**Fluorescence lifetime imaging detects long-lifetime signal associated with reduced pyocyanin at the surface of *Pseudomonas aeruginosa* biofilms and in cross-feeding conditions**

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Fluorescent emission spectra of all fluorophores, including florophores with emission sepctra outside of the FLIM range

Flourescent spectra and lifetime signal for chemically and electrochemically reduced pyocyanin

Fluorescent intensity signal at the surface vs. bottom of the biofilm and similar lifetime signal for different frame counts

FLIM distributions for *P. aeruginosa* biofilms compared to uninoculated media

FLIM phasor signal for different bacteria

Comparison of FLIM signal for biofilms with and without a coverslip

Pairwise t-test results for phasor coordinates for different biofilm depths

Pairwise t-test results for phasor coordinates for cross-feeding conditions

**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 evolve strategies to tolerate chemical gradients associated with infections. The DIVER (Deep Imaging Via Enhanced Recovery) microscope can image auto-fluorescence and fluorescence lifetime throughout highly- scattering samples, enabling the study of naturally-forming chemical gradients throughout intact biofilms. Using the DIVER, a long fluorescent lifetime signal associated with reduced pyocyanin, a molecule for electron cycling in low-oxygen, was detected in low-oxygen conditions – at the surface of *Pseudomonas aeruginosa* biofilms and in the presence offermentation metabolites from *Rothia mucilaginosa,* which co-colonizes infected airways with *P. aeruginosa*. These findings underscore the utility of the DIVER microscope and fluorescent lifetime in non-invasively studying bacterial physiology within complex environments, which could inform on more effective strategies for managing chronic infection.

**Keywords:**

Fluorescence, fluorescence lifetime, biofilm, *Pseudomonas aeruginosa*, pyocyanin, phenazines

To colonize an infection site, bacteria adapt to the chemical conditions associated with infected tissue, including oxygen depletion (1–3). These low-oxygen environments are commonly found in wound infections and the airways of persons with cystic fibrosis (CF), caused by the rapid consumption of oxygen from host cells (4) and bacterial biofilms (1).

The opportunistic pathogen, *Pseudomonas aeruginosa,* survives in low-oxygen environments through anaerobic respiration via denitrification (5, 6) and secreting phenazines (7, 8), which are colorful, electron-recycling molecules that contribute to disease progression (7–12). Production of the phenazine, pyocyanin, is promoted by low oxygen, in addition to other conditions associated with hypoxia. Specifically, pyocyanin production is associated with high cell densities (13, 14) and the presence of fermentation metabolites produced by anaerobes that co-colonize infection sites (15, 16). Pyocyanin can also be retained in biofilms by *P. aeruginosa*-derived extracellular DNA (17, 18), distributing pyocyanin both inside and outside of the biofilm and enabling electron cycling in local environments with low oxygen. Furthermore, pyocyanin has been linked with increasing *P. aeruginosa* tolerance to antibiotics in low oxygen environments (19).

Due to its role in low-oxygen survival and disease progression, there has been interest in detecting pyocyanin in models that recapitulate infection-relevant conditions. Pyocyanin is a blue-pigmented, non-fluorescent molecule in its oxidized form (12, 20). The reduced form of pyocyanin is fluorescent, and the emission spectrum overlaps with other fluorophores including NADH and pyoverdine (21). Hyperspectral imaging can unmix multiple fluorophores (21). However, the limited imaging depths of commercial microscopes poses challenges to spatially resolving signals throughout naturally-formed chemical gradients. The DIVER (Deep Imaging Via Enhanced Recovery) (22–24) is a custom-made microscope that measures fluorescent intensity and fluorescent lifetime throughout high-scattering samples.

The fluorescent lifetime refers to the exponential decay of the fluorescent intensity and can be used to identify multiple fluorophores contributing to a signal. Fluorescent lifetime imaging microscopy (FLIM) data can be represented using phasors, a fit-free approach to analyze FLIM data (25). Longer lifetimes are near the phasor origin (G=0, S=0), while shorter lifetimes are near the right-hand corner (G=0, S=1). Fluorophores with single exponential decays are located on the universal circle of the phasor, and combinations of different fluorophores fall on the line connecting the coordinates of pure species. The distance of the signal from each contributor can be used to calculate relative contribution. In samples consisting of three or less species, the relative abundance of each species can be calculated algebraically with one harmonic measurement (25).

**RESULTS & DISCUSSION:**

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

The 2-photon emission spectra of eight fluorophores were characterized with a hyperspectral imaging microscope (740 nM excitation and emission range of 400-690 nm). The fluorophores included ubiquitous metabolites such as coproporphyrin, flavin adenine dinucleotide and nicotinamide adenine dinucleotide, in addition to pyoverdine and phenazines *–* 1-hydroxyphenazine, phenazine-1-carboxylic acid, 5-methyl phenazine-1-carboxylic acid, phenazine-1-carboxamide, pyocyanin (**Fig. S1**). Chemically and electrochemically reduced pyocyanin had a peak emission at 520 nm (**Fig. S2, Fig. 2A**). The peak emission at 520 nm differed from a previous study, which found chemically-reduced pyocyanin had a peak at 475 nm, despite using identical reduction methods (21).

Based on spectral analyses (**Fig. 2A, S1**), four of the eight fluorophores (NADH, reduced pyocyanin, reduced 1-hydroxy-phenazine, and pyoverdine) can be captured by the FLIM acquisition parameters used in downstream experiments, which included a blue emission filter (400-500 nm). While the spectrum of the pyocyanin precursor, 5-methyl phenazine-1-carboxylic acid, was not measured in this study due to commercial unavailability, previous studies have reported an emission peak at 620 nm (21, 26). The fluorescence of 5-methyl phenazine-1-carboxylic acid would likely contribute minimally to signal detected by the blue emission filter.

A graph of a normalized fluorescence

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**Figure 2: Fluorescent spectra and lifetime phasor of some of the fluorescent metabolites produced by *P. aeruginosa***(two-photon excitation = 740 nm). (**A**) Florescence emission spectra normalized by max peak intensity of fluorophores which emit in the 400-500 nm window (grey-shaded box). (**B**) Fluorescence lifetime phasor of fluorescent solutions, collected with a Schott BG-39 filter and NADH-targeted optical bandpass emission filter (400-500 nm). Points represent the phasor G and S coordinates from a fluorescence lifetime image, colored by the number of pixels. The large shapes represent the mean G and S values for each fluorescent solution. The mean G and S values for LDH NADH and OLS were determined from previously reported fluorescence lifetimes (27–30). LDH = Lactate Dehydrogenase, OHPhz = 1-hydroxyphenazine (DTT reduced), OLS = Oxidized Lipid Signal, PVD = pyoverdine, PYO = pyocyanin (TCEP reduced).

The FLIM phasor positions of solutions of free NADH, reduced 1-hydroxyphenazine, reduced pyocyanin, and pyoverdine are on the universal circle, suggesting the signals originate from a single exponential decay (**Fig. 2**). For reference, also depicted are the previously-reported phasor coordinates of lactate dehydrogenase (LDH) bound NADH (29) and an oxidized lipid signal (OLS) (28, 30). Notably, reduced pyocyanin had a long lifetime signal (mean G= 0.02, S= 0.1; lifetime >10 ns) with a distinct phasor position (**Fig. 2**).

The emission spectra and lifetime of pyocyanin shifted with increasing TCEP concentrations, indicative of a longer fluorescence lifetime as the solution was further reduced (**Fig. S2**). Pyocyanin was nearly on the FLIM universal circle when mixed with a 1:1 ratio of TCEP in an anaerobic chamber (**Fig. 2, S2B**).

**Longer fluorescence lifetimes at the surface of *P. aeruginosa* biofilms when limiting oxygen exposure**

To recapitulate slower bacterial growth observed in infections (31, 32), colony biofilms were grown for 72h 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 using the DIVER microscope (22–24). Laser power was increased with imaging depth to compensate for signal attenuation (**Fig. S3A-B**). Prolonged laser exposure did not significantly impact the fluorescence lifetime signal, as the phasor coordinates were similar when comparing the first frame to all frames for a given sample (**Fig. S3C**). Acquiring multiple frames for a single image reduced noise in the FLIM phasor signal (**Fig. S3D**).

Two strainswere cultured: wildtype (WT) and a phenazine double mutant *P. aeruginosa* PA14 *∆phzA1-G1*/ *∆phzA2-G2* (∆*phz*). The phasor position of *P. aeruginosa* cultures was distinct from uninoculated media, the exception being for ∆*phz* strains in ASM, likely due to high amounts of NADH in ASM (**Fig. S4, Fig. 3**). The FLIM phasor signal of the WT strains shifted with biofilm depth in both ASM and M9 succinate, as demonstrated by a shift in G and S distributions (**Fig. 3A-B).** The mean G and S values also had a depth-dependent shift for the WT strain **(Fig. 3C; Table S1**). Specifically, WT PA14 had significantly different phasor G and S values at the surface of the biofilm compared to the 100-100 µm measurements in ASM and the 200-1000 µm measurements in M9 succinate (t-test, FDR < 0.05; **Table S1**). The depth-dependent lifetime shift was not observed in the *∆phz* cultures (**Fig. 3, Table S1**). The surface of the WT biofilm was dominated by a longer lifetime species. The long fluorescent lifetime signal associated with the WT *P. aeruginosa* cultureswas also not observed in cultures of other microbial genera (**Fig. S5**) (33). Examples of fluorescent intensity images, normalized by max intensity (**Fig. 4A**), with the FLIM phasor signal color-mapped on top of the image (**Fig. 4B-C**) indicate the FLIM signal is similar both within and outside of cell clusters, suggesting secreted molecules are largely contributing to the fluorescent lifetime signal.

Given that other phenazines emit low fluorescent signal within the emission filter range and reduced 1-hydroxyphenazine has a short fluorescence lifetime distinct from reduced pyocyanin (**Fig. 2, S1**), reduced pyocyanin is likely producing the long lifetime signal found at the surface of WT *P. aeruginosa* biofilms (**Fig. 3, 4**).

We initially hypothesized thatthere would be higher levels of reduced pyocyanin deeper in the biofilm (7–9), reasoning there would be less oxygen exposure. However, the long lifetime signal associated with reduced pyocyanin was prominent at the surface of WT biofilms. The reduced pyocyanin signal was prominent when biofilms were prepared for imaging with a coverslip (**Fig. S6**), and there was high density of *P. aeruginosa* growth at the biofilm surface (**Fig. 3, 4**). The oxygen consumption rate of the dense bacterial populations at the biofilm surface is likely high, and the bacteria could have reduced a pool of pyocyanin for electron recycling. This model agrees with previous observations that population density controls phenazine biosynthesis (34, 35) and oxygen is required for pyocyanin biosynthesis (36). Although it may seem counterintuitive that oxygen is necessary for synthesis, pyocyanin has the highest affinity for oxygen out of other studied phenazines (10). In locally anoxic conditions, *P. aeruginosa* couples pyocyanin reduction with oxidation of glucose and pyruvate, which generates ATP and increases anaerobic survival (8, 37, 38). The reduced pyocyanin is secreted and oxidized extracellularly (8, 9), and a portion of the pyocyanin is retained in the biofilms when bound to extracellular DNA (17, 18).

**A diagram of a number of pixels

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**Figure 3: Fluorescence lifetime at different depths of *P. aeruginosa* PA14 biofilms.** (**A**) Fluorescence lifetime phasor of *P. aeruginosa* PA14 WT and *∆phz* strains grown in ASM or M9 succinate soft agar for 72h. Three replicates of biofilms were imaged every 100 µm from the biofilm surface (0 µm) to the bottom (1000 µm) (33 images per strain and media type, 132 images total). The scatter plot points are G and S values of pixels from fluorescence lifetime images, where the color indicates number of pixels at a given G,S coordinate. For reference, mean G and S values of fluorescent solutions from Figure 2 are displayed as black shapes. (**B**) One-dimensional distribution of phasor G and S values, where line type and color are indicative of biofilm depth (0 µm = biofilm surface). The grey dotted line is the uninoculated M9 succinate or ASM media (2 images). The G and S distributions from the surface of the WT biofilms were significantly shifted to the left of the *∆phz* biofilm surfaces in both media types (Wilcoxon rank sum test, p-value < 2.2e-16). (**C**) Mean G and S values for each image, colored by biofilm depth. The gray crosses represent uninoculated ASM and M9 succinate. The G and S values for WT PA14 were significantly different at the biofilm surface than at other measured depths (200-1000 µM for M9 succinate and 100-1000 µM for ASM; **Table S1**). *∆phz* = *P. aeruginosa* PA14 *∆phzA1-G1/∆phzA2-G2*; does not produce PYO or OHPhz. LDH = Lactate dehydrogenase, OHPhz = 1-hydroxyphenazine, OLS = Oxidized Lipid Signal, PVD = pyoverdine, PYO = pyocyanin, ASM = artificial sputum medium, suc = succinate.

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**Figure 4: Fluorescence intensity and lifetime images at the surface and 1000 µM deep into *P. aeruginosa* PA14 biofilms.** (**A**) Examples of auto-fluorescent intensity images of *P. aeruginosa* PA14 WT and *∆phz* strains grown in ASM or M9 succinate soft agar for 72h. Scale bar = 20 µm. (**B**) The fluorescence lifetime image at the surface and 300-1000 µM deep into *P. aeruginosa* biofilms. In the image (**B**) and corresponding phasor (**C**), each pixel in the image is colored based on phasor position by overlaying a color map in the phasor in panel C. For reference, mean G and S values of pure fluorescent solutions from Figure 2 are displayed as black shapes (**C**). The same color map was used for all lifetime images, where blue is indicative of a longer lifetime near the origin of the phasor (G=0, S=0) and orange is indicative of shorter lifetime near free NADH (lifetime = 0.4 ns; G=0.96=, S=0.19). ASM = artificial sputum medium, suc = succinate.

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

Although incapable of anaerobic fermentation, *P. aeruginosa* can co-colonize infection sites with other microbes that ferment in low-oxygen environments, including *R. mucilaginosa.* To investigate observations that *P. aeruginosa* increases pyocyanin production in the presence of fermentation metabolites (15, 16), *P. aeruginosa* PaFLR01 was grown with supernatant from *Rothia mucilaginosa (*M9 suc + sup), in M9 media alone (M9 suc), or in ASM (the background media of the *Rothia* supernatant) (**Fig. 5**). The S distribution of *P. aeruginosa* grown in M9 suc + sup was significantly shifted to the left towards reduced pyocyanin for (**Fig. 5**).

In previous studies, it was determined that *P. aeruginosa* metabolized pyruvate and lactate generated by *R. mucilaginosa* in the supernatant (39). 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.

A close-up of a graph

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**Figure 5: *P. aeruginosa* PaFLR01** **fluorescence lifetime shifts when cross-fed *Rothia* supernatant.** (**A**) Fluorescence lifetime phasor of *P. aeruginosa* PaFLR01 grown in three media types under hypoxic conditions. Scatter plot points are G and S coordinates of pixels from fluorescence lifetime images of PaFLR01 grown in hypoxic ASM (14 images), M9 suc (8 images), or M9 suc + sup (7 images). For reference, mean G and S values of pure fluorescent solutions are displayed on the universal circle as black shapes. The two-dimensional G and S distributions were significantly different for each pairwise comparison (ASM vs. M9 suc, ASM vs. M9 suc+sup, M9 suc vs. M9 suc+sup; Fasano-Francheschini Test, p-value < 2.2e-16). (**B**) The one-dimensional distributions of S from the image data in panel A are represented in each plot and colored by media condition. M9 suc + sup S distributions were significantly shifted to the left of the M9 suc and ASM distributions (Wilcoxon rank sum test, p-value < 2.2e-16). The blue line represents the lower S limit where only reduced pyocyanin is expected to be detected. **C**) Examples of fluorescent intensity images from the cross-feeding experiment. Scale bar = 3 µm. (**D**) The fluorescence lifetime image (top) from three example samples, colored by position on the phasor (bottom). The pixel color in the images corresponds with the color of pixels in the phasor. Blue is indicative of a longer lifetime near the origin of the phasor. LDH = Lactate Dehydrogenase, OHPhz = 1-hydroxyphenazine, OLS = Oxidized Lipid Signal, PVD = pyoverdine, PYO = pyocyanin, ASM = artificial sputum medium, suc = succinate, sup = supernatant from *Rothia mucilaginosa*.

**Conclusion.**

To our knowledge, this is the first study to measuremetabolites throughout an intact *P. aeruginosa* biofilm. We detected a long fluorescent lifetime signal associated with reduced pyocyanin at the surface of intact biofilms and in the presence of fermentation metabolites. Given the significance of pyocyanin in disease progression and *P. aeruginosa* physiology, the DIVER FLIM approach shows promise as a tool to advance understanding of infections and treatment strategies in infection-relevant models.

**Limitations and future directions.**

Due to the large number of fluorescent species produced by *P. aeruginosa,* detection of every fluorophore was not feasible. In this study, any fluorescent (30)Due to the large number of detected fluorophores, theadditional (40, 41)

**METHODS**

**Chemicals and bacterial media.**

Ten millimolar stocks of pyocyanin (Sigma-Aldrich P0046) and 1-hydroxyphenazine (OHPhz) (Fisher H0289100MG) were dissolved in 20% ethanol and methanol, respectively. Phenazine-1-carboxamide (PCN) (Sigma-Aldrich AMBH2D6F8130) and phenazine-1-carboxylic acid (PCA) (Fisher 2538-68-3) were dissolved in chloroform to 4.5 mM. Dissolved phenazine stocks were stored at -20˚C.

Artificial sputum and M9 minimal media with 40 mM succinate soft agar in large petri dishes (150x15 mm) were used to grow *P. aeruginosa* biofilms. Both media recipes were modified from Gao et al. (39) to include 0.28% final agar concentration.

**Bacterial strains and growth.**

*P. aeruginosa* PA14 and the phenazine knockout *phzA1-G1/A2-G2* were obtained from Dianne Newman (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 (39). The biofilms were grown in an aerobic incubator at 37˚C for 3 days.

For the cross-feeding experiment, Rothia mucilaginosa RmFLR01(15, 39) was grown in liquid (ASM) for 48h. *Rothia*-derived supernatant was then filtered and cross-fed to a cystic fibrosis isolate, *P. aeruginosa* FLR01, in liquid M9 succinate for 72h in hypoxic conditions (2% oxygen). For a control, *P. aeruginosa* FLR01 was also grown in liquid M9 succinate media or ASM alone. After the 72h incubation, 5 µL of the *P. aeruginosa* FLR01 culture was transferred to a slide and mixed with 5 µL of 1% pre-warmed agar, heated to approximately 55°C in an hypoxic chamber (2% oxygen). To maintain an hypoxic environment the coverslip was then sealed onto the slide with nail polish. The slide was removed from the chamber and imaged within 30 minutes.

**Chemical and electrochemical reduction of phenazines**

Prior to reduction, dissolved phenazine stocks were moved from the -20˚C to a Coy Anaerobic Chamber (0% oxygen).

For chemical reduction of pyocyanin, 75 to 600 µM stocks of pyocyanin were diluted in 1X MOPS buffer with TCEP concentrations ranging from 0.1 mM to 125 mM (pH 7).

For electrochemical reduction, a fresh stock of 821 µM of pyocyanin was prepared in ammonium acetate 0.1M KCl MOPS buffer and electrochemically reduced following Wang and Newman (10). The electrochemical cell consisted of a glassy carbon working electrode, platinum wire counter electrode, and Ag/AgCl reference electrode. The voltage was set to -0.345V, and the reaction proceeded in an anaerobic chamber overnight until the current reached zero.

The three other phenazines only underwent chemical reduction. For 1-hydroxyphenazine, 500 micromolar stocks were diluted in 1XMOPS buffer with pH 7-buffered DTT as the reducing agent. Five hundred micromolar stocks of phenazine-1-carboxamide and phenazine-1-carboxylic acid were diluted in 1x MOPS buffer with 5 mM TCEP.

**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, pure solutions were transferred to a clean slide. 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 ASM and M9 succinate soft agar were cut with a sterile razor and placed onto a MATTEK dish (P35G-1.5-14-C) with the biofilm surface facing the coverslip. To compare the impact of oxygen on the spectral and lifetime signal, the biofilm samples were (1) placed in a dish and immediately imaged or (2) were placed in between two coverslips and sealed in the dish with tape for 2h at room temperature.

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 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 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 SimFCS4. 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 biofilms on the DIVER microscope.**

Intact WT *P. aeruginosa* PA14biofilms were grown in ASM for three 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 (Olympus LUMPLFLN 40XW Objective). Z-stacks were obtained on a custom-made microscope at the Laboratory for Fluorescence Dynamics, the DIVER (Deep Imaging Via Enhanced Recovery) (22–24). 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). 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 mW.

**Fluorescence lifetime analysis and visualization.**

Fluorescent lifetime data analysis was performed in SimFCS v4-20200515 software (42). The pixels in the fluorescent images were median-filtered. Plots and statistical analyses were performed in python and R (<https://github.com/tgallagh/PseudomonasFLIM>).

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