Final Report for grant DE-SC0019013

The Eliceiri lab in close collaboration with the Otegui group has built a novel multimodal imaging platform to characterize cell wall and metabolites in plants. The system has been used and will be use more in ongoing work to classify and study the silicification effects of cellulosic and lignified cell walls, cutin deposition, and accumulation of anthocyanins and other metabolites under stress conditions (Otegui, 2021). Our main emphasis in the funded period was imaging of maize and *Arabidopsis thaliana* to functionalize and deploy the multimodal system. But the platform is now ready for many other studies.

Our main emphasis has been to formulate optical fingerprints to identify metabolic markers. This question can be broken down into three essential steps of a) system design, b) fingerprinting and c) validation using a model system. a) We have successfully made a working imaging platform and improved existing methods to achieve multimodal imaging. The primary optical fingerprints based on fluorescence (lifetime, intensity, spectrum, polarization) and scattering (second harmonic generation) have been used to distinguish different metabolic and environmental factors. b) The fingerprints need to be validated based on biochemical and correlative microscopy methods to ensure the response. We used the FLIM of anthocyanins to validate the lifetime-based fingerprinting. A big-data management pipeline was developed based on ImageJ and lifetime analysis algorithms (SLIM Curve and others) to achieve the ability to quantitatively study the data. Future work will be full incorporating stimulated Raman scattering, we did preliminary implementation with loaned equipment and results were promising. As well we plan to apply the system to future studies in maize and bioenergy crops.

Acquisition pipeline

The first step was to develop the multimodal imaging system and establish a dataflow pipeline for handling large data. With the intention of a quick yield of results, we divided the development into two microscope platforms. 1) Main system: Employed the software developments and deployed a rapid FLIM using two hybrid-detectors and polarization optics to identify anthocyanin response. This system uses a Titanium Sapphire laser (MIRA900, Coherent) and timing electronics of Picoquant TimeHarp260. 2) A support system facilitated high content screening using larger imaging optics (using an Insight laser, Spectra-Physics, Timing Electronics-SPC150, Becker Hickl timing, and a hybrid detector HPM100-40). Four portable units are also used with these systems to get other modalities of imaging. 1) Portable spectral imaging using time-resolved contrast and optical fiber dispersion. 2) Portable widefield polarimetry imaging using a color camera and (low power) fast polarization control. 3) Portable SHG detection using a reflector and time-resolved collection 4) Color camera for pigment quantification. The portable units are hardware coupled to the main system and can be used on another system as well. With registration tools and fast data transfer, we can use multiple microscopes to achieve fast multidimensional collection. The support system is mainly used for large data screening using FLIM. This fast screening is carried out by either the rapid FLIM module or BH 150 module, both systems capable of imaging an entire leaf at speeds of 4fps (256x256) or 4sec (1024x1024) image. However, a high photon number is required for accurate FLIM fitting, which in-turn requires imaging up to 20-60sec to accrue 10000 photons in a pixel. With the hybrid detectors and custom algorithms, we can screen faster and mounted 6x6 sample can be screened in less than 5mins by compromising the photon strength.

Rapid FLIM: Anthocyanin contrast in as a readout of plant stress

A high content lifetime-based screening was developed using larger-collection optics and time-resolved collection. Anthocyanin presents a short lifetime (~100-200ps) that can be distinguished in a sample under the FLIM modality (Chacko et al., 2021). The lifetime curves are displayed such that each pixel is colored based on the mean lifetime measured in that pixel: hence more anthocyanin will present in red and lesser will present in blue. We screened anthocyanin fingerprints in a large collection of Arabidopsis mutants to separate the two species using FLIM and rapid FLIM modality (Figure 1). This distinction was possible on both Main and support systems, but we found screening should be separated from the multimodal system due to the compromise in single molecule sensitivity needed for anisotropy.

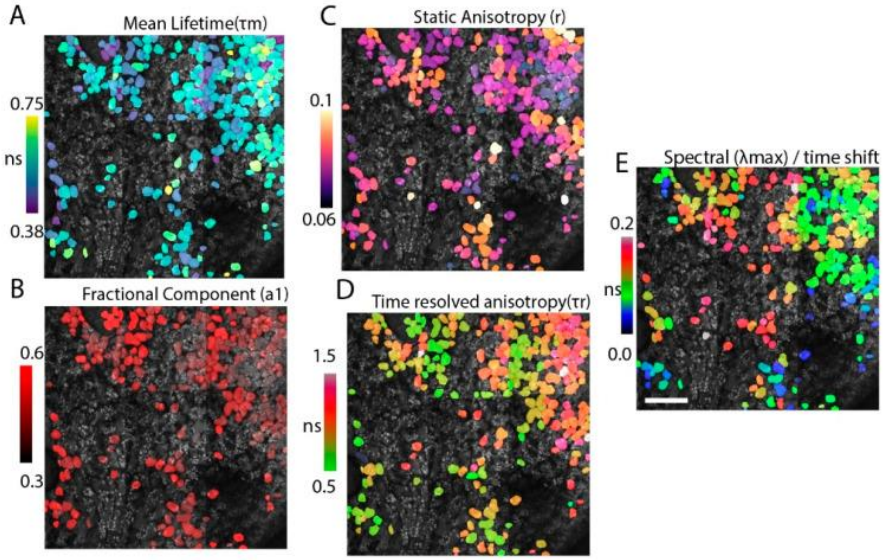

Figure 1: Multiparametric Collection of fluorescence from Arabidopsis cotyledons accumulating anthocyanins. The contrast from different fluorescence modalities is compared here. The images are segmented for cotyledon epidermal cells, and each parameter per-cell is colored and overlaid on the intensity image (in grayscale). (A) the lifetime curves are fit using a 5 × 5 kernel size and fit to multiexponential fits. The mean lifetime is colored in this scheme. (B) The fractional component of the smallest lifetime species (anthocyanins) is shown. (C) The static anisotropy parameter (r) derived as a ratio of depolarized light to total emission light is shown here. (D) The time-resolved anisotropy curve (r(t)) is fit to multiexponential fit and plotted for the mean rotational time. Note that these values are higher than anthocyanin lifetime, and only the smallest (green) values represent anthocyanins. (E) The peak-wavelength parameter is derived from the fiber-shift calculation and overlaid as previous panels. Panels C and B are ratio-metric quantities without units. All five panels show different parameters, showing different contrast between cells.

New computational tools for analysis of FLIM data

We have also developed computational tools for integrating FLIM analysis with image and data processing. We created FLIMJ, an ImageJ plugin and toolkit that allows for easy use and development of extensible image analysis workflows with FLIM data. FLIMJ allows for FLIM fitting routines with seamless integration with many other ImageJ components, and the ability to be extended to create complex FLIM analysis workflows. Building on ImageJ Ops also enables

FLIMJ's routines to be used with Jupyter notebooks and integrate naturally with science-friendly programming in, e.g., Python and Groovy (Gao et al., 2021).
We also introduced a new nonparametric empirical Bayesian framework for FLIM data analysis (NEB-FLIM), leading to both improved pixel-wise lifetime estimation and a more robust and computationally efficient integral property inference. This framework is based on a newly proposed hierarchical statistical model for FLIM data and adopts a novel nonparametric maximum likelihood estimator to estimate the prior distribution (Wang et al., 2019).

Anisotropy: time-resolved pH stress sensor for endogenous markers
Fluorescence Anisotropy or Polarization of a fluorophore gives the information of dipole orientation of the molecule. We have implemented a polarization controlled excitation and orthogonal polarization detection to collect the time-resolved anisotropy curve of a fluorophore. We tested this unit on an endogenous fluorescent coenzyme, NADH. The mitochondrial membrane-bound molecule has restricted anisotropy, and when pH stress is applied, the drop in anisotropy quantifies the total decrease in NADH bound to the inner mitochondrial membrane. This test was done on eukaryotic cell lines to visualize a fast response, but the method is translatable to similar systems which change its degree of freedom of motion.

Cost efficient Forward: Backward SHG detection using time-resolved detection.
SHG is a directed optical phenomenon, majorly favoring the forward emission of the illumination photons. The ratio of forward: backward emitted photons can give valuable information on the scale of the ordering of molecules. In order to collect both forward and backward collection, we used a reflector past the sample. The light is collected using timing electronics, which can distinguish the photons from direct incidence and photons reflected after an extra optical path.

Spectral detection using time-resolved detection of Fiber-based dispersion of emission.
Spectral Imaging is made possible in the visible range using dispersion from a 30m optical fiber and separating the dispersion information in the time-resolved delay suffered by each emission photon. This method achieves a spectral resolution of 10nm and has real-time spectral contrast, also, the lifetime contrast achieved without fiber. The spectral (λ) lifetime (τ) information can be used together as λ (max)-τ (mean) 2D histogram for fingerprinting (Sagar et al., 2019; Chacko et al., 2021).

The current system for hyperdimensional imaging: Anisotropy-Spectral-Lifetime Imaging
The Main system is currently able to measure hyper-dimensional imaging Microscopy (HDIM). The fingerprinting validation is currently done only using FLIM and SHG. Spectral and Anisotropy data is currently being merged.

Applications:
We have used this system to measure:
a) Anthocyanin distribution for correlative analysis based on spectral fingerprints and metabolism (Chacko et al., 2021).

b) Chloroplast FLIM for stress characterization

The synchronization of chloroplast retrograde signaling and transcellular communication by vascular cells is required for high light–responsive genes produced in leaf tissues. We examine FLIM as a technique to study chlorophyll characteristic at a single chloroplast level to address specific proteins of interest. We compared the response of high-level light stress in WT and GFP-expressing plant cells (Lee et al., 2023). Figure 2 shows results to establish FLIM as a tool to study chlorophyll characterization in the presence of a GFP-tagged protein to study signaling and photodamaging radiation.

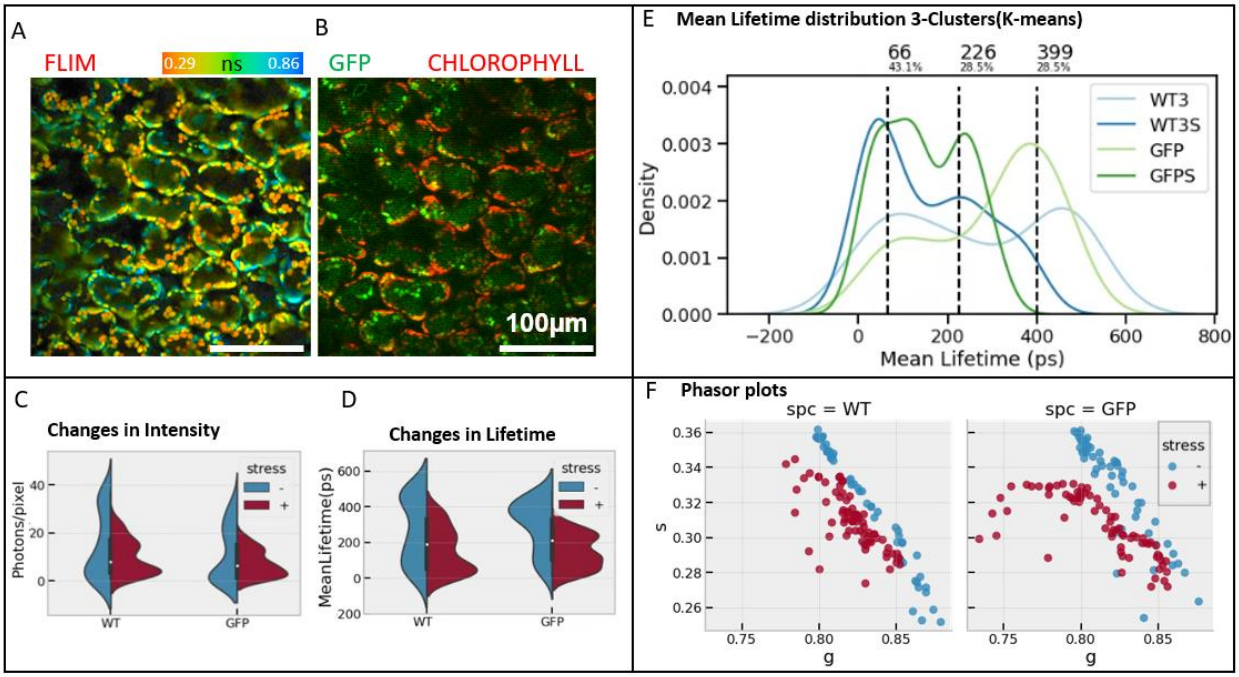

Figure 2. Imaging Chloroplasts: A) Mean Lifetime Image of chlorophyll fluorescence from leaf cells. Single chloroplasts show mean fluorescence lifetime values in the range of 0.29ns to 0.86 ns. B) GFP tags can be used to track and image specific proteins of interest without changing the chlorophyll signature. The red color represents chlorophyll intensity (as mapped on panel A), while the green color represents chloroplasts containing a specific protein of interest. C, D) Plant cell imaging comparison between stressed and control sample. The FLIM or intensity characteristic of the chlorophyll is unaffected by the presence of GFP. In panel C, the intensity is compared, and in panel D, the mean lifetime (picoseconds) is compared. E) Using a K-Means clustering technique, the FLIM model for tri-exponential fit for chlorophyll fluorescence was examined to reveal three separable clusters with mean lifetimes of 66ps, 226ps, and 399ps and fractions of 43%, 28 %, and 28%, respectively. WT3 (Wildtype fitted using 3comp), WT3S (WT3+stress), GFP (GFP-tagged leaf), and GFPS (GFP-tagged leaf) are shown in the legend (GFP-tagged leaf under stress). These findings show distinct differences in stressed leaves, which are graphically depicted in panel F as phasor plots of the data.

We analyzed Arabidopsis leaves exposed to high light to examine the change in fluorescence lifetime signature of chlorophyll inside chloroplasts. Previous studies characterized the pH, CO_2 and water levels play important roles in the FLIM signature of chloroplasts. FLIM can give molecular level insight into the dynamics of photosystem II, biotic/abiotic stress responses and address questions about microscopic chlorophyll binding to macroscopic effects in chlorotic systems. Figure 2 examines the FLIM distribution of two sets of leaves under high-level light conditions (as an abiotic stress). Panel A and B compares FLIM signature of chlorophyll to the distribution of GFP stained chloroplasts in the same leaf. We studied multiple leaves at a higher magnification, generating FLIM Z-stacks that are analyzed for predefined ROIs. Panels C and D

compares the intensity and lifetime values of these leaves from multiple images (n=1 leaf, 30+ z-slices). We developed a boundary-based plant registration scheme to image similar areas of the leaves and compared the FLIM outputs using a k-means clustering method. The decays were fit to tri-exponential decays (ncomp=3) and analysis showed the presence of three distinguishable clusters (in a 8D space with t1,t2,t3,a1%,a2%,a3%,tm,intensity). The mean values of these distributions are noted in the figure Panel E. We also demonstrate the use of fast phasor-based methods which readily identify the stress conditions at <100ms speed per FLIM dataset. The images are collected using a WI 40X,1.25NA Nikon objective lens mounted on a Bruker Prairieview microscope. We used a Spectra physics Insight laser at 890nm excitation suitable for exciting both GFP and chlorophyll. We used bandpass emission filters 630/69 for chlorophyll and 525/50 for GFP.

c) As we have now developed CRISPR-CAS9-edited lines of maize with mutations in silicon transporter, we will soon initiate the imaging of silicification of cellulosic and lignified cell walls

To summarize, we have developed a system to image plant tissues that combine: 1) novel rapid FLIM imaging system for HCS screening; 2) A cost efficient forward:backward SHG detection using FLIM; 3) A cost efficient Spectral imaging using FLIM 4) fast anisotropy imaging using hybrid detectors; 5) First HDIM system capable of generating optical fingerprints for plant samples; 6) Established software and Data analysis pipeline for processing and handling these large datasets.

Publications resulting from grant DE-SC0019013

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