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**Figure 1: The fluorescence lifetime phasor.**

A simplified representation of the transformation of fluorescence exponential decays (left) into the fluorescence lifetime phasor (right). A Fourier transform is used to calculate the modulation (M) and phase shift (A) relative to the laser pulse excitation source. M and A are represented graphically for two pure fluorophores (orange dash line, blue dash-dot line) and a sample containing a mix of the two species (green solid line). The phasor G and S coordinates are the cosine and sine components of the Fourier transforms. Species closer to the origin of the phasor have long lifetimes, whereas species on the right corner of the phasor have short lifetimes. The fractional contribution of fluorescent species 1 (orange square) and species 2 (blue circle) to a sample (green diamond) can be determined if the lifetime of the pure species is known.



**Figure 2:**

(A) Two-photon emission spectra, normalized by the max peak intensity, of some of the fluorescent metabolites produced by P. aeruginosa. The Zeiss LSM-880 FLIM emission filter is shaded in gray. The DIVER FLIM emission filter is wider: 400-500 nm.

(B) Fluorescence lifetime phasor of pure fluorescent species (first harmonics). Points represent the phasor g and s coordinates from a fluorescence lifetime image, colored by the number of pixels in a bin. The large shapes represent the arithmetic mean g and s values for each fluorescent species. MDH = Malate Dehydrogenase, PVD = pyoverdine, PYO = pyocyanin.

* **Figure 1: schematic of phasor representation of lifetime (code: schematic.rmd)**
* **Figure 2 (code: spectral\_flim\_phasor.Rmd, Phasor\_fig2.py, Spectral\_fig2.py) :** 
  + **A. Two-photon emission spectra of fluorophores**
  + **B. 1st harmonics phasor of fluorophores**
* Table S1:
  + 2 photon spectral conditions – dyes
  + FLIM conditions – dyes
  + DIVER – bacterial cultures
* Figure S1: Oh Phz Spectra (not fluorescent, low fluorescence relative to pyocyanin, NADH)
* Figure S2: 2nd harmonics phasor of flurophores
* Figure S3: TCEP reduction of pyocyanin
  + A. Spectra of pyocyanin chemical reduction
  + B. 1st harmonics phasor of Pyocyanin chemical reduction
* Figure S4: Electrochemical reduction of pyocyanin – phasor and pictures
* **Figure 3:** 
  + **A: Pa vs other bacteria phasor (ASM data)**
  + **B: Single Cell Examples**
* **Figure 4: Pa phasor (1st harmonics) in liquid M9 media and ASM – aerobic vs hypoxic**
* **Figure 5:** 
  + **A. Pa biofilm intensity**
  + **B. Pa biofilm phasor**

color\_map\_1<-c(

"Enzyme-bound NADH"="#E9D8A6",

"MDH NADH"= "#001219",

"Free NADH"= "#001219",

"PVD"="#CA6702",

"PYO"="#0A9396",

"PYO (Reduced)" = "#0A9396",

"FAD" = "#EE9B00" ,

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"MDH NADH"=22,

"Free NADH"=23,

"PVD"=24,

"PYO"=25 ,

"PYO (Reduced)" = 25 ,

"FAD" = 21 ,

"CPX" = 22 )

**TEXT:**

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

The two-photon fluorescence emission spectra of *P. aeruginosa* fluorophores were characterized (NADH, enzyme-bound NADH, FAD, pyoverdine, pyocyanin, 1-hydroxy-phenazine, copoprophoryin) (**Fig. S3.1**) and agreed overall with previously published spectra (12). Different reduction methods of pyocyanin changed the fluorescence spectra and lifetime phasor results. The resulting pyocyanin population likely consisted of a mix of the radical and reduced form, but the FLIM phasor analysis suggests our FLIM setup primarily acquired the reduced form (**Fig. S3.2**).

Four of the seven species were captured by the FLIM DIVER acquisition parameters, which included an emission filter targeted towards NADH (400-500 nm): NADH, enzyme-bound NADH, and reduced pyocyanin, and apo-pyoverdine (**Fig. S3.1**). The FLIM and HIM phasor components for the pure fluorescent species were determined, and pyocyanin had a distinct FLIM and HIM phasor signature (**Fig. 3.2**). To compare the detected fluorescent species across both methods, the HIM spectral window was truncated to 410-500 nm to exclude measurements of species not captured with our FLIM acquisition settings (**Fig. S1**).