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# Adding Polarimetric Multiphoton Imaging to the W. M. Keck 3-Dimensional Fusion Microscope



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## Abstract

The W. M. Keck 3-Dimensional Fusion Microscope's (3DFM) multimodal imaging platform provides researchers the unique ability to capture and process images from 5 co-registered optical imaging modalities without moving the specimen. Here, we detail the addition and first experimental results of a novel 6th modality, multiphoton fluorescence anisotropy, which allows for dynamic studies of macromolecule binding interactions. We have begun the application of Fluorescence Lifetime Intensity Microscopy (FLIM) techniques using a Time Correlated Single Photon Counting (TCSPC) module to measure the time-dependent decay of polarized fluorescence. Calculation of the time-dependent fluorescence anisotropy decay provides information related to the rotational dynamics of the excited fluorophores. Data corresponding to the calibration of the excitation and emission polarization planes, sample images of fluorescence anisotropy measured from a randomly labeled sample, and initial results of fluorescence anisotropy decay experiments are presented.

## Fluorescence Anisotropy is a Measure of Molecular Mobility

Fluorescence anisotropy<sup>1</sup> is a ratiometric measurement technique that takes advantage of the intrinsic absorption and emission dipoles present in all fluorescent molecules. Fluorescent molecules absorb the excitation light and emit fluorescence more efficiently along the polarization plane parallel to their respective dipoles than other polarization planes.<sup>1</sup> Fluorescence anisotropy,  $A$ , is defined as:

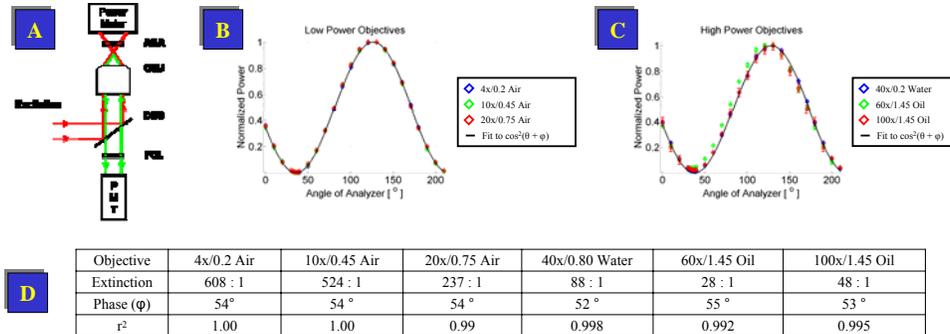
$$A = \frac{I_{\parallel} - I_{\perp}}{I_{\parallel} + 2I_{\perp}}$$

where  $I_{\parallel}$  and  $I_{\perp}$  are the intensities measured parallel and perpendicular to the excitation polarization plane. Molecules rotationally stationary with respect to their fluorescence lifetime will preferentially emit fluorescence polarized parallel to their emission dipole ( $A = 1$ ) while molecules in rotational motion occurring on shorter timescales than their fluorescence lifetime will emit randomly polarized fluorescence ( $A = 0$ ). Traditionally, fluorescence anisotropy measurements have been used to determine the rotational mobility of molecules resulting from a chemical reaction. Recently, fluorescence anisotropy measurements have been made utilizing confocal microscopes to image steady state distributions of fluorescent macromolecules in heterogeneous environments *in vitro*.<sup>2,3</sup> However, several engineering challenges remain before the full potential of fluorescence anisotropy measurements within a heterogeneous environment can be realized.

## Fluorescence Lifetime Provides Additional Anisotropic Information

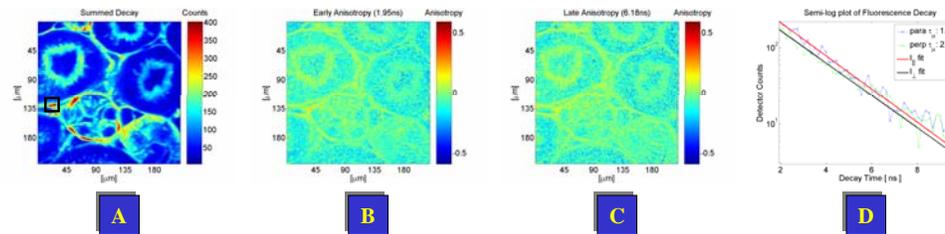
Current fluorescence anisotropy imaging utilizes confocal fluorescence polarization microscopes (CFPM) to image 3-dimensionally steady-state anisotropic distributions of fluorophores. CFPMs differ from traditional confocal microscopes by employing polarized excitation light to achieve single photon excitation; simultaneous discrimination between orthogonal emission polarization states is provided by a polarizing beamsplitter (PBS) and a dual confocal detector apparatus.<sup>2,3</sup> This setup measures average anisotropic distributions at fixed points in time. However, fluorophores have a characteristic decay lifetime dependent on the local environment and the ability of the excited fluorophore to transfer energy to that environment. Fluorescence decay lifetimes are on the order of nanoseconds. Fluorescence Lifetime Imaging Microscopy (FLIM) employs advanced time-discrimination electronics to measure the decay time of individual fluorophores present within the sample.<sup>4</sup> Successful attempts to integrate FLIM within a CFPM to study fluorescence anisotropic decay have been made recently.<sup>4</sup> Here we begin an investigation to incorporate multiphoton excitation<sup>5</sup> within a polarization sensitive FLIM system on the W. M. Keck 3-Dimensional Fusion Microscope.

## 3DFM Polarization Calibration: A Test of Malus' Law



A.) Optical layout: Femtosecond pulses of polarized Near Infrared (NIR) excitation light (red lines) reflect from a dichroic beamsplitter (DBS) and are focused to a point by an objective lens (OBJ). An analyzer (ANA) is placed after the focal point of the objective and an optical power meter measures the throughput as the angle of the ANA is rotated relative to the polarization plane of the excitation. B, D.) Normalized power measurements vs. angle of ANA for three low power, air objectives with Malus' Law,  $\cos^2(\theta + \phi)$ , fit. C, D.) Normalized power measurements vs. angle of ANA for three high power, immersion objectives with Malus' Law,  $\cos^2(\theta + \phi)$ , fit. To reduce complicating refractive effects, immersion media was not used to couple the objectives to the ANA. All of the tested objectives display the expected Malus' Law behavior. The extinction values for the high power objectives are found to be approximately one order of magnitude less than those found for the low power objectives. The decrease in extinction values indicate depolarization effects, possibly due to inherent birefringence and/or the difference in Fresnel reflection coefficients between the orthogonal polarization states resulting from the steep lens curvatures necessary to provide high magnification.

## Preliminary Multiphoton Anisotropic Decay Results



A.) Parallel emission image of mouse kidney section stained with Alex Fluor® 488 WGA. The fluorophore was assumed to be randomly bound. The sample was excited at 930nm via multiphoton excitation and imaged with a 60x/1.45 Oil immersion objective. FLIM imaging was performed with the Becker & Hickl SPC-830 kindly provided by Boston Electronics. B.) Fluorescence anisotropy 1.95 ns after excitation. Anisotropic regions closely aligned with the bound fluorophores are clearly visible. Regions of unexpected negative anisotropy due to system noise fluctuations are found in regions where no fluorophores are present. C.) Fluorescence anisotropy 6.18 ns after excitation shows little change from the earlier time bin. This result indicates that the fluorophores did not execute rotational motion on the timescale of the fluorescence decay. This conclusion is supported by examination of individual pixel timecourses. D.) The blue data points correspond to the detector counts measured parallel to the excitation within the region identified by the box in A.), the green data points correspond to perpendicular counts, the red line is the exponential decay fit to the parallel data, and the black line is the exponential decay fit to the perpendicular data. The semilog plot illustrates that the anisotropy remains nearly constant on the timescale investigated as implied by B.) and C.).

## Results

- Creation of a simple calibration routine to enable robust knowledge of the excitation and emission planes of polarization, thereby providing the ability to perform longitudinal experiments.
- Development of MATLAB analysis code to facilitate visualization and quantitative manipulation of anisotropy data.
- Investigation of a rotationally stationary fluorophore in a heterogeneous environment.

## Conclusions/Future Work

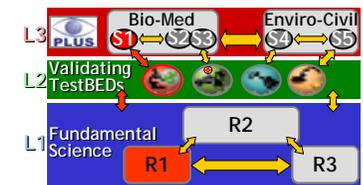
- With the demonstration of time independent anisotropy, a successful application of the technique within a time dependent sample would further prove its utility to biomedical imaging.
- Integration of advanced dual-exponential decay fitting algorithms will provide improved fits to the decay data.
- Further study of the high power, immersion objectives will determine accurate extinction values and further establish confidence in their polarization maintaining abilities and applicability within precision anisotropy experiments.

## Opportunities for Technology Transfer

Successful demonstration of time resolved fluorescence anisotropy measurements acquired with single photon excitation FLIM has been shown previously.<sup>4</sup> Integration of multiphoton excitation to time resolved anisotropy measurements would make the technique more attractive within biological research due to multiphoton excitation's lower photobleaching and photodamage effects *in vivo* and *in vitro* as compared to single photon excitation.

## References

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