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Xiaotong Yuan, Varun Mannam, Scott Howard, "3D time resolved multiphoton fluorescence lifetime imaging microscopy of nano-crystalline agricultural treatments on living plant tissue," Proc. SPIE 12384, Multiphoton Microscopy in the Biomedical Sciences XXIII, 123840A (25 April 2023); doi: 10.1117/12.2650736

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Event: SPIE BiOS, 2023, San Francisco, California, United States

3D Time Resolved Multiphoton Fluorescence Lifetime Imaging Microscopy of Nano-Crystalline Agricultural Treatments on Living Plant Tissue

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ABSTRACT

Molecular imaging tools that can image plant metabolism and effects of external agricultural treatments in the micro-environment of plant tissues are significant for further understanding plant biology and optimizing the formulation of new agricultural products. Mass spectrometry, a common tool used by plant biologists, is unable to resolve nano-crystalline active ingredients (AIs) on the leaf surface nor achieve 3D molecular imaging of living plants. To address that, multiphoton microscopy (MPM) and fluorescence lifetime imaging microscopy (FLIM) are combined to achieve sub-cellular, depth-resolved fluorescence lifetime of both AIs and intrinsic proteins/pigments (e.g., chlorophyll and/or cytosolic NADH) after the herbicide treatment application. Here we present a method using a custom-designed, high-speed MPM-FLIM system, "Instant FLIM", to achieve real-time, unlabeled 3D functional molecular imaging of intrinsic proteins and pigments in optically thick and highly scattering plant samples with the application of external treatments. To validate the capability of MPM-FLIM to measure intrinsic proteins and pigments within plant tissues, we present the results of unlabeled bluegrass blades samples. To demonstrate simultaneous imaging of 3D molecular plant tissue and the agricultural AI nano-crystals deposition and formation, we evaluate the performance of the MPM-FLIM by applying commercial herbicide product to gamagrass blade sample. Additionally, to measure the herbicide-induced cellular-level functional responses within living plant tissues, 3D time-resolved molecular MPM-FLIM imaging of hemp dogbane leaf with herbicide is performed. Results demonstrate MPM-FLIM is capable of 3D simultaneous functional imaging of label-free living plant tissues and the quantitative measurements of the location and formation of AI nano-crystals within the plant tissues.

Keywords: Fluorescence lifetime imaging microscopy (FLIM), multiphoton microscopy (MPM), highly scattering plant samples, agricultural active ingredients, 3D time and depth MPM-FLIM imaging

1. INTRODUCTION

Molecular imaging tools that can be used to image plant metabolic activities and the cellular effects of the micro-environments brought by the external agricultural treatment are essential for understanding basic plant biology and optimizing agricultural products. Due to the cellulose structure of the cell walls and the organelle distribution of a plant leaf, plant tissues are usually highly scattering, which makes the application of depth-resolved confocal and light sheet microscopes difficult.[1,2] In agriculture, mass spectrometry (MS) is commonly used by plant biologists for molecular imaging to measure the spatial distributions of molecules in plant tissues due to its great sensitivity and selectivity.[3,4] However, MS imaging technique is typically limited by low resolution and shallow penetration depth, as well as the incapability of real-time measurement and analysis, which prohibits achieving sub-cellular resolution and resolved agricultural treatment active ingredients that typically are nano-crystalline structures. These limitations can be overcome by fluorescence microscopes with enhanced imaging resolution and 3D imaging techniques. In order to better understand the spatial organization and distribution of the plant tissues and explore how the small molecules (e.g., agricultural active ingredients) in the external chemical treatment affect the mechanisms and the cellular environments, as well as to improve and optimize the function and formulation of novel agricultural active ingredients (AIs), it is important to visualize and map their distributions or effects *in vivo* within the plant tissues. Therefore, non-invasive, fluorescence microscopy

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Multiphoton Microscopy in the Biomedical Sciences XXIII, edited by Ammasi Periasamy, Peter T. C. So, Karsten König, Proc. of SPIE Vol. 12384, 123840A
 · 1605-7422 · doi: 10.1117/12.2650736

imaging techniques can be used in plant imaging to qualitatively and quantitatively explore dynamic events both in the laboratory and in the field. Multiphoton microscopy (MPM) has been widely used as an invaluable tool in biological and medical imaging applications because of its capability of visualizing cellular and sub-cellular dynamic events in living cells and tissues.[5,6] In this work, we will be focusing on a specific case of MPM: two-photon microscopy, for its advantage of deeper penetration and less photodamage due to the reduced scattering effects from employing near-infrared (NIR) excitation.[7] Fluorescence lifetime imaging microscopy (FLIM) is also a powerful tool in biomedical research and studies.[8] Due to its high sensitivity to the molecular environments, FLIM has been commonly used in imaging living animals to estimate the metabolic responses and explore the fundamental molecular interactions within cells and tissues.[9] FLIM is capable of not only identifying the existence and location of fluorophores, but also the state of the emitters (e.g., protein bound or unbound stats, or quantitative measures of the micro-environment). Additional information about the FLIM system and FLIM measurements are provided in these references.[10–12] FLIM has also been performed in plants to study the sub-cellular localization of specific proteins and pigments like chlorophyll/NADH and anthocyanins, as well as to detect the physiological status during photosynthesis process.[13,14] Although previous work has shown that FLIM combined with MPM can be used to monitor changes in the fluorescence lifetime of chlorophyll after applying herbicide treatment,[15] the slow frame rates of typical MPM-FLIM prohibits the 3D acquiring complete 3D stacks over a long term which would be needed to acquire a depth and time resolved study.

In this work, we utilize a custom-designed high-speed frequency-domain MPM-FLIM system, "Instant FLIM",[16] to achieve real-time, label-free 3D functional molecular imaging of intrinsic proteins and pigments in optically thick plant samples while simultaneously imaging the formation of agricultural treatment nano-crystals on the surface and at depth within a leaf during treatment.

2. RESULTS AND DISCUSSION

As a proof of concept, preliminary results are shown first to validate the performance of the system and build up some fundamental knowledge of the appearances and optical properties of plant samples. First, the intrinsic FLIM results of bluegrass blades are shown in both intensity and composite in Fig. 1. The top row shows the 2D optical plane of a whole 3D stack of the grass sample, containing many chloroplasts in the palisade layer with the lifetime from around 0.5-0.8 ns. The bottom row shows the results at a different plane showing the upper epidermis structures, and the cell walls are indicated by longer lifetimes (1-1.4 ns). This figure shows how the bluegrass plant looks like under instant FLIM system and indicates the system's ability to separate plant structures by their respective responses.

In this work, we focus on the commercial herbicide products. Each treatment can be formulated in two types: emulsifiable concentrate (EC) and suspension concentrate (SC). EC has AIs dissolved in a solvent and emulsifiers while SC has insoluble solid AIs dispersed in a medium (e.g., water/air).[17] One of the major differences between EC and SC solution is that SC solution contains tiny AI particles and EC solution does not. To demonstrate simultaneous imaging of 3D plant tissue and agricultural herbicide AI nano-crystal deposition and formation after application, we applied commercial SC herbicide to gamagrass blade sample and image the leaf to compare the results before and after to investigate the work mechanism of the herbicide and its molecular effects on plant tissues. Fig. 2 presents the results as maximum z-projections of the 3D intensity and composite stacks, where the intensity and lifetime are respectively mapped to pixels' brightness and hue. The results of gamagrass prior to the application of herbicide is shown in Fig. 2(a). The epidermis (including stomata) structures are clearly present and exhibit sub-cellular lifetime values ranging fom 0.9-1.2 ns. As discussed previously, shorter lifetime (≈ 0.9 ns) features are typically associated with chlorophyll, and slightly longer lifetime (≈ 1.2 ns) structures are usually associated with cytosolic proteins (e.g., NADH). Fig. 2(b) shows the SC herbicide residue deposited on a glass slide. The AIs are contained in the form of particles with random and non-uniform shapes and display near zero lifetimes, as expected. After applying approximately 10 microliters of the herbicide treatment via micro-pipette to the gamagrass blade sample, the results of the plant tissues after 40 minutes are shown in Fig. 2(c). Note that this commercial herbicide is selective and only expected to have herbicidal function on dicot plants, while the sample used here is a monocot plant, sub-cellular lifetime does not appear to be affected. However, simultaneous imaging of AI location and cellular structure does identify aggregation of SC material around and in stomatas, penetrating these voids in plant tissue to a depth of 96 μm.

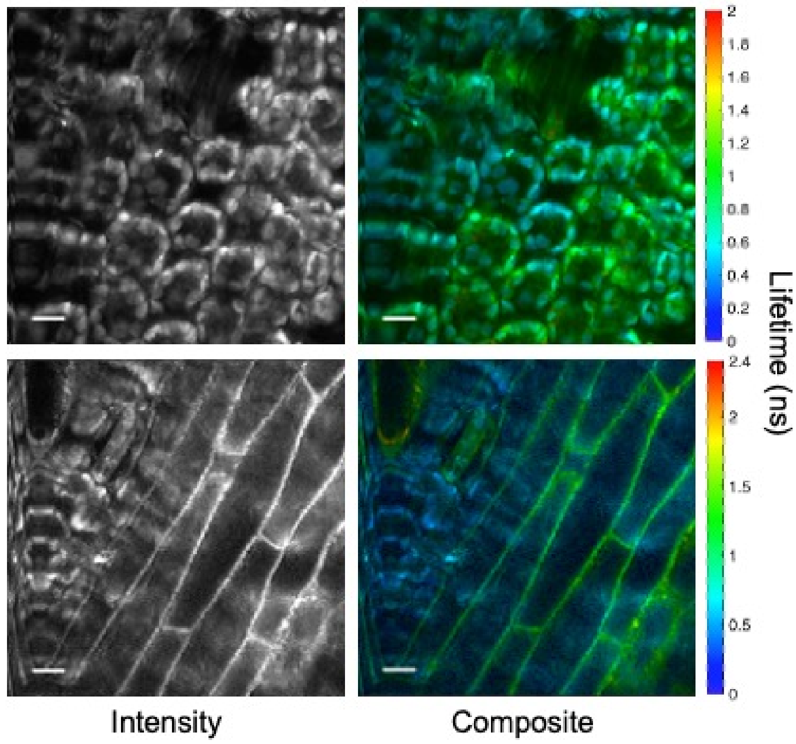

Figure 1. Intrinsic instant FLIM images of the bluegrass blades. Intensity images on the left column and composite images on the right column, respectively. Excitation wavelength: 800 nm; sample power: 19 mW (top row), 16.4 mW (bottom row). Top row shows a 2D optical section of a 3D stack of the grass including chloroplasts; bottom row shows 2D results of the flipped side, cell walls are present and indicating longer lifetimes compared to chloroplasts. Scale bar, 5 μm.

To see the molecular functional changes within the plant tissues, we applied the herbicide in EC formulation to dicot plant, hemp dogbane leaf. This experiment was designed as a control experiment containing both untreated and treated field of views (FOVs) to keep other factors that might trigger the lifetime changes (e.g., natural plant aging and dying) constant, in order to illustrate the herbicide-induced functional changes in plants more precisely. The results are shown in Fig. 3. The selected untreated and treated FOVs are about 76 um deep with respect to the top surface of the plant structure. Two 1ul drops of the herbicide EC solution with 1:100 dilution was added to the treated leaf only, no external treatment was applied to the untreated leaf. Both untreated and treated regions were imaged every one hour for three times after the application of the treatment. An increasing trend of fluorescence lifetime values of the plant cells is present in the treated results. The circled region of interests (ROIs) in the 3-hours-after treated image show longer lifetime structures that do no exist in the untreated ROIs, which indicates the cellular-level effects inside the plants due to the external herbicide treatment. The increase of the lifetime could potentially indicate the catabolism of chlorophyll inside the plant tissues or other mechanism leading to the decay and senescence of plant cells and tissues. This experiment validated the performance of the MPM-FLIM system and its capability to monitor the micro-environment changes within plant tissues.

3. CONCLUSION

In summary, this work is to explore highly scattering plant tissues and their cellular-level responses to the external agricultural treatments using intrinsic, unlabeled MPM-FLIM system to instantaneously perform label-free, 3D *in vivo* time resolved molecular imaging for the first time in plant imaging field. The preliminary MPM-FLIM results of living plants have been presented and demonstrated, which validated the capability of the instant FLIM system to measure intrinsic protein and pigment inside the plant samples. The ongoing experimental studies of the molecular effects brought by the external agricultural treatments, as well as the formulation and penetration capability of agricultural AI nano-crystals to up to μm in depth during treatment demonstrated that MPM-FLIM enables 3D, real-time, simultaneous depth and time resolved functional imaging of living plant tissues and the quantitative evaluations of the penetration profiles of different AIs, which is essential for optimizing the function of the agricultural products.

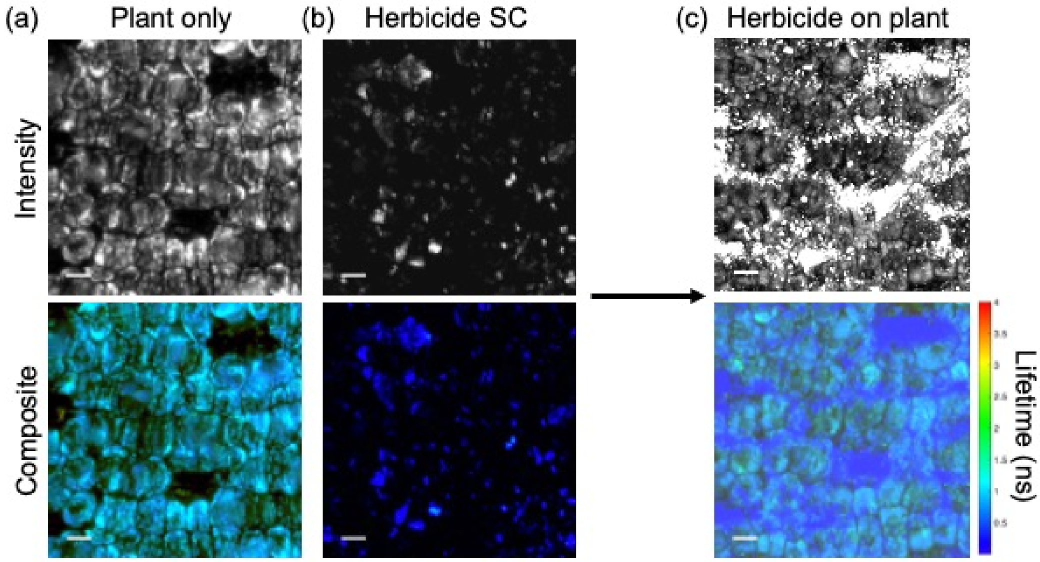

Figure 2. Intensity and composite results showing (a) maximum z-projections of the 3D stacks of the gamagrass blade sample; (b) the crystals of the commercial herbicide in SC formulation and (c) maximum z-projections of the 3D stack showing the simultaneous imaging of AI and plant tissues. Excitation wavelength: 880 nm; sample power: 23 mW. Scale bar, 5 μm.

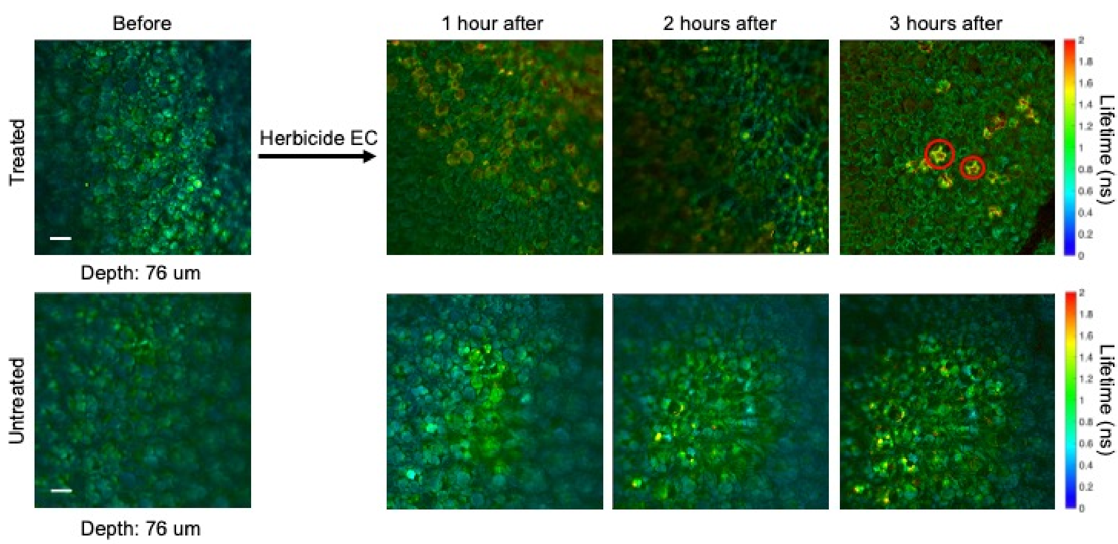

Figure 3. The instant FLIM measurements of the effect of herbicide EC on hemp dogbane leaf over time. The lifetime of the treated region has an increasing trend compared to the untreated region. Excitation wavelength: 800 nm; sample power: 43.8 mW (first column); 37 mW (second and third column); 13.7 mW (fourth column, treated result) and 19 mW (fourth column, untreated result). Scale bar, 5 μm.

ACKNOWLEDGMENTS

This work was supported by Center for Bioanalytic Metrology (CBM) National Science Foundation (NSF) under grant 1916601, and for valuable advice provided by the CBM Industry Advisory Board.

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