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Photobleaching Kinetics of Fluorescein in Quantitative Fluorescence Microscopy

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INTRODUCTION

Photobleaching is a dynamic process in which fluorochrome molecules undergo photo-induced chemical destruction upon exposure to excitation light and thus lose their ability to fluoresce. The photobleaching phenomenon has been the basis of many fluorescence measurement techniques developed and successfully applied since the 1970's.

The mechanisms of photobleaching in biological objects are not yet well-understood. In microscopy, fluorochrome molecules are chemically bound to targets of interest (such as DNA, RNA, protein, or other cellular components) in which the chemical micro-environment is very complex, often differs from one specimen to another, and is very difficult to control. Although a single-exponential (or first-order) process is often used as a basis for the photobleaching techniques in microscopy, the experimental data from many studies deviate from a pure single-exponential function $I = Be^{-kt}$ (Benson et al. 1985, Koppel et al. 1989, and Rigaut et al. 1991).

The study reported here is aimed at a systematic analysis of photobleaching kinetics in microscopy. It focuses on the photobleaching process of fluorescein alone.

PHOTOCHEMICAL AND PHOTOPHYSICAL BACKGROUND

Lindqvist and co-worker (Lindqvist 1960 and Kasche et al. 1964) demonstrated that the triplet excited state fluorescein molecules became depopulated via two major pathways: the reaction between a triplet and another triplet or a ground state dye molecule (Dye-Dye mechanism); and the reaction between a triplet dye molecule and an oxygen molecule (Dye-Oxygen mechanism). These reactions, in turn, were shown to produce the transient semi-reduced and semi-oxidized forms of the dye triplet, and then became non-fluorescent photo-product or were reverted back to the ground state molecules.

To study the photobleaching behavior of fluorescein, all of the photochemical reactions from the studies of Lindqvist and Kasche are incorporated into a model described by the following six coupled differential equations:

$$\frac{d}{dt}[N_s(t) = [k_d N_s*(t) + k_1 N_{T*}(t) + k_2 N_{T*}^2(t) + k_3 N_{T*}(t) N_s(t) + k_6 N_{T*}(t) N_x(t) + k_7 N_{T*}(t) N_R(t) + k_8 N_{T*}(t) N_{o2}(t)] - [k_a N_s(t) + k_5 N_{T*}(t) N_s(t)]$$

$$\frac{d}{dt}[N_{s*}(t) = k_d N_s(t) - [k_d N_{s*}(t) + k_{isc} N_{s*}(t)]$$

$$\frac{d}{dt}[N_{T*}(t)] = k_{isc} N_{s*}(t) - [k_1 N_{T*}(t) + k_2 N_{T*}^2(t) + k_3 N_{T*}(t) N_s(t) + 2 k_4 N_{T*}^2(t) + k_5 N_{T*} N_s(t) + k_6 N_{T*}(t) N_x(t) + k_7 N_{T*}(t) N_R(t) + k_8 N_{T*}(t) N_{o2}(t) + k_9 N_{T*}(t) N_{o2}(t)] \quad \text{Eq. 1}$$

$$\frac{d}{dt}[N_x(t)] = k_4 N_{T*}^2(t) + k_5 N_{T*}(t) N_s(t) + k_9 N_{T*}(t) N_{o2}(t)$$

$$\frac{d}{dt}[N_R(t)] = k_4 N_{T*}^2(t) + k_5 N_{T*}(t) N_s(t)$$

$$\frac{d}{dt}[N_{o2}(t) = -k_9 N_{T*}(t) N_{o2}(t)$$

where S, S*, T*, X and R refer to the singlet ground, singlet excited, triplet excited, semi-oxidized and semi-reduced state of fluorescein, respectively, and N refers to the molecular population. In this system, k_1 to k_9 are those derived by Lindqvist and Kasche (Lindqvist 1960, Kasche 1964) for the pH range in the current study. The non-linearity introduced by the bimolecular processes makes it extremely difficult to find an analytical solution for the system in Eq. 1. Instead, an efficient iterative numerical method has to be used to study the photobleaching kinetics of fluorescein.

MATERIALS AND METHODS

Study of Kinetics by Mathematical Simulation

The photobleaching kinetics was monitored by numerically solving the system of ODE's using the highly-efficient software package "Livermore Solver for Ordinary Differential Equations" (LSODE) (Hindmarsh 1983) and was customized for the current study. A strategy of a dynamically adjusted step size took into consideration the drastically different time scales originating from the nanosecond singlet excited state lifetime and micro- to milli-second triplet state lifetime (a so-called stiff problem).

Simulation of the photobleaching process of free fluorescein in solution and bound fluorescein in microscopy was accomplished by setting the appropriate initial conditions and recording the population change of each energy state over time. For the simulation, only the excitation intensity and fluorochrome concentration were varied to closely resemble the experimental conditions, and all other rate constants are intrinsic to fluorescein for the pH used in the present study, and were quoted directly from Lindqvist.

Free Fluorescein in Solution

A fluorescein sodium solution of 0.01 μM was then placed in three cuvettes: (1) control sample, which was not bleached, (2) air-saturated sample, and (3) deoxygenated sample, from which oxygen was purged by flushing argon gas for 15 minutes. A Leitz DM epi-fluorescence microscope with a 100W mercury arc lamp and a 450-490 nm excitation filter block was used as a bleaching light source.

Bound Fluorescein in Microscopy

Fluorescein surface-labeled microspheres were centrifuged onto standard microscope slides. The slides were air-dried in the dark and embedded in PBS in the absence of anti-fading agents.

Ficol-isolated human lymphocytes on glass slides were *in situ* hybridized and fluorescein molecules were directly (without antigen-antibody complex or spacer molecules) attached to probes specific for the centromeric region of chromosome 1. The preparation was embedded under a cover slip in PBS.

RESULTS AND DISCUSSIONS

The experimentally derived bleaching curves and simulated kinetic curves demonstrated that the simulation very closely resembled the bleaching behavior observed in the actual experiment, both for free and bound fluorescein. For simulated air-saturated fluorescein solution, the ground state depletion was completely described by a single-exponential function with the least-square residual very close to zero. The build-up of the bleached dye population was largely due to the D-O mechanism.

For both the experimentally-derived and the simulated conventional fluorescence microscopy case, the kinetic curve of the ground state depletion deviated significantly from a single-exponential function. The build-up of the bleached dye population was predominantly due to the D-D mechanism.

Since the D-O mechanism consists of only one pseudo-unimolecular reaction leading to the bleached dye molecules, the bleaching behavior will be a single-exponential process in the absence of all D-D reactions. In the simulation of fluorescein in solution, where N_S is low and $N_S << N_{O2}$, the probability of a reaction between dye molecules is very low. This probability is governed only by the concentration (or the intermolecular distance) of the reacting molecules, since the rate of reaction is constant at a given temperature. The photobleaching in this case is primarily caused by the reaction between a dye molecule and an oxygen molecule, since the intermolecular distance between an oxygen molecule and a dye molecule is smaller than that between the dye molecules. Photobleaching then shows a single-exponential behavior.

On the other hand, in microscopy where fluorescein molecules are bound to targets of a very small surface area or volume, the average in-

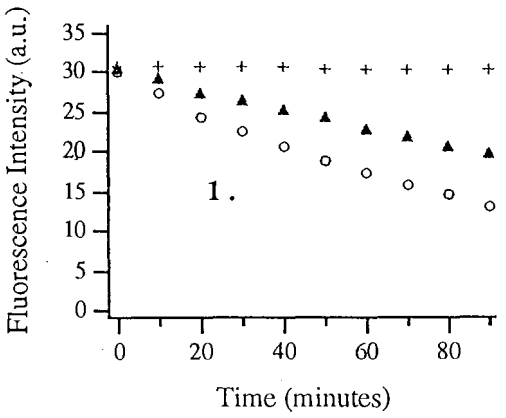

Fig. 1. The photobleaching curve of free fluorescein solution.

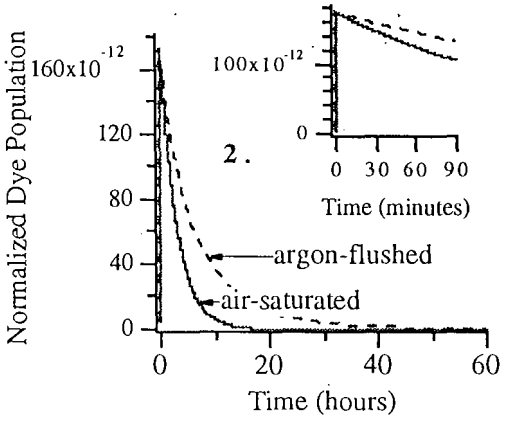

Fig. 2. Simulation for free fluorescein in air-saturated and argon-flushed solutions.

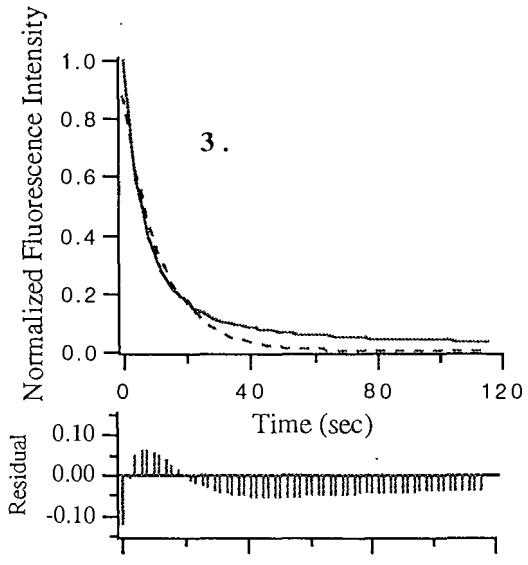

Fig. 3. The photobleaching curve of the centromeric region of chromosome 1 in a human lymphocyte. (– data, --- single-exponential fit)

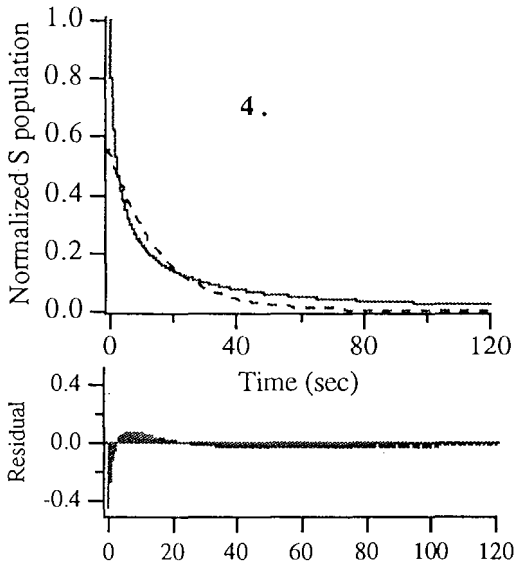

Fig. 4. Simulation for bound fluorescein in conventional fluorescence microscopy. (– simulated data, --- single-exponential fit.)

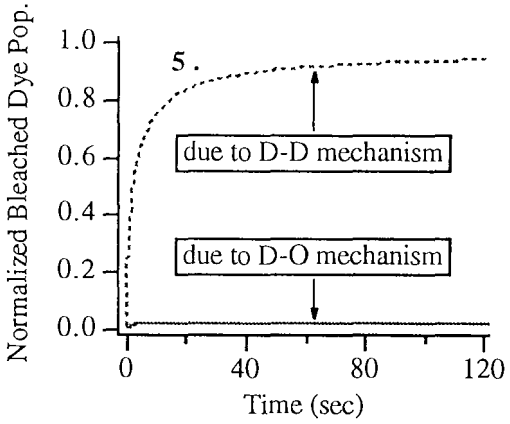

Fig. 5. Formation of the bleached molecules due to the different bleaching mechanisms (for the same case as in Fig. 4.)

termolecular distance of fluorescein molecules is smaller than the distance between a fluorescein molecule and oxygen. A triplet dye molecule is therefore more likely to react with another dye molecule than in solution. The high local density of fluorescein molecules promotes the proximity-induced D-D reactions. Since the D-D mechanism involves more than one bimolecular reaction (and thus more than one exponential term) leading to the bleached dye molecules, the bleaching behavior will not be single-exponential even in the absence of the D-O reactions.

CONCLUSION AND WORK IN PROGRESS

The present study has addressed a fundamental question about the photobleaching mechanism of fluorescein in microscopy. It has demonstrated through both experimental and theoretical methods that the single-exponential behavior is only a special case of the bleaching process when the average intermolecular distance of fluorescein is significantly greater than the average distance to oxygen, and that the photobleaching process of bound fluorescein in microscopy is in general not a single-exponential process. Further studies are being carried out to provide additional photophysical evidence for the role of the triplet in the photobleaching of fluorescein in microscopy, the results of which will be presented at the conference. The improved understanding of photobleaching mechanism in microscopy will have a direct application in quantitation of fluorescence emission.

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