Focal Points

Application Note FP-151

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Measuring Molecular Mobility with Macro Fluorescence Imaging

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Abstract

We demonstrate that molecular mobility can be measured by macro imaging with steady state fluorescence anisotropy. A BioSpectrum Imaging System (Fig. 1) with overhead UV light, emission filter and a cooled CCD camera with additional linear polarizers can perform fluorescence anisotropy macro imaging. The BioSpectrum system produces results comparable to other anisotropy instruments on top of the capability to perform macro imaging which had been frequently applied to quantify molecular expression in vivo. We applied this technique to examine GFP in live plants and observed systematic differences in different areas non-invasively.

Fig. 1 - BioSpectrum Imaging System

Background

The cylinders in Fig. 2 represent fluorescent molecules with red arrows indicating their optical orientation. The excitation of the fluorescent molecules (painted in light blue instead of dark) correlates with the alignment between the red and black dashed arrows which represent the polarization of the light. The purple shade and the green arrow each illustrate the excitation illumination and the emission of fluorescence. The polarization of the emission relates to the orientation of the molecules as well. Typically there is a nano-second time gap between the two events and is statistically described as fluorescence lifetime. Rotational displacement occurs during the time gap which leads to the difference between excitation and emission polarization which can be measured by using linear polarizers. Measured value can be denoted as anisotropy (*r*) or polarization (*p*).

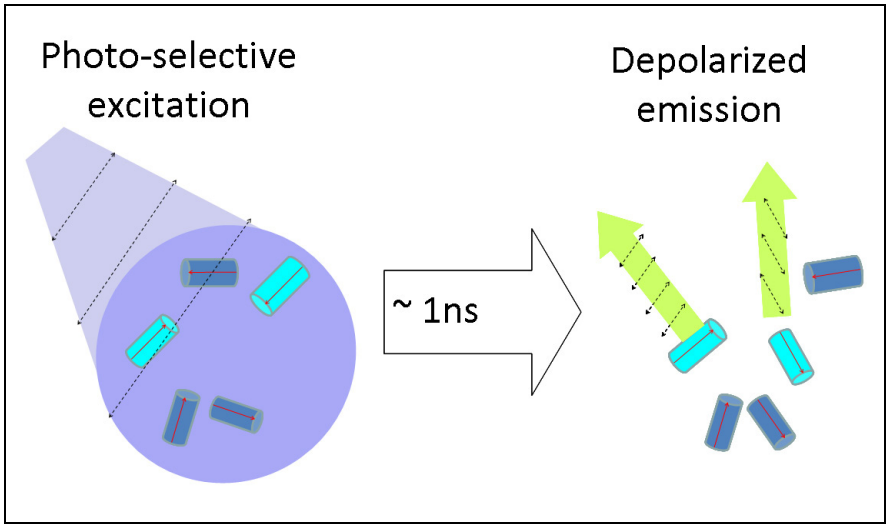


Fig. 2

Methods

A BioSpectrum Imaging System was modified as shown in the Fig. 3 illustration. The UV lamp provided 365nm illumination for excitation UV passing through the linear polarizers masked over the lamp surface. The emission filter used was a 520nm bandpass filter with a 40nm band width. The spectral configuration targets the wavelength of GFP and FITC.

The system was fully controlled by the systems' VisionWorks®LS Software. The motorized filter wheel provides the options to capture without polarizers, with horizontal or vertical linear polarizer. Wide-spectrum (white light) images were captured with the UV lamp off and flooding the imaging cabinet with room light. A low F number (1.2) 50mm lens was used to provide both the macro imaging field of view (16x16cm) and recover as much light as possible when the polarizers unavoidably reduced the signal strength.

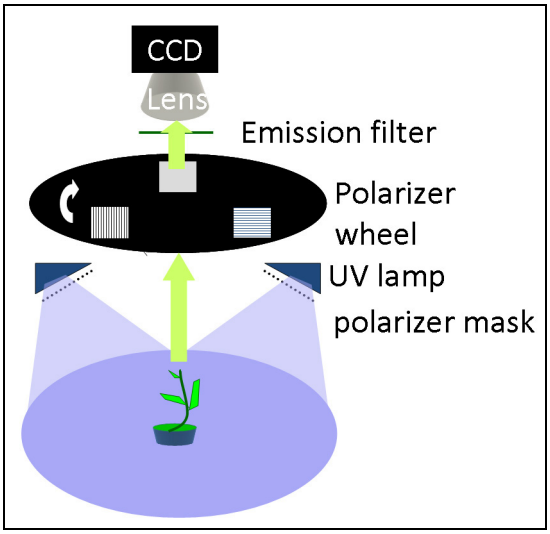


Fig. 3 - System Diagram

Depending on the angle of the incident light, the transmission of linear polarizers varies. To compensate for the uneven detection efficiency when imaging a large sample, a correction factor (*G*) is created by rationing the detected intensities of a non-polarized emission sample using horizontal and perpendicular polarizers at every point of the image.

$$G(x,y) = I_h(x,y) / I_p(x,y)$$

The corrected anisotropy is calculated according to the following equation:

$$r = \frac{I_h - GI_p}{I_h + 2GI_p}$$

Results

A controlled, mobility assay was first examined. A series of glycerol/water mixture from 0% to 60% glycerol by weight was prepared as the solvent of known viscosity values. A drop of concentrated fluorescein and 1M NaOH was added to the solvent to create about 20 μ M of high pH fluorescein solutions which has a well-known lifetime value of 4.05ns. The prepare solutions were filled in a standard 92-well plate and imaged shown in Fig. 4.

Assuming the fluorescein molecules are spherical, the linear fit to the correlation time to viscosity plot gives an estimation on the molecular size. The measured value of fluorescein's radius was 0.49nm.

The same method was applied to measure macroscopic viscosity changes in live Nicotinana benthamiana expressing GFP delivered using modified tobacco mosaic virus.

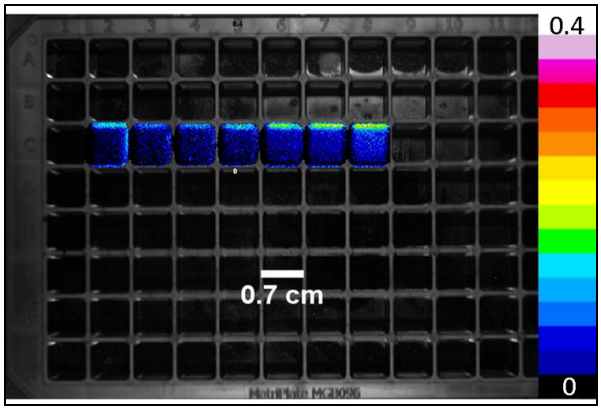


Fig. 4 - White light image (black and white) with anisotropy image (color) overlaid, threshold by fluorescence intensity

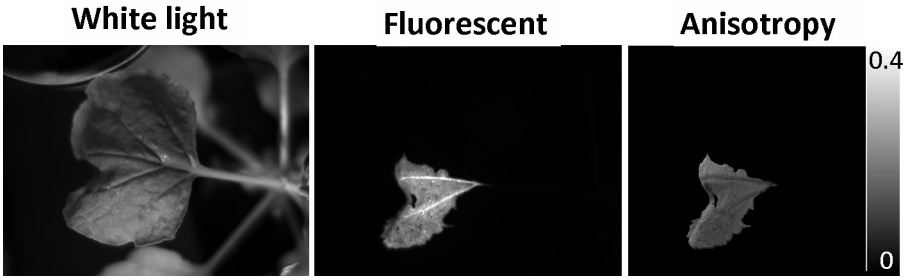
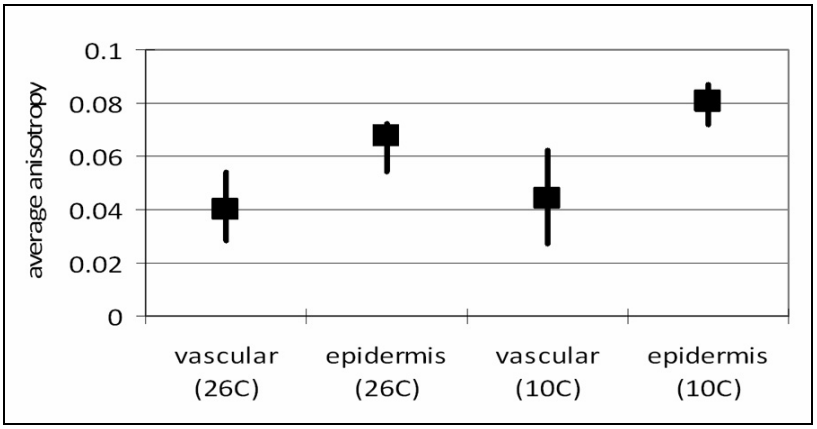


Fig. 5

The environmental temperature was changed to fluctuate the internal viscosity of the plant. The anisotropy images (Fig. 5) suggests that the viscosity increases as temperature declines, however, the effect is more pronounced in the mesophyll than in the vascular system.



Conclusion

This anisotropy nature of the fluorescence had been exploited to investigate

- **Changes in environmental viscosity**
- **Molecular binding**
- **Changes in fluorescence lifetime**

on fluorometers and microscopic platforms. Our experiments showed that FAMI have the potential to perform high quality anisotropy measurements with the additional benefits of large field-of-view which compliments both high through-put, in-vitro assay and in-vivo, non-invasive studies.