**Title ideas:**

* **Visualization of *Pseudomonas aeruginosa* pyocyanin reduction at the surface of biofilms**

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**Abstract:**

Understanding bacterial physiology in real-world environments is a challenging, yet necessary endeavor to effectively treat infection. Studying bacteria in infection-relevant conditions requires non-invasive approaches, such as fluorescence lifetime imaging microscopy (FLIM). FLIM of auto-fluorescent metabolites, including NADH, can be used to track changes in cellular metabolism. Here, FLIM of bacterial cultures and biofilms was performed on the DIVER, a custom-made instrument originally designed for deep tissue imaging. The metabolism of *Pseudomonas aeruginosa*, a ubiquitous opportunistic pathogen, shifted throughout a biofilm and in coculture-like conditions. Specifically, the reduced form of a redox-active metabolite, pyocyanin, was detected at the surface of *P. aeruginosa* biofilms, where growth was dense and oxygen consumption was possibly high. In addition, *P. aeruginosa* cultures had higher levels of reduced pyocyanin in the presence ofmetabolites produced by *Rothia mucilaginosa,* an oral microbe reported to cocolonize the cystic fibrosis airways. Taken together, FLIM can be used to detect shifts in bacterial metabolism and the presence of pyocyanin in conditions associated with airway infections.

I**ntroduction**

To persist in an infection, bacteria adapt to chemical gradients. Infection environments can consist of steep drops in oxygen, which arise from low penetration and cellular consumption of oxygen (1, 2). A common opportunistic pathogen, *Pseudomonas aeruginosa* must survive in hypoxic conditions, in environments such as wounds and the airways of persons with cystic fibrosis (CF). *P. aeruginosa* can persist in low-oxygen by respiring anaerobically via denitrification (4, 5) and secreting phenazines (6–8)1/23/22 7:54:00 PM. Phenazines are colorful, redox-active molecules that recycle electrons. Pyocyanin, the final product in the phenazine synthesis pathway, has the highest affinity for oxygen out of the phenazine family (9). In the oxidized form, pyocyanin has a blue pigment and is toxic to other cells (10, 11). *P. aeruginosa* uses oxidized pyocyanin to metabolize glucose into acetate, thereby generating more energy and reducing pyocyanin in the process. The reduced form of pyocyanin is fluorescent, but the emission spectrum of pyocyanin overlaps with other fluorescent metabolites, including NADH and pyoverdine (12).

Hyperspectral imaging microscopy can unmix pyocyanin from fluorophores like NAHD and pyoverdine (12). However, the spatial production and reduction of pyocyanin in naturally-formed oxygen gradients, such as those found in biofilms and sputum, has not been characterized, due to limits in the imaging depths of commercial microscopes. The DIVER (13, 14) is a custom-made microscope designed for deep tissue imaging that can measure fluorescence 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. The lifetime of free NADH is short (0.4 ns), whereas the lifetime of enzyme-bound NADH reportedly ranges from 3 to 8 ns (ref).

FLIM data can be transformed and represented on phasors, a powerful, fit-free approach to analyze fluorescence lifetime data (20, 21) (**Fig. 1**). For lifetime images, the response of the fluorophore to the excitation source is determined by Fourier transformation of exponential decay traces to obtain the modulation (M) and phase () at different harmonics (20). The sine and cosine coefficients of the transform make up the y and x-axes of the lifetime phasor. Pure species (with single exponential decays) are located on the universal circle (**Fig. 1A**). Samples containing a combination of the fluorescent species fall on a line connecting the sample signal to the pure components, where the distance from the sample signal to the pure component is proportional to fractional contribution of that species,

Here, the DIVER was used to characterize the fluorescence lifetime of *P. aeruginosa* in infection-relevant conditions. FLIM of *P. aeruginosa* biofilms indicated an accumulation of reduced pyocyanin at the surface of the biofilm, where bacterial density and oxygen consumption were high. In addition, FLIM detected reduced pyocyanin when *P. aeruginosa* was cross-fed fermentative metabolites produced by *Rothia mucilaginosa*. Taken together, FLIM phasor analysis can be used to track the spatial changes in reduced pyocyanin and other fluorescent metabolites.

**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 (12). However, the emission spectra of pyocyanin and 1-hydroxyphenazine were broader than previously published spectra. Interestingly, the spectra of chemically and electrochemically reduced pyocyanin had two peak wavelengths: 475 and 520 nm, while another study reported reduced pyocyanin has a single florescent peak around 475 nm. In addition, the fluorescent spectra of pyocyanin shifted depending on the reduction method used (Fig. S3, S4). Specifically, the florescent spectra shifted towards longer wavelengths with higher concentrations of reducing agent (Fig. S3). Taken together, this suggests the chemically and electrochemically reduced pyocyanin solutions actually consist of multiple species(Fig. S3).

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

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

Fluorescence lifetime data can be represented on the phasor, where longer lifetime species are located towards the origin (G=0, S=0) and shorter lifetime species approach the righthand corner (G=0, S=1) (**Fig. 1**). The fluorescence lifetime of solutions of free NADH, pyoverdine, reduced 1-hydroxy-phenazine, and reduced pyocyanin are represented on the phasor. The FLIM phasor position of the solutions are on (or near) the universal circle, suggesting the captured signals each come from one species with a single exponential decay (**Fig. 2**). The phasor position of Malate\_Dehydrogenze (MDH) bound NADH and an oxidized lipid signal (OLS) associated with fatty acid oxidation, are also depicted (ref). Worth noting, reduced pyocyanin had a long lifetime signal with a phasor position distinct from the other fluorophores (**Fig. 2**). Furthermore, this long lifetime signal (>10 ns) has only been identified in *P. aeruginosa* cultures and not in other microbial cultures (Fig. S1) (ref).

The fluorescence lifetime of reduced pyocyanin shifted depending on local conditions. Specifically, pyocyanin’s fluorescence lifetime increased with increasing concentrations of TCEP (Fig. S3, S4). 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, S3, S4). Taken together, the spectral and phasor data suggest that chemically and electrochemically-reduced pyocyanin solutions contain multiple forms, but the FLIM settings (400-500 nm emission filter) likely capture only one form.

**Longer fluorescence lifetimes at the surface of *P. aergunisa* 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 the DIVER FLIM (13, 14). Two strains of *P. aerignosa* PA14were cultured: wildtype (WT) and a phenazine double mutant (∆phz), (ref) which does not produce any phenazines, including 1-hydroxy-phenaine and pycoyanin (see methods). Laser power was increased with deeper imaging in the sample to compensate for signal attenuation from scattering and absorption (Table S). The measured total fluorescence intensity was similar throughout the biofilm depths, suggesting effective excitation delivery (**Fig. S**). The FLIM phasor signal of all cultures was distinct from uninoculated media (Fig. S). Cell density decreased with biofilm depth, indicating more growth at the biofilm surface (**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 ∆phzcultures (Fig. 3, 4). The WT biofilm surface FLIM signal was dominated by a longer lifetime species, with G and S values shifted more to the left than the ∆phz strains (Wilcoxon rank sum test, p-value < 2.2e-16 for both ASM and M9 suc comparisons). The WT fluorescence lifetime signals in both media types were near that of the reduced pyocyanin signal.

We found that this long lifetime signal associated with the WT cultures was observed when a coverslip was placed on top of the biofilm samples. FLIM of the biofilm surface without a cover slip was acquired 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. In addition,

This suggests limitation in oxygen is driving the lifetime signal of *P. aeruginosa*  towards reduced pyocyanin.

Further confirmed by growing

***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,* and increases production of pyocyanin in the presence of fermentation metabolites(ref). *P. aeruginosa* PaFLR01 was grown with the supernatant from *R. mucilaginosa (*M9 suc + sup), in M9 media alone (M9 suc), or in the background media of the *Rothia* supernatant (ASM). 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; Table 1). This suggests the lifetime signal shifts towards reduced pyocyanin in the presence of *Rothia* derived metabolites.

**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; however, some of these metabolites are also toxic and may contribute to disease progression. Bacterial biofilms have little oxygen beneath the surface. *P. aeruginosa* synthesizes and secretes redox-active pyocyanin to recycle electrons in low-oxygen (8). We sought to determine the redox state of *P. aeruginosa-*produced pyocyanin throughout biofilms and in the presence of fermentation metabolites, and utilized fluorescence imaging to study phenazines and other fluorescent metabolites in infection-relevant condtions.

**FLIM and HIM unmixing results varied for fluorophores.**

FLIM phasor unmixing of the first harmonic was used to determine the contribution of four species - reduced pyocyanin, apo-pyoverdine, NADH, and enzyme-bound NADH - to fluorescent signals in *P. aeruginosa* biofilms (**Fig. 3.2**). To validate the unmixing results from FLIM, which is an underdetermined system for four fluorophores, we implemented an orthogonal method with HIM phasor-based unmixing of two harmonics. By incorporating additional harmonics, the system is sufficiently constrained and can theoretically be solved with HIM unmixing. However, there was very little modulation of intensity in the emission acquisition window (410-500 nm). The HIM data were truncated for two reasons: (1) to directly compare predictions from the HIM and FLIM approaches and (2) to avoid introducing additional fluorescent species, such as FAD and other phenazines (12), and potentially confounding the HIM analyses (**Fig. S3.1**).

The HIM and FLIM-predicted fractional contributions did not correlate overall (**Fig. 3.4-3.5**). Pyoverdine and NADH FLIM predictions contradicted with that of HIM. FLIM predicted large contributions from pyoverdine, while HIM predicted larger contributions from NADH in the same samples. The spectral phasor positions of pyoverdine and free NADH are close, likely contributing to the discordance between HIM and FLIM predictions. In addition, the fluorescence lifetime of pyoverdine (4 ns) and enzyme-bound NADH (reported to range from 1.7-9 ns (19, 23)) could overlap depending on local conditions. In contrast, reduced pyocyanin had a distinct HIM spectral phasor position and FLIM phasor fingerprint with a long fluorescence lifetime (>10 ns) (**Fig. 3.2**). We proceeded with the FLIM unmixing method to determine if pyocyanin fractional contributions shifted throughout *P. aeruginosa* biofilms.

**Reduced pyocyanin was localized at the biofilm surface in our system.**

To recapitulate slower bacterial growth observed in infections (24, 25), colony biofilms were radially grown for five days in artificial sputum medium with soft agar. The radial center of the colony was imaged axially to capture the different depths in the oldest population of the biofilm. Reasoning that natural gradients would form with less oxygen exposure deeper in the biofilm, we initially hypothesized that *P. aeruginosa* would produce more pyocyanin in the hypoxic core of the biofilm (7, 8, 26). However, in our system, reduced pyocyanin dominated the FLIM signal at the surface of the biofilm (**Fig. 3.6, 3.7**), and the pyocyanin-dominant signal was only observed when imaging with a coverslip placed on top of the sample.

The highest density of *P. aeruginosa* growth was at the surface and was associated with the reduced pyocyanin FLIM signal (**Fig. 3.6**). After oxygen was limited by the introduction of a coverslip at the surface, it is likely dense bacterial populations utilized a pool of pyocyanin for electron recycling. Our biofilm pyocyanin model agrees with previous studies showing that population density controls phenazine biosynthesis (27, 28) and oxygen is required for pyocyanin biosynthesis (29). 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 (8, 30, 31). The reduced pyocyanin is secreted and oxidized extracellularly (8, 26). A portion of the pyocyanin can be retained in the biofilms by *P. aeruginosa*-derived extracellular DNA that binds to phenazines (32, 33), distributing pyocyanin both inside and outside of the biofilm and enabling electron cycling.

**Limitations and future directions.**

The phasor approach has several benefits, including a clear visualization of the data and reliable deconvolution of the instrument response function (34). The phasor transform effectively applies a bandpass filter to the data, compressing the complete time-domain (or wavelength-domain) signal into two numbers, the G and S components. The first harmonic contains the low frequency components of the signal, representing an approximation to the shape of the lifetime (or spectrum) trace with a single sine or cosine function. The addition of higher harmonics further refines the shape of the signal. The approach decreases the influence of high-frequency noise, giving an advantage over direct least-squares unmixing approaches.

A consideration in the application of the simultaneous spectral-lifetime unmixing method is that the spectral range of acquisition must be nearly identical for the two measurements. For accurate unmixing, the spectral ranges need to have broad enough modulation in the pure species. If the spectral range is too narrow (as in our measurements), the difference in the shape between pure species is negligible.

One of the challenges in unmixing the FLIM and HIM images using spectrum and lifetime simultaneously is that each pixel in the FLIM image must be aligned with the HIM image. With our instrument, the Zeiss LSM 880, acquisition of FLIM images was delayed by a couple minutes relative to spectral acquisition due to the need to switch to a different data acquisition software. During this time, bacteria in the sample can produce new metabolites.

The unmixing method yields the fractional contributions of fluorophores in each pixel in an image, and its accuracy is dependent on the fluorophores used as the references. While several studies on bacterial FLIM have been conducted, the lifetime of NADH when bound to bacteria enzymes has not been well-characterized. We used 2.8 ns to represent all enzyme-bound NADH in our unmixing program, but the lifetime of NADH changes in different enzymes and local environments and poses a challenge to NADH FLIM studies (19, 23, 35).

Finally, the fractional contributions are proportional to the relative concentrations of various metabolites. With appropriate instrumentation and characterization of fluorophores, it is possible measure the absolute concentration of molecules (36).

The phasor is commonly used to unmix two or three fluorescent species using linear algebra (**Fig. 3.1**). Additional fluorescent species can be unmixed if additional harmonics are incorporated into the phasor analyses, because the orthogonality of the Fourier transform guarantees that G and S components for each harmonic are independent observations.

Understanding spatial changes in can be used to assess bacterial activity, treatment susceptibility, and infection progression.

**Conclusions.**

Here, we compared two orthogonal measurements to determine the presence and relative amount of reduced pyocyanin in *P. aeruginosa* biofilms. Although our implementation was far from perfect, it put forward a framework to combine hyperspectral imaging and lifetime imaging and map out the concentrations of different fluorophores in a sample with high molecular specificity.

**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. (37) 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 or M9 succinate soft agar plates. The biofilm colonies were grown aerobically at 37˚C for 5 days.

**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 (38).

**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) (13, 14, 39). 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 (17). 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 (34). 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.

**Unmixing of fluorescence lifetime and hyperspectral data.**

To represent the fluorescence lifetime data in phasor space, the intensity trace is Fourier transformed to obtain the s and g coordinates at a harmonic n,

Similarly, the spectrum is transformed with the equations

The pure fluorophores predicted to be present in the sample are characterized by spectral and lifetime imaging, creating a basis set of k - pure components in the lifetime and spectral phasor space, at each harmonic n:

The measured signal in each pixel of the image is given by

In the absence of FRET or other non-linear effects, and if the spectral window for FLIM acquisition is the same as the spectral window for hyperspectral acquisition, the measured signal is modeled as the sum of the each of the components of the basis set, multiplied by the fractional contribution fj , (193),

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The fractional contributions best describing the signal are found by least squares global optimization:

With the constraint that the sum of all fractions f is 1,

The optimization routine used in this work is simplicial homology global optimization (41). The number of harmonics that can be used in the unmixing algorithm is determined by the resolution of the spectral or lifetime instrument. If too many harmonics are used, the data become too noisy and the model breaks down. The maximum number of harmonics that can be used is limited by the Nyquist frequency of the instrument as well as the signal modulation. If there is no modulation in the signal, the phasor coordinates become smaller than the variance introduced by the noise, and unmixing becomes impossible.

The unmixing program is open-source and available at: <https://github.com/tgallagh/HyperFluo>.

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