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# A new approach to polarized fluorescence using phase and modulation fluorometry

## I. Theory with reference to hindered and anisotropic rotations

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**Abstract.** The usual method to obtain fluorescence anisotropy decay parameters from frequency domain measurements is to determine the phase angle difference between the perpendicular and parallel components of the polarized emission and the ratio of their modulated amplitudes ("differential method"). Here, an alternative method is introduced analogous to a time-resolved study of the fluorescence anisotropy ("Sine-Cosine Transform method"), in which the intensity difference is studied in the frequency domain. It is shown that the differential method is better at low steady-state fluorescence anisotropy, while the Sine-Cosine Transform method is preferable at high anisotropy values and even more preferable, if the probe rotation is strongly anisotropic or hindered, giving rise to multiple rotational diffusion phenomena.

**Key words:** Fluorescence anisotropy, phase fluorometry

## Introduction

Phase fluorometry studies of polarized fluorescence can be used to determine rotational characteristics of molecules in isotropic solvents and lipid membranes (Weber 1978). Mantulin and Weber (1977) have shown that the in-plane and out-of-plane rotational rates for perylene in propylene glycol are different. Lakowicz and Prendergast (1978) have provided evidence for a hindrance of the diphenylhexatriene rotation in lipid bilayers. In both studies differential phase fluorometry was used at two modulation frequencies, 10 and 30 MHz.

Recently, the potential of the technique has increased enormously, since continuously variable frequency phase and modulation fluorometers have become available (Gratton and Limkeman 1983; Jameson et al. 1984). Gratton and coworkers have shown that this advanced instrumentation permits measurements with high precision of rotational correlation times in the nanosecond time range and that it is feasible to distinguish between sophisticated rotator models with one or more exponential terms (Lakowicz et al. 1985). They employed an extension of a method introduced by Weber (1977), in which they measured not only the phase-angle difference between the perpendicular and parallel polarized components of the modulated emission, but also the corresponding modulation ratio (Lakowicz et al. 1985). In the present paper we will call this approach "the differential method". Here, we introduce an alternative method in which the phase and modulation of the parallel and perpendicular components of the modulated emission are measured. From these phases and modulations the sine and cosine transform of the intensity-difference is constructed. This analysis is analogous to studying time-resolved fluorescence anisotropy in the time domain using excitation with short flashes, because there the intensity difference between the parallel and perpendicular components is studied as well (Wahl 1969). We will call our approach the "Sine-Cosine Transform method".

It is the purpose of this paper to outline the theory of polarized phase modulation fluorescence, to introduce our "Sine-Cosine Transform method" for studying rotation and orientation and to compare it with the "differential method".

## Principles of polarized phase-modulation fluorescence

The exciting light is modulated sinusoidally at high frequencies, typically in the megahertz range. The

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