**Title: Fluorescence lifetime imaging detects long-lifetime signal associated with reduced pycoyanin at the surface of *P. aeruginosa* biofilms and in cross-feeding conditions**

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**Abstract:**

Understanding bacterial physiology in real-world environments requires non-invasive approaches and is a challenging yet necessary endeavor to effectively treat infectious disease. Bacteria evolved strategies to tolerate chemical gradients associated with infections. For example, the opportunistic pathogen *Pseudomonas aeruginosa* utilizes the redox-active metabolite, pyocyanin, as an alternative electron acceptor in low-oxygen. The reduced form of pyocyanin, in addition to other metabolites produced by *P. aeruginosa,*  are fluorescent, and imaging *P. aeruginosa* in biofilms or clinical samples is necessary to understand how *P. aeruginosa* responds to the local chemical environment. The DIVER, a custom-made instrument originally designed for deep tissue imaging, was used to image *P. aeruginosa* auto-fluorescence and the fluorescence lifetime throughout biofilms and aggregates. Using the DIVER fluorescence lifetime instrument, the reduced form of pyocyanin was detected at the surface of *P. aeruginosa* biofilms, where growth was dense and oxygen consumption was likely high. In addition, *P. aeruginosa* cultures had higher levels of reduced pyocyanin in the presence offermentation metabolites produced by, *Rothia mucilaginosa,* which co-colonizes the cystic fibrosis airways with *P. aeruginosa*.

I**ntroduction**

To colonize an infection-site, bacteria must adapt to the chemical gradients associated with many infections, including steep drops in oxygen that arise from low penetration and cellular consumption of oxygen (1, 2). Hypoxic conditions found in wounds and the airways of persons with cystic fibrosis (CF) pose a challenge to opportunistic pathogens, such as *Pseudomonas aeruginosa.*

*P. aeruginosa* can persist in low-oxygen by respiring anaerobically via denitrification (3, 4) and secreting phenazines (5–7). Phenazines are colorful, redox-active molecules that recycle electrons (6–10). Pyocyanin, the final product in the phenazine synthesis pathway, has the highest affinity for oxygen out of the phenazine family (9). *P. aeruginosa* uses oxidized pyocyanin to metabolize glucose into acetate, thereby generating more energy and reducing pyocyanin in the process (7).

In addition to low oxygen, factors such as quorum-sensing and the presence of other microbes can stimulate pyocyanin production. Specifically, fermentation metabolites produced by other microbes can drive *P. aeruginosa* pyocyanin production. This includes the fermentation products lactic acid and butanediol (11, 12), which are produced by microbes that co-colonize infection sites with *P. aeruginosa*, such as *Rothia mucilaginosa* (13, 14)*.*

In the oxidized form, pyocyanin has a blue pigment and is not fluorescent (15, 16). However, the reduced form of pyocyanin is fluorescent, and the emission spectrum of reduced pyocyanin overlaps with other fluorescent metabolites, including NADH and pyoverdine. The overlap in emission spectra makes it challenging to resolve the fluorescent signal of pycoyanin using standard single-channel fluorescent microscopy (17).

One approach to unmix pyocyanin from other fluorophores is hyperspectral imaging (17). However, the spatially dependent production of reduced pyocyanin in naturally formed oxygen gradients, such as those found in biofilms and infections, has not been characterized, due to limits in the imaging depths of commercial microscopes. The DIVER (18, 19) is a custom-made microscope designed for deep tissue imaging that can measure fluorescence intensity and lifetime. The fluorescence lifetime is the exponential decay of the fluorescence intensity of a fluorophore and can be used to determine the composition of multiple fluorophores contributing to a fluorescent signal. For example, fluorescence lifetime imaging microscopy (FLIM) of NADH is often used to study the respiratory state of cells, because the fluorescence lifetime of NADH shifts depending on local conditions. Specifically, the reported lifetime of free NADH is short (0.4 ns), whereas the lifetime of enzyme-bound NADH ranges from 1.7 to 9 ns (20–22).

FLIM data can be transformed and represented on phasors, a powerful, fit-free approach to analyze fluorescence lifetime data (23, 24) (**Fig. 1**). For lifetime images, the response of the fluorophore to fluorescent excitation is determined by Fourier transformation of exponential decay traces to obtain the modulation (M) and phase () for a given harmonic (23). The sine and cosine coefficients of the transform make up the y- and x-axes of the lifetime phasor. Fluorescent species with longer lifetimes are located towards the origin (G=0, S=0) of the phasor, while species with shorter lifetimes are located near the right-hand corner (G=0, S=1). Pure species with single exponential decays are located on the universal circle on the phasor (**Fig. 1A**). Shifts in the FLIM phasor position of samples containing multiple fluorophores is indicative of a change in the relative abundances of species. For example, the FLIM phasor has been used to track shifts in cellular respiration by imaging NADH, which has a longer lifetime when bound to enzymes (**Fig. 1B**). Another powerful feature of the phasor is the ability to project phasor analyses onto fluorescence intensity images. Specifically, the phasor coordinates of a fluorescent dye or sample can be color mapped onto the image, facilitating visualization of spatially-dependent lifetime signals (**Fig. 1C**).

Here, the DIVER was used to characterize the fluorescence lifetime of *P. aeruginosa* biofilms in infection-relevant conditions. By projecting FLIM phasor analyses onto fluorescent images, a long lifetime-signal associated with reduced pyocyanin was detected at the surface of the biofilm, where bacterial density was high. In addition, the fluorescence lifetime signal shifted towards reduced pyocyanin when *P. aeruginosa* was cross-fed fermentative metabolites produced by *R. mucilaginosa*.

**RESULTS:**

**Spectral characterization of *P. aeruginosa* fluorophores.**

The two-photon fluorescence emission spectra of *P. aeruginosa* fluorophores were characterized with a hyperspectral imaging microscope (excitation = 740 nm, emission window = 400-690 nm) (Fig. S1, Fig. 2). Most of the spectra agreed with previous publications (17). However, the emission spectra of pyocyanin and 1-hydroxyphenazine were broader than previously published data (17), and the spectra of chemically and electrochemically reduced pyocyanin had two peak wavelengths: 475 and 520 nm. In contrast, other reports found reduced pyocyanin has a single florescent peak around 475 nm (17). In addition, the fluorescent spectra of pyocyanin shifted depending on reduction method (Fig. S2, S3). Specifically, the florescent spectra shifted towards longer wavelengths with higher concentrations of reducing agent (Fig. S2). The spectra suggest the chemically and electrochemically reduced pyocyanin solutions consist of multiple fluorescent subspecies (Fig. S3).

**FLIM phasor characterization of *P. aeruginosa* fluorophores.**

Based on the spectral analyses (Fig. 2, S1), four out of the eight tested fluorophores (NADH, reduced pyocyanin, 1-hydroxy-phenazine, and pyoverdine) can be captured by the FLIM acquisition parameters used in downstream experiments, which included a two-photon excitation wavelength of 740 nm and a blue emission filter (400-500 nm).

Fluorescence lifetime data can be represented on the phasor (**Fig. 1**). The FLIM phasor positions of the solutions of free NADH, reduced 1-hydroxyphenazine, reduced pyocyanin, and pyoverdine are on or near the universal circle, suggesting the captured signals are from one species with a single exponential decay (**Fig. 2**). The previously reported phasor coordinates of lactate dehydrogenase (LDH) bound NADH (25) and an oxidized lipid signal (OLS) (26), are also depicted. Worth noting, reduced pyocyanin had a long lifetime signal (mean G= 0.02, S= 0.1; lifetime >10 ns) with a phasor position distinct from the other tested fluorophores (**Fig. 2**). The long lifetime signal has only been identified in *P. aeruginosa* cultures and not in other microbial cultures (Fig. S4) (27).

The fluorescence lifetime of reduced pyocyanin shifted depending on local conditions. Specifically, pyocyanin’s phasor position shifted left with increasing concentrations of TCEP (**Fig. S2A**). The FLIM phasor position of pyocyanin was nearly on the universal circle when pyocyanin was mixed with a 1:1 ratio of TCEP (Fig. 2, S2B). Taken together, the spectral and phasor data suggest that chemically and electrochemically reduced pyocyanin solutions contain multiple subspecies. However, the FLIM acquisition settings used here (400-500 nm emission filter) capture mainly the reduced form.

**Longer fluorescence lifetimes at the surface of *P. aeruginosa* biofilms**

The fluorescence intensity and lifetime were acquired throughout different depths of three-day old *P. aeruginosa* biofilms grown in artificial sputum medium (ASM) or M9 succinate agar using a custom-made microscope at the Laboratory for Fluorescence Dynamics, the DIVER (Deep Imaging Via Enhanced Recovery) (18, 19, 28). Two strains of *P. aeruginosa* PA14were cultured: wildtype (WT) and a phenazine double mutant *P. aeruginosa* PA14 *∆phzA1-G1*/ *∆phzA2-G2* (∆*phz*)(29), which does not produce phenazines. Laser power was increased with deeper imaging in the sample to compensate for signal attenuation from scattering and absorption. The measured total fluorescence intensity was similar throughout the biofilm depths, suggesting effective excitation delivery (**Fig. S5**).

The phasor position of cultures was distinct from uninoculated media (**Fig. S6**), indicating the fluorescence signal from cultures was above background. Cell density decreased with biofilm depth (**Fig. 3**). The FLIM phasor signal of the WT strains shifted with biofilm depth in both ASM and M9 succinate (**Fig. 3, 4**). However, the depth-dependent lifetime shift was not observed in the *∆phz* cultures (**Fig. 3, 4**). The surface of the WT biofilm was dominated by a longer lifetime species, with G and S values shifted more to the left than those of the *∆phz* strains (Wilcoxon rank sum test, p-value < 0.05 for both ASM and M9 suc comparisons). The WT fluorescence lifetime signals in both media types were near the reduced pyocyanin signal. Notably, the long lifetime signal associated with the WT cultures was prominent when a coverslip was placed the surface of the biofilm samples to facilitate imaging with higher-resolution water objectives. When imaged with an air objective, the lifetime signal of the biofilm with a coverslip was closer to reduced pyocyanin than that of the sample without a cover slip (**Fig. S7**), suggesting that oxygen at the surface of the biofilm was consumed.

***P. aeruginosa* fluorescence lifetime shifts in the presence of *Rothia* metabolites**

*P. aeruginosa* often co-colonizes infections with other microbes such as the fermenter, *Rothia mucilaginosa*. In the presence of fermentation metabolites, *P. aeruginosa* increases production of phenazines (11, 12). The CF strain, *P. aeruginosa* PaFLR01, was grown with supernatant from *R. mucilaginosa (*M9 suc + sup), in M9 media alone (M9 suc), or in the background media of the *Rothia* supernatant (ASM) (**Fig. 5**). The G and S distributions of *P. aeruginosa* grown in M9 suc + sup were significantly shifted to the left of the M9 alone and ASM cultures (Wilcoxon rank sum test, p-value < 2.2e-16, **Fig. 5A**). *P. aeruginosa* lifetime signal shifted towards reduced pyocyanin in the presence of *Rothia-*derived metabolites (**Fig. 5A, 5C**).

**Discussion**

Oxygen is scarce in many environments, and in the context of chronic infections, hypoxia drives microbes to produce redox-active metabolites to act as alternative electron acceptors. Bacterial biofilms have little oxygen beneath the surface (30). In low oxygen. *P. aeruginosa* synthesizes and secretes redox-active pyocyanin to recycle electrons (6). Pyocyanin production has also been associated with additional low-oxygen conditions, such as fermentation metabolites produced by co-colonizing microbes (11, 12). Pyocyanin can facilitate *P. aeruginosa* survival in hypoxic conditions (7) while contributing to disease progression (15, 16). We sought to determine the redox state of pyocyanin through *P. aeruginosa* throughout biofilms and in the presence of fermentation products, by utilizing fluorescence imaging to study phenazines and other fluorescent metabolites in infection-relevant conditions.

**Reduced pyocyanin had a distinct long lifetime signal.**

FLIM phasor analysis can be used to unmix the fluorescence components in a sample if (1) the fluorophores contributing to that signal are known and characterized and (2) the number of unsolved components is less than the number of orthogonal measurements. In our system (excitation = 740 nm, emission window = 400-500 nm), at least five fluorophores were detected (free NADH, enzyme bound NADH, reduced pyocyanin, pyoverdine, 1-hydroxy-phenazine) and could not all be unmixed. However, due to its distinct long lifetime signature, any shifts in the fluorescence lifetime 1-hydroxy-phenazine were assumed to be associated with reduced pyocyanin.

**The fluorescence lifetime shifted towards reduced pyocyanin at the biofilm surface.**

To recapitulate slower bacterial growth observed in infections (31, 32), colony biofilms were grown for three days in soft agar. The radial center of the colony, or point of inoculation, was imaged axially to capture the different biofilm depths in the oldest population of the biofilm. Reasoning that there would be less oxygen exposure deeper in the biofilm, we initially hypothesized that *P. aeruginosa* would produce more reduced pyocyanin deeper in the biofilm (6–8). However, in our system, reduced pyocyanin dominated the FLIM signal at the surface of the biofilm (**Fig. 3,4**). The shift towards reduced pyocyanin was more prominent when samples were imaged with a coverslip (which facilitated higher-resolution imaging with a water objective), albeit the longer lifetime trend was observed both with and without a coverslip (**Fig. S7**).

The highest density of *P. aeruginosa* growth was at the surface and was associated with the reduced pyocyanin FLIM signal (**Fig. 3,4**). The oxygen consumption rate of the dense bacterial populations at the biofilm surface is likely high, and the bacteria at the surface could have reduced a pool of pyocyanin for electron recycling. Our biofilm pyocyanin model agrees with previous studies showing that population density controls phenazine biosynthesis (33, 34) and oxygen is required for pyocyanin biosynthesis (35). Although it may seem counterintuitive that oxygen is necessary to synthesize an alternative electron acceptor, pyocyanin has the highest affinity for oxygen out of other studied phenazines (9). In locally anoxic conditions, *P. aeruginosa* couples pyocyanin reduction with oxidation of glucose and pyruvate, which generates ATP and increases anaerobic survival (7, 36, 37). The reduced pyocyanin is secreted and oxidized extracellularly (7, 8). A portion of the pyocyanin can be retained in the biofilms by *P. aeruginosa*-derived extracellular DNA that binds to phenazines (38, 39), distributing pyocyanin both inside and outside of the biofilm and enabling electron cycling.

**Cross-feeding interactions drive the fluorescent lifetime signal towards reduced pyocyanin.**

Although incapable of anaerobic fermentation, *P. aeruginosa* can co-colonize infection sites with other microbes that ferment in low-oxygen environments, including *R. mucilaginosa.* Fermentation products, such as butanediol and lactate, are metabolized by *P. aeruginosa* and promote pyocyanin production (11, 12, 40). In agreement, the fluorescence lifetime signal shifted towards reduced pyocyanin when *P. aeruginosa* was cross-fed *R. mucilaginosa*-derived supernatant. In previous studies with the same growth conditions, it was determined that *P. aeruginosa* metabolized pyruvate and lactate generated by *R. mucilaginosa* in the supernatant (1). The presence of fermentation metabolites may be used by *P. aeruginosa* as an indicator of low oxygen, driving production of pyocyanin before oxygen is completely depleted.

**Conclusion.**

Understanding bacterial physiology in conditions that mimic infections, such as chemical gradients, is an important step forward in improving treatment. Fluorescence lifetime imaging microscopy with the DIVER microscope was used to track changes in *P. aeruginosa* metabolism in naturally forming oxygen gradients in biofilms and in the presence of reducing chemicals. Specifically, we detected a long lifetime signal associated with reduced pyocyanin at the surface of biofilms and in the presence of fermentation metabolites.

**Limitations and future directions.**

The FLIM phasor approach allows the relative abundance of fluorescence metabolites to be determined by using linear algebra to unmix the fluorescence lifetime signal. This technique requires (1) prior knowledge of the fluorophores that can are present in the sample and (2) orthogonal measurements for a mix of five or more fluorescent species. This study relied on previous studies characterizing the fluorescent spectra of *P. aeruginosa* to initially identify fluorescent species that could be captured by the DIVER FLIM instrument*.* Due to the high number of fluorescent species produced by *P. aeruginosa,* quantitative analysis was not possible. However, any shifts in lifetime longer than the reported oxidized lipid signal of 7.8 ns(1) were assumed to be associated with reduced pyocyanin, which had a long lifetime signal >10 ns.

To fully determine the relative abundance and potentially concentration of fluorophores, additional measurements, such as higher harmonics of the phasor or additional spectral windows, are required.

**Methods and Materials**

**Chemicals and bacterial media.**

HPLC-grade pyocyanin was ordered from Sigma-Aldrich (P0046). 10 mM stocks were dissolved in 20% ethanol and stored at -20˚C. Artificial sputum and M9 minimal media with 40 mM succinate soft agar were used to grow *P. aeruginosa* biofilms. The recipes for both media types were modified from Gao et al. (40) to include 0.28% final agar concentration. To visualize biofilm colony growth over time, the agar was prepared in large petri dishes (150x15 mm).

**Bacterial strains and growth.**

*P. aeruginosa* PA14 and the phenazine knockout *phzA1-G1/A2-G2* were obtained from Dianne Newman’s lab at California Institute of Technology. For biofilm imaging, the bacteria were grown overnight on Todd-Hewitt agar, and individual colonies were inoculated into the center of the artificial sputum (ASM) or M9 succinate soft agar plates(1). The biofilm colonies were grown aerobically at 37˚C for 5 days.

For the cross-feeding experiment, Rothia mucilaginosa RmFLR01(1, 2) was grown in (ASM) for 48h. *Rothia*-derived supernatant was then filtered and cross-fed to a cystic fibrosis isolate P. aeruginosa FLR01 in M9 succinate for 72h in hypoxic conditions (2% oxygen). For a control, P. aeruginosa FLR01 was also grown in M9 succinate media or ASM alone. After the 72h incubation, P. aeruginosa FLR01 was transferred to a slide and suspended in 1% warm agar.

**Chemical reduction of 1-hydroxyphenazine and pyocyanin and electrochemical reduction of pyocyanin.**

Five hundred micromolar stocks of pyocyanin were diluted in 1X MOPS buffer with concentration gradients of TCEP ranging from 0.1 mM to 125 mM (pH 7). A fresh stock of 821 µM of pyocyanin was prepared in ammonium acetate 0.1M KCl MOPS buffered solution and electrochemically reduced following the protocol developed by Wang and Newman (9). The electrochemical cell consisted of a glassy carbon working electrode, platinum wire counter electrode, and Ag/AgCl2 reference electrode. The voltage was set to -0.345V, and the reaction proceeded in an anaerobic chamber overnight until the current reached zero. For 1-hydroxyphenazine, 500 micromolar stocks were prepared and diluted in 1XMOPS buffer with pH 7-buffered DTT as the reducing agent (41).

**Hyperspectral and fluorescence lifetime imaging on Zeiss LSM-880.**

To characterize the emission spectra and fluorescence lifetime of NADH, FAD, pyoverdine, reduced pyocyanin, and reduced 1-hydroxyphenazine, solutions were transferred to a clean slide. The reduced pyocyanin and 1-hydroxyphenazine were prepared in a Coy anaerobic chamber and sealed with iSpacers to avoid oxygen exposure (<https://www.sunjinlab.com/)>.

WT PA14 and *∆phz* biofilms grown in the artificial sputum and M9 succinate soft agar were cut with a sterile razor and placed onto a MATTEK dish (Part No: P35G-1.5-14-C) with the surface of the biofilm on the coverslip. To compare the impact of oxygen on the spectral and lifetime signal, the biofilm samples were (1) placed in a dish open to air and immediately imaged or (2) were placed in between two coverslips and sealed in the dish with tape for 2h at RT to promote oxygen consumption.

The pure fluorophore solutions and biofilm surfaces were imaged on an inverted Zeiss LSM-880 with an ISS Spartan3 FLIMbox, BH HPM-100-40-Hybrid detector, and a Spectra Physics Mai Tai titanium sapphire laser. The fluorophores were excited with 2-photon excitation at 740 nm and laser power ranging from 1-10 mW. For the hyperspectral imaging, emission ranging from 410-695 nm was collected with 9 nm step resolution over 32 channels. One frame was collected per sample with a pixel dwell time of 4 µs. The spectra were analyzed on Zeiss Zen software, and .lsm files were exported for downstream unmixing steps. After collecting the spectra, fluorescence lifetime of the same sample was obtained by switching the light path to the FLIMbox detectors. The sample was excited with the same wavelength and laser power as the spectral images. The emission was filtered with a 495 nm LP dichroic and Semrock 442/46 nm BrightLine single-band bandpass filter (CFW-BP01-Clin-25). The fluorescence lifetime data was acquired using simFCS software v4. To obtain enough fluorescence lifetime signal for the downstream analysis, 10-30 frames were collected per sample, with a frame size of 256x256 pixels and pixel dwell time of 32 µs.

**Z-stack fluorescence lifetime of WT PA14 biofilms on the DIVER microscope.**

Intact WT *P. aeruginosa* PA14biofilms were grown in ASM for five days and prepared for z-stack imaging by adding a large coverslip on top of the surface of the biofilm, and then imaged with a 0.8 NA 40x Water objective. Z-stacks were obtained on a custom-made microscope at the Laboratory for Fluorescence Dynamics, the DIVER (Deep Imaging Via Enhanced Recovery) (18, 19, 28). The DIVER is a Nikon Eclipse TE2000-U microscope equipped with a wide-area 18x18 mm photomultiplier tube (PMT) (Hamamatsu R7600P-300) which enhances photon collection. Samples were excited with 2-photon excitation at 740 nm using a Tsunami Spectra-Physics Ti:Sapphire laser (80 MHz). The emission was filtered with a Schott BG-39 filter and NADH-targeted optical bandpass filter (400-500 nm). The fluorescence lifetime data was collected with SimFCS v4 software. Z-stacks were automatically acquired every 100 µm from the surface of the biofilm to 1 mm deep. The laser power was increased with an exponential function for deeper sample imaging, with the power ranging from 1-58 mM.

**Fluorescence lifetime analysis and visualization.**

The fluorescence lifetime data was analyzed in SimFCS v4 software using the phasor approach (42). The phasor approach uses a cosine-sine discrete fast Fourier transform to transform raw fluorescence lifetime traces onto a two-coordinate polar phasor plot. The resulting G and S coordinates are the cosine and sine components of the transform for a given frequency (80 MHz). For the FLIM phasor analyses, images were processed following Ranjit et al (43). The images were first masked using fluorescence intensity thresholds to exclude pixels with background signal. The resulting images contain the fluorescence lifetime phasor coordinates for each pixel. For the single cell or cluster analyses, individual cells or group of cells were selected as regions of interest, and the average g and s values were calculated.

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