Title
INTRACELLULAR DYE HETEROGENEITY DETERMINED BY FLUORESCENCE LIFETIMES

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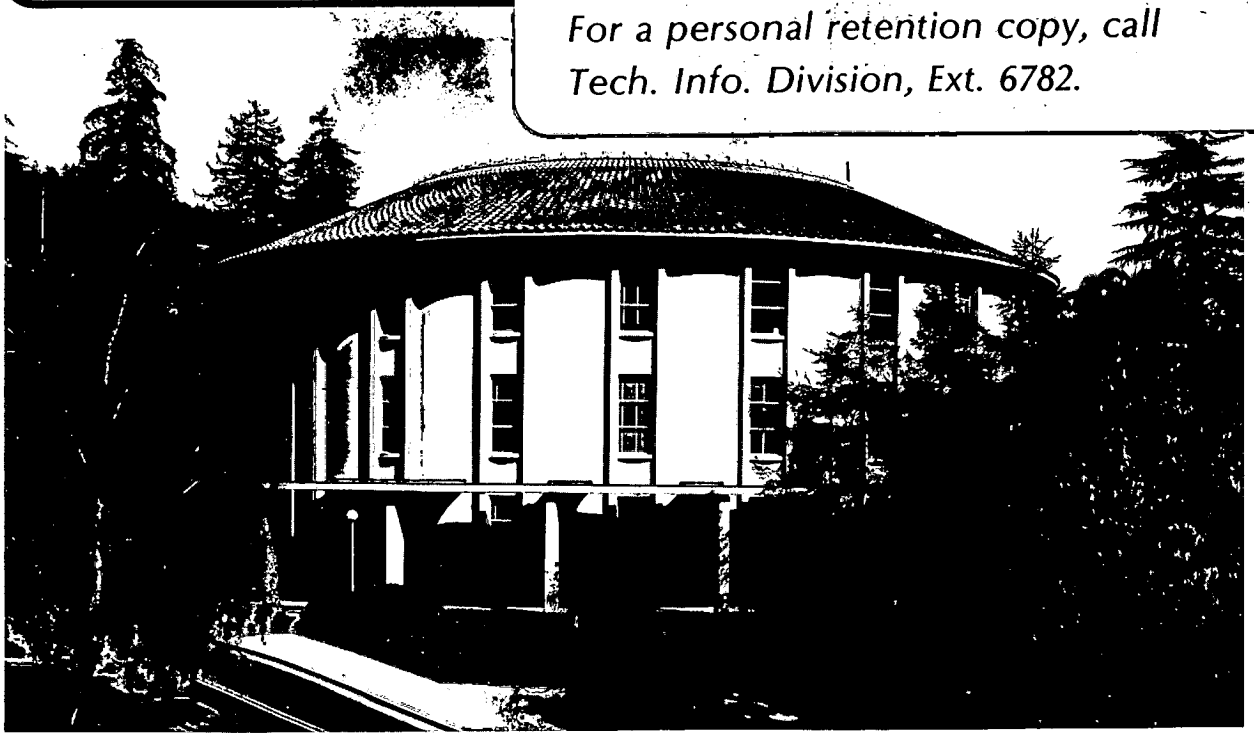

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DISCLAIMER

INTRACELLULAR DYE HETEROGENEITY DETERMINED BY FLUORESCENCE LIFETIMES

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The use of molecular probes for quantitation of cellular phenomena requires knowledge of probe locations. For fluorescent molecules, locations are usually determined by inspection under a fluorescence microscope or by subcellular fractionation. The former method of detection suffers from the limitation of spatial resolution while the latter requires destructive manipulations. We have used the environmental dependence of fluorescence lifetimes to quantitatively measure the heterogeneity of fluorescence in cells by determining the lifetimes of two rhodamine derivatives as functions of time and spatial distribution. Thus, effective monitoring of intracellular fluorophore compartmentalization and its time evolution in viable cells is possible by use of the presently reported technique.

Fluorescence is the phenomenon of radiative decay from an excited molecular singlet state to the ground state. The detailed nature of the molecular environment influences its rate of decay (1). For example, increased proximity to or concentration of a molecular species which can accept energy from the excited state of a probe molecule results in a shortening of the latter's fluorescence lifetime; this is observed in photosynthetic organisms (2). Additionally, characteristics of the surrounding medium such as polarity, dielectric constant, pH, metal ion concentration, viscosity, or hydrophobicity may also affect excited state lifetimes (3).

We have used this principle of variation in lifetimes with environment to measure quantitatively the fraction of

fluorescence in different cellular compartments. The Madin-Darby Canine Kidney (MDCK) cell line, epithelial in origin (4) and grown as adherent monolayers on glass coverslips in Modified Eagle's Medium (MEM) containing 10% fetal calf serum and 20 mM Hepes buffer at pH 7.4 and 310 mosm, was chosen as our cellular system. We observed that after brief periods of incubation with different dyes, particular patterns were seen under the fluorescence microscope (Fig. 1a). Based on the known sensitivities of fluorescent molecules to their environments, we predicted that partitioning of such molecules among physically and/or chemically distinct cellular compartments would give rise to a distribution of fluorescence lifetimes.

Fluorescence lifetimes were measured by the single photon counting method using a previously described apparatus (5,6) which contains a Spectra Physics synchronously-pumped mode-locked dye laser (SP 171 argon ion laser, SP 362 mode locker, and modified SP 375 dye laser operating with Rhodamine 6G) with a 12 nsec. pulse separation and a pulse full-width at half maximum of ca. 8 psec. Excitation of samples was at 580 nm and emission was detected at 650 nm with a cooled RCA C31034A photomultiplier. Cells on coverslips in cuvettes filled with phosphate buffered saline (PBS) were counted long enough to acquire either 10,000 or 2,500 photons in the peak channel of a 1024-channel Northern NS636 multichannel analyzer. The response function of the apparatus was deconvolved from the raw data leading to the true decay. These data were then fitted with an assumed decay law approximated by a sum of exponentials. Minimization of the

deviations from this sum were guided by determining the least number of terms for convergence (7).

For the initial experiment we incubated MDCK cells with rhodamine-B (8 ug/ml of serum-free MEM) for five minutes at room temperature. Measurements obtained immediately after washing the cells several times with dye-free medium indicated the presence of more than one fluorescent component, the predominant one (95-96%) having a lifetime of 1.90 nsec. (Measurement of the dye-containing medium gave a lifetime of 1.74 nsec.) A two-component fit with a second lifetime approximately 0.28 nsec. showed deviations due only to statistical noise whereas the one-component fit showed deviations well above the noise level (fig. 2a,b). A three-component fit with 1.90 nsec. as the predominant and two shorter (0.53 and 0.14 nsec.) lifetimes with almost equal amplitudes (.022 and .025, resp.) showed a deviations' plot not significantly different than that of the two-component fit. Thus, the molecule has at least two environments which lead to two significantly distinguishable lifetimes.

We next exposed cells to Collarein. This is a fluorescent phospholipid analog that we have recently synthesized in which a lissamine rhodamine-B is attached to cardiolipin (details to be published elsewhere). It was designed for the property of exclusive localization in the plasma membrane and examination under the fluorescence microscope indicates this to be the case (fig. 1b). The fluorescence decay curve of Collarein is fit with a single exponential having a lifetime of 1.85 nsec. When a two-component fit was attempted, the two components collapsed into

one, indicating that only a single exponential is required to describe the fluorescence decay (fig. 2c,d).

To demonstrate the potential of this technique for studying the kinetics of cellular compartmentalization, we performed fluorescence lifetime measurements at thirty minute intervals. In the case of rhodamine-B a reciprocal linear relationship between fractions of the short and long lifetime components with time was observed; additionally, the fluorescence yield decreased monotonically (fig. 3). Since we observe under the fluorescence microscope a time-dependent redistribution of cellular fluorescence, specifically, vesicular accumulations of fluorescence exclusive of the nuclear region (fig. 1a), with a time course in parallel with changes observed in the fluorescent lifetime measurements, it is reasonable to ascribe the short lifetime component to those molecules which have become concentrated in the observed vesicles. Similar results were observed when lifetime measurements were performed on a series of acellular, graded (from 8 to 800 ug/ml) solutions of rhodamine-B in PBS; fluorescence lifetimes decreased with increasing fluorophore concentrations. Thus, we believe the shortened lifetime is a consequence of mutual energy transfer. Other parameters of fluorescent molecules, e.g., absorption or emission wavelength shifts may accompany relocation, but they are less accessible to precise quantitation.

For cells incubated with Collarein, single exponentials gave the best fits to data acquired at thirty minute intervals over a four and one-half hour period; here again the total fluorescence

continuously decreased over time. Thus, the visual observations of Collarein with a single cellular location (fig. 3b) and intensity decreasing continuously with time, due to catabolism or conversion to an otherwise nonfluorescent state, are consistent with lifetime measurements.

We are extending lifetime measurements to molecules known to intercalate into nucleic acids. Decay curves with complex behavior of the fluorescence time course have been observed and may indicate energy transfer along the DNA helix. Thus, extensions of this technique may prove to be extremely useful in nucleic acid structural work as well as in cell cycle kinetic studies.

Acknowledgements

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Fig. 1: Fluorescence micrographs of MDCK cells stained with (a) rhodamine-B and (b) Collarein.

Fig. 2: Fluorescence decay curves of MDCK cells containing fluorophores. Curves labeled E(t) are the excitation profiles induced by the laser pulse. Curves labeled F(t) are the experimental fluorescence decay (noisy) and the calculated fit (smooth) essentially superimposed. Deviations' plots are below. Fits for: (a) rhodamine-B, single exponential, (b) rhodamine-B, sum of two exponentials, (c) Collarein, single exponential, (d) Collarein, sum of two-exponentials.

Fig. 3: The amplitudes of long lifetime (□-□-□-□), short lifetime (0-0-0-0) and fluorescence yield (X-X-X-X) as functions of time after incubation with rhodamine-B).

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fig. 1

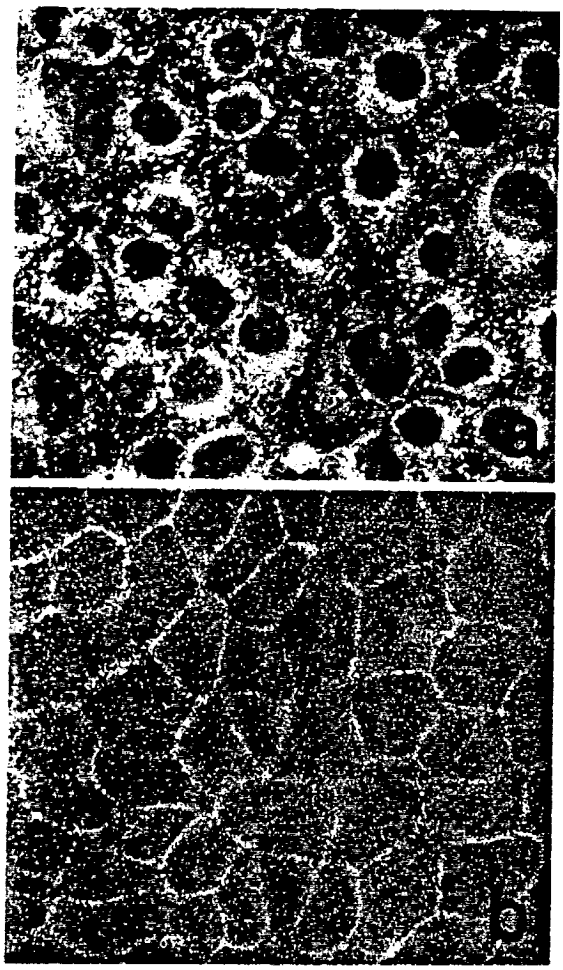
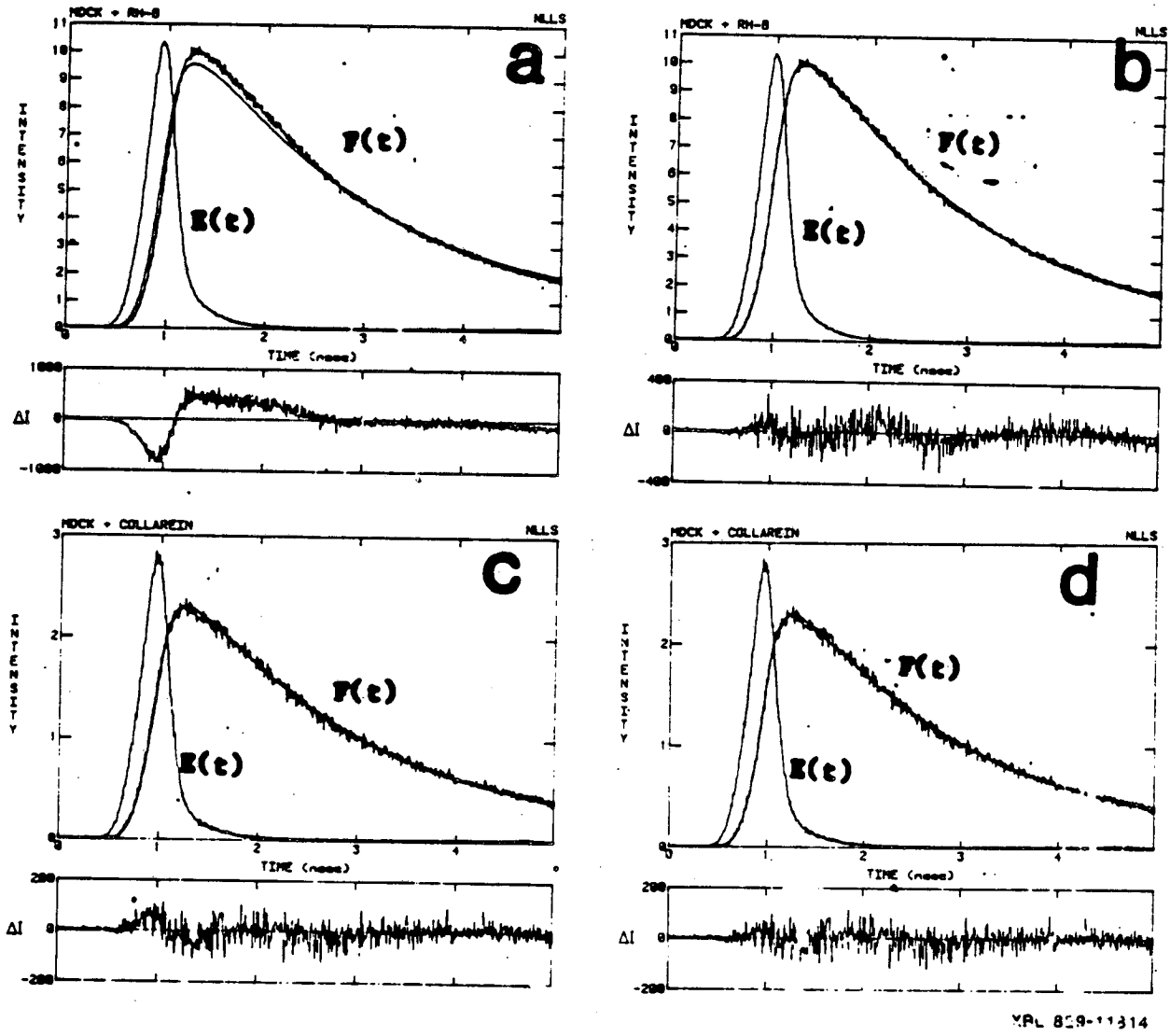


fig. 2


MDCK + RH-8
MLLS
a
F(t)
E(t)
INTENSITY
TIME (nsec)
ΔI
MDCK + RH-8
MLLS
b
F(t)
E(t)
INTENSITY
TIME (nsec)
ΔI
MDCK + COLLAREIN
MLLS
c
F(t)
E(t)
INTENSITY
TIME (nsec)
ΔI
MDCK + COLLAREIN
MLLS
d
F(t)
E(t)
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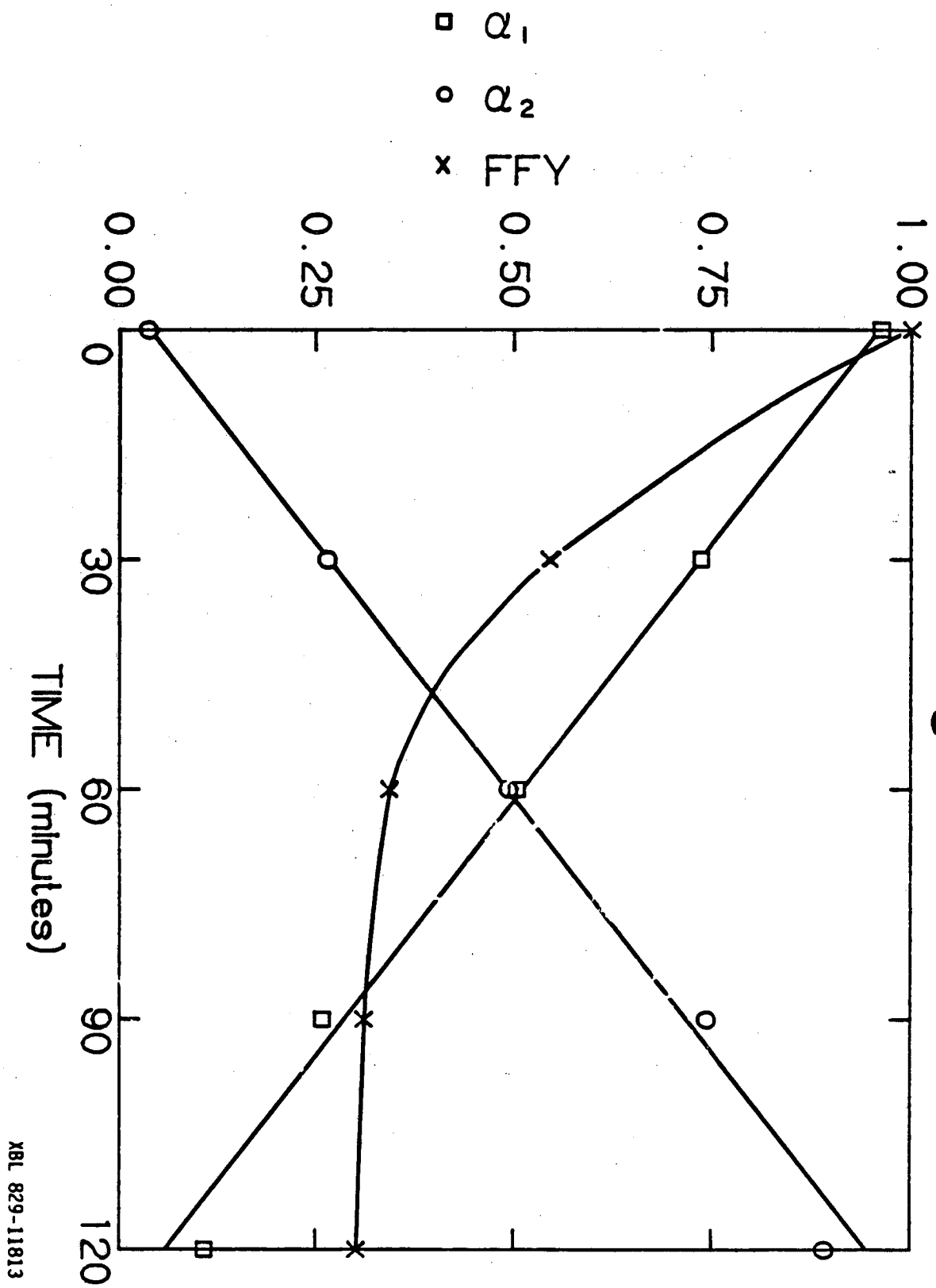
α1
α2
FFY
0.00
0.25
0.50
0.75
1.00
0
30
60
90
120
TIME (minutes)
XBL 829-11813

fig.3

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