <5 μL) was added to 50 μL electrocompetent cells and incubated for 5 to 10 minutes at room temperature (RT), transferred to a electroporation cuvette (1 mm gap) and pulsed at RT with: 21 kV/cm, 100 Ω and 25 μF.

Cells were immediately recovered in 1 mL prewarmed TSB 500 mM sucrose and incubated for 1 to 2 hours at 37°C, before being spread with volumes of 10 μL, 100 μL and the remaining volume, which was spun down for 2 minutes at maximal speed and resuspended in remaining approximately 100 μL, on TSA chloramphenicol (10 μg/mL) plates and incubated at 37°C overnight until visible colonies appeared.

5.3 | Development of biofilms

Biofilm formation was allowed to occur in presterilized polycarbonate optical quality glass-bottom cell cultures dishes (Ibidi, LRI Instrument AB, Lund, Sweden). Aliquots of 2 mL of bacteria/TSB inoculum containing approximately 10⁸ cells/mL were transferred into each petri dish. The plates were incubated under aerobic atmosphere at 37°C for an initial incubation period of 24 hours. After the first 24 hours, the liquid medium was carefully aspirated from the dish and replaced with fresh sterile TSB to remove planktonic cells while maintaining stable adhered biofilm culture to the dish. TSB was replaced every 24 hours for the remainder of the incubation time of each sample.

5.4 | Biofilm viability imaging

Biofilms grown for 48 to 72 hours in the conditions described were removed from incubation and stained with 1 mL FilmTracer Biofilm stain (Fisher Scientific, Gothenburg, Sweden), prepared as suggested by the supplier, in PBS (pH 7.4). FilmTracer live/dead staining uses 2 fluorescent nucleic acid stains, SYTO9, penetrating both viable and nonviable bacteria, and propidium iodide, penetrating only bacteria with damaged membranes. Staining was used to visualize the viability of the biofilm grown. Biofilms were stained for 15 minutes protected from light at RT. The stain was carefully aspirated from the petri dish and the biofilm was carefully washed with 1 mL PBS (pH 7.4). Prior to imaging, 2 mL fresh PBS (pH 7.4) was added to the petri dish for imaging with a 20× water-immersion objective.

5.5 | Photosensitizer preparation

**Curcumin-HPγCD:** A cyclodextrin complex solution of curcumin was prepared using HPγCD provided by CycloLabs (Budapest, Hungary) and curcumin (Sigma-Aldrich, Stockholm, Sweden). The HPγCD-curcumin complex was prepared as a method to enhance water solubility and photochemical stability of the compound [25]. HPγCD was dissolved to a concentration of 112 g/L in 0.18 mol/L sodium hydroxide solution. Curcumin was added to a concentration of 15 g/L. The solution was agitated and after complete dissolution of curcumin, the pH was adjusted to 6.0 with a combination of 1 M citric acid and 1 M hydrochloric acid (HCl). The final solution contained 12 g/L curcumin and 93 g/L cyclodextrin in 20 mM sodium citrate, 100 mM NaCl solution. Final preparations of solution of 10 and 40 μM were prepared in PBS adjusted to pH 6.1 with HCl. **Curcumin-ethanol:** Curcumin was dissolved in 99.7% ethanol to a concentration of 3.75 mg/mL. The stock solution was diluted in PBS adjusted to pH 6.1 with HCl to the final concentrations of 10 and 40 μM.
5.6 Two-photon laser scanning microscopy

2PE was obtained using a multiphoton microscope (LSM 710 NLO microscope, Carl Zeiss, Jena, Germany) equipped with mode-locked femtosecond pulsed Mai Tai DeepSee laser (SpectraPhysics, Staehnzdorf, Germany) operating at 80 MHz, tunable in the wavelength region 700 to 1100 nm. A Plan-Apochromat 20x water immersion objective (NA 1.0) was used in the experiments. Fluorescence was registered with descanned (internal) detectors with a fully opened pinhole. Emission spectra were acquired by using the spectral detector on the system collecting with a 9.7 nm step in the emission range of 416 to 727 nm. Excitation scan was performed using a built-in excitation scan macro. For imaging of biofilms stained only with curcumin solutions, the excitation wavelength was set to 750 nm and emission recorded in 1 channel corresponding to 523 to 624 nm. For imaging of biofilms stained with FilmTracer, excitation wavelength was set to 850 nm, and emission recorded in 2 channels corresponding to 485 to 571 nm, and 591 to 727 nm for SYTO9 (green stain, viable cells) and PI (red stain, damaged cells), respectively. Images were acquired with a pixel dwell time of 1.58 μs. The image frame size was 1024 × 1024 pixels for all images presented. Imaging processing was performed with ZEN (Carl Zeiss), ImageJ (U.S. National Institutes of Health, Bethesda, Maryland) and MATLAB (The MathWorks, Inc., Natick, Massachusetts).

5.7 One-photon spectroscopy

UV/Vis absorption spectra were recorded using a Cary UV-Vis spectrophotometer (Agilent Technologies AB, Kista, Sweden). Fluorescence spectra were acquired by fluorescence spectrophotometer (Cary Eclipse, Varian AB, Bromma, Sweden). Spectral measurements were performed with 10 μM concentration of curcumin or 10 μM curcumin-HPγCD complex diluted in 99.7% ethanol. Absorption and emission spectra measurements are corrected for solvent background.

5.8 One-photon PDI

*Staphylococcus epidermidis* bacteria were cultured as previously described. Biofilms were grown for 48 hours in optical quality glass bottom petri dishes (35 cm diameter, NO 1.5 glass thickness, Ibidi, LRI Instrument AB). Samples were irradiated under an UV light source for 10 minutes corresponding to 5 J/cm². Following irradiation, samples were stained with FilmTracer stain, prepared and employed as described previously. Samples were washed carefully with fresh, sterile PBS (pH 7.4) with 2 mL PBS (pH 7.4) left in the dish for MPM imaging. Images and spectral characterization were acquired as described in “2-photon laser scanning microscopy” section. Images were analyzed as described previously.

5.9 Two-photon PDI

2PE PDI was performed by MPM as described previously. About 30 minutes prior to 2-photon photoinactivation experiments, growth media of 48 hours grown biofilms prepared as described in “biofilm formation” was removed and biofilm cultures were washed with fresh sterile PBS (pH 7.4). Curcumin solutions were prepared as described above and cultures were incubated with 1 mL of the solutions. Immediately prior to imaging cells were washed with PBS (pH 7.4) and 2 mL PBS was added to the culture for imaging with a 20x water-immersion objective.

The laser wavelength was set to 750 nm for photoinactivation; ideal excitation wavelength for both preparations of curcumin was determined using 2PE excitation scanning (Figure S2). Photoinactivation was performed using the photobleaching function of the microscope with the following procedure: an ROI for localized photoinactivation was chosen within the larger biofilm structure and this region was subjected to 5 cycles of 15 bleaching iterations at 15% laser power (~30 mW). Following irradiations, biofilms were stained with FilmTracer and images and spectral characteristics were acquired as previously described.

5.10 Airy scan microscopy

*Sample preparation: S. epidermidis* bacteria were grown as described previously; 2 mL aliquots of bacteria in suspension was added to glass bottom microscopy petri dishes (MatTek, Ashland, Maine) and biofilms were allowed to grow for 24 hours. Immediately prior to imaging, biofilms were washed carefully with PBS (pH 7.4). Biofilms were stained with HPγCD-curcumin or EtOH-curcumin solution, prepared as previously described, for 15 minutes. Biofilms were washed carefully with sterile PBS (pH 7.4) then stained with 1 mL of 10 μg/mL FM4-64 dye solution (excitation/emission = 558/734 nm, Thermo Fisher, Gothenburg, Sweden), prepared as suggested by manufacturer, for 1 minute. The final stain was removed and biofilms were washed carefully 2× with sterile PBS (pH 7.4) and kept in 2 mL sterile PBS for imaging. *Imaging:* Images were acquired in a Carl Zeiss LSM 880 Airyscan microscope using the “Fast Mode” application with a Plan-Apochromat 63x/1.4 oil objective. Images were collected with pixel dwell time 1.05 μs and image size 1000 × 1000 pixels corresponding to 37.49 × 37.49 μm. Laser excitation was split into 2 channels: 561 nm at .98% laser power, for excitation of FM4-64, and 405 nm at .93% laser power, for excitation of curcumin.

5.11 Statistical analysis

Statistical analysis was performed using excel data analysis, all viability experiments were performed in 3 replicates, error bars represent SD in percentage.
ACKNOWLEDGMENTS
The authors would like to acknowledge the Martin Andersson group, Chalmers University of Technology, for use of bacterial culture facilities and donation of the bacterial strain used in these studies and Prof. Alex Horswill, University of Colorado Anschutz Medical Campus, for donation of the fluorescent plasmids used. We also thank the Center for Cellular Imaging, University of Gothenburg, for use of infrastructure and kind support from the staff, in particular Dr Julia Fernandez-Rodriguez for assistance in super-resolution microscopy. Financial support was obtained from the Marie Curie Program #608407 Cyclon-HIT (FP7-PEOPLE-ITN-2013), the Swedish Research Council (621-2011-5189) and the Centre for Antibiotic Resistance Research (CARe) at the University of Gothenburg.

AUTHOR BIOGRAPHIES
Please see Supporting Information online.

ORCID
Hanna Thomsen http://orcid.org/0000-0001-6719-0919
Fabrice E. Graf http://orcid.org/0000-0003-1132-5348
Anne Farewell http://orcid.org/0000-0002-0841-4908
Marica B. Ericson http://orcid.org/0000-0002-5987-5915

REFERENCES
[1] T. F. C. Mah, G. A. O’Toole, Trends Microbiol. 2001, 9, 34.
[2] L. Hall-Stoodley, J. W. Costerton, P. Stoodley, Nat. Rev. Microbiol. 2004, 2, 95.
[3] N. Hatiby, T. Bjarnsholt, M. Givskov, S. Molin, O. Ciofu, Int. J. Antimicrob. Agents 2010, 35, 322.
[4] T. Bjarnsholt, K. Kirketerp-Møller, P. O. Jensen, K. G. Madsen, R. Phipps, K. Krogfelt, N. Hatiby, M. Givskov, Wound Repair Regen. 2008, 16, 2.
[5] T. G. St. Denis, T. Dai, L. Izikson, C. Astrakas, R. R. Anderson, M. R. Hamblin, G. P. Tegos, Virulence 2011, 2, 509.
[6] D. De Beer, R. Srinivasan, P. S. Stewart, Appl. Environ. Microbiol. 1994, 60, 4339.
[7] B. D. Hoyle, J. Alcantara, J. W. Costerton, Antimicrob. Agents Chemother. 1992, 36, 2054.
[8] M. C. Gomes, S. M. Woranovicz-Barreira, M. A. F. Faustino, R. Fernandes, M. G. P. M. S. Neves, A. C. Tomé, N. C. M. Gomes, A. Almeida, J. A. S. Cavaleiro, A. Cunha, J. P. C. Tomé, Photochem. Photobiol. Sci. 2011, 10, 1735.
[9] A. B. Hegge, E. Bruzell, S. Kristensen, H. H. Tønnesen, Eur. J. Pharm. Sci. 2012, 47, 65.
[10] P. F. Sperandio, Y. Y. Huang, M. R. Hamblin, Recent Pat. Antinfect. Drug Discov. 2013, 8, 108.
[11] D. M. A. Vera, M. H. Haynes, A. R. Ball, T. Dai, C. Astrakas, M. J. Kelso, M. R. Hamblin, G. P. Tegos, Photomed. Laser Surg. 2012, 88, 499.
[12] A. Tavares, C. M. B. Carvalho, M. A. Faustino, M. G. P. M. S. Neves, J. P. C. Tomé, A. C. Tomé, J. A. S. Cavaleiro, Â. Cunha, N. C. M. Gomes, E. Alves, A. Almeida, Mar. Drugs 2010, 8, 91.
[13] W. C. M. A. de Melo, P. Avci, M. N. de Oliveira, A. Gupta, D. Vecchio, M. Sadasivam, R. Chandran, Y.-Y. Huang, R. Yin, L. R. Perussi, G. P. Tegos, J. R. Perussi, T. Dai, M. R. Hamblin, Expert Rev. Anti Infect. Ther. 2013, 11, 669.
[14] C. A. Gordon, N. A. Hodges, C. Marriott, J. Antimicrob. Chemother. 1988, 22, 667.
SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

**FIGURE S1** Two-photon microscopy (2 PM) images and spectra of *Staphylococcus epidermidis* biofilms expressing dsRED and BFP (B, C) dsRED, λexcitation = 1000 nm (A, D) BFP, λexcitation = 760 nm. 2 PM images collected in z-stacks of 1 μm depth and represented in 3D.

**FIGURE S2** Two-photon excitation spectra of curcumin (10 μM, in PBS with 1% EtOH) and curcumin-CD (10 μM, in PBS) solutions. Solutions were excited at varying wavelengths from 700 to 900 nm at 10 nm intervals, emission was collected and reported as emission intensity at each represented wavelength.

**FIGURE S3** Effect of photodynamic inactivation against *Staphylococcus epidermidis* biofilms using curcumin and curcumin-CD as photosensitizers with UV-blue light irradiation with dosage 5 J/cm². Biofilms were grown in 96 well plates for 72 hours and treated with curcumin and curcumin-CD for 30 minutes, followed by illumination with UV light. An identical 96 well plate was treated with the same solutions but kept in the dark, outside of incubation, as a control experiment run parallel to the illuminated samples. Following illumination, biofilms were removed from the plate surface, resuspended in tryptic soy broth, and plated on tryptic soy agar plates in a serial dilution. Plates were incubated in 37°C for 24 hours and colony forming units were counted, displayed here as CFU/ml. Experiments were performed in replicates of 3; error bars represent SD based on the entire population.

How to cite this article: Thomsen H, Graf FE, Farewell A, Ericson MB. Exploring photoinactivation of microbial biofilms using laser scanning microscopy and confined 2-photon excitation. *J. Biophotonics*. 2018:11:e201800018. [https://doi.org/10.1002/jbio.201800018](https://doi.org/10.1002/jbio.201800018)