A Novel Fluorescence Ratiometric Method Confirms the Low Solvent Viscosity of the Cytoplasm

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ABSTRACT Two homologous indocyanine dyes, Cy3.18 and Cy5.18, can be used as a ratio pair for fluorometric determination of solvent viscosity. Succinimidyl ester derivatives of these dyes can be attached to inert carrier macromolecules, such as Ficoll 70, for measurement of intracellular or intravesicular solvent viscosity. When the viscosity of the solvent was varied by various methods, the fluorescence intensity ratio (Cy3/Cy5) in a mixture of Cy3.18-Ficoll 70 (Cy3F70) and Cy5.18-Ficoll 70 (Cy5F70) in solution was found to be solely a function of solvent viscosity and was insensitive to other solvent parameters such as dielectric constant, temperature, and the ability of the solvent to form hydrogen bonds. Most important, it was insensitive to the presence of large macromolecules, such as proteins, which increase the shear viscosity but have little effect on solvent viscosity. Following microinjection into the cytoplasm of living tissue culture cells, no binding of Cy3F70 or Cy5F70 to intracellular components was detected by fluorescence recovery after photobleaching. Fluorescence intensity ratio imaging of Cy3F70 and Cy5F70 in non-motile interphase CV1 and PtK_1 cells showed that the solvent viscosity of cytoplasm was not significantly different from water and showed no spatial variation.

INTRODUCTION

A long standing debate in the field of cellular biophysics concerns the state of water within living cells (for reviews, see Refs. 1 and 2). On one hand, proponents of a hypothesis extensively outlined by Ling (3), believe that, due to ordering of water on charged and hydrophobic surfaces, much of intracellular water has altered solvent properties and lower mobility compared to bulk water (4–6). Others argue that the interaction of water with intracellular surfaces is limited to a thin shell of hydration a few water molecules thick, and that the rest of intracellular water has the properties of bulk water (7, 8). If a significant fraction of intracellular water is indeed bound or ordered, the solvent viscosity of the cell interior will be elevated and its solvent properties altered, with important consequences for cellular metabolism. Although a variety of methods have been employed to address this question over the last two decades, the experimental results have not completely clarified the issue.

Of the techniques used to study water mobility, proton NMR has the advantage of being noninvasive. However, due to the ambiguity inherent in fitting relaxation curves to two or more components with the time constant and the fractional contribution of each component as adjustable parameters, the same data has been used to argue both points of view (5, 7). Measurements of the rotational correlation times of small electron spin probes indicate an intracellular viscosity of two to eight times the value for bulk water, but the potential binding of these probes to intracellular components was not assessed (9, 10). In addition, since both the NMR and the electron spin resonance experiments were performed on populations of cells in suspension, the subcellular localization of the probes was unknown and the data could have been biased by high viscosity in one compartment. Recently, Fushimi and Verkman (11) used time-resolved fluorescence polarization microscopy to map subcellular solvent viscosity and reported that the viscosity of the cytoplasm was indistinguishable from bulk water. Although circumstantial evidence points toward the validity of their measurements, this result is not unambiguous because a significant fraction of their probe was bound to intracellular components, and fitting of the relaxation curves required estimation of the relative fractions of bound and unbound probe. We have developed a novel fluorescence intensity ratio imaging method for mapping intracellular solvent viscosity using a pair of homologous indocyanine dyes, Cy3.18 and Cy5.18. The method is straightforward to implement in any laboratory with a cooled charge-coupled device camera or other linear detector and an image processor capable of pixel-by-pixel image division. Solvent viscosity can be determined directly from the fluorescence intensity ratio of the two dyes without the need for curve fitting and with no adjustable parameters.

Cy3.18 and Cy5.18 differ only in the length of the polymethine chain linking the two indole rings, and in this difference lies the utility of Cy3.18 and Cy5.18 as a ratio pair for measuring solvent viscosity. In Cy5.18 the indole rings are separated by a five-carbon chain, and the rings can assume a coplanar configuration that is very stable and relatively rigid. In Cy3.18, the polymethine chain contains only three carbons and steric hindrance prevents the rings from assuming a coplanar configuration, resulting in a more flexible molecule that can twist about the polymethine chain

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(12, 13). The relative rigidity of the molecule has important consequences for the quantum yield of fluorescence because internal conversion due to intramolecular motion competes with fluorescence for dissipation of the excited state energy after a photon is absorbed. Since the lifetime for internal conversion is several orders of magnitude shorter than for fluorescence, molecules such as Cy3.18 tend to dissipate most of the excited state energy by internal conversion before significant fluorescence can occur. However, as the viscosity of the solvent increases, molecular motion is slowed and the quantum yield of fluorescence increases significantly, making Cy3.18 a potential indicator of solvent viscosity (12, 13). Due to the more rigid configuration of Cy5.18, internal conversion is negligible, the primary mode of de-excitation is by fluorescence, and changes in viscosity of the solvent should have little effect on the quantum yield. In principle, this makes Cy5.18 a good ratio partner for Cy3.18, because it can be used to normalize for solvent effects other than viscosity. For measurements in vessels of nonuniform diameter, including living cells, Cy5.18 can also be used to normalize for pathlength (14).

In this manuscript, we demonstrate that the Cy3/Cy5 fluorescence intensity ratio is indeed a function of solvent viscosity and show that it is insensitive to dielectric constant, temperature, solvent hydrogen bonding, and the presence of large macromolecules, including proteins. We describe the procedure for labeling Ficoll 70 with Cy3.18 and Cy5.18 and show that Cy3-Ficoll 70 (Cy3F70) and Cy5-Ficoll 70 (Cy5F70) do not bind with high affinity to cytoplasmic components following microinjection into living cells. Values of cytoplasmic solvent viscosity obtained from the fluorescence intensity ratio of Cy3F70 to Cy5F70 confirm the conclusions of Fushimi and Verkman (11) that the viscosity of the aqueous cytoplasm does not differ from water.

MATERIALS AND METHODS

Synthesis of dyes and labeling of ficolls

The synthesis of Cy3.18 and Cy5.18 and their succinimidyl esters (designated .OSu) is described elsewhere (15). For microinjection experiments, we attached Cy3.18.OSu and Cy5.18.OSu to aminated Ficoll 70 (AECM-F70). Use of Ficoll 70 as a carrier molecule minimizes uptake into vesicles and other nonspecific interactions of the dyes with intracellular components and allows independent sampling of the nuclear and cytoplasmic compartments. Because of the degree of overlap between the emission spectrum of Cy3.18 and the excitation spectrum of Cy5.18, it was necessary to avoid energy transfer by putting the dyes on separate Ficoll molecules. Ficoll 70 was aminated as previously described (16). A ninhydrin assay with glycine as a standard indicated that carrying out the first step of this procedure at 25°C resulted in approximately three to four amino groups per mol of Ficoll in the final product. For fluorescent labeling, 100 mg of AECM-F70 was dissolved in 2 ml of 10 mM carbonate-bicarbonate buffer, pH 9.2. Five mg of Cy3.18.OSu (M_r 935) or 6 mgs Cy5.18.OSu (M_r 1522) or 20 mg fluorescein isothiocyanate (FITC; Isomer I) was dissolved in 200 μl of dimethylformamide and added to the AECM-F70 solution dropwise with stirring. (For double-labeling experiments, half as much Cy5.18 was used, leaving half the amino groups available for the second fluorophore.) The mixture was allowed to react at room temperature for 1 h and unreacted dye was removed by desalting on a 0.5- × 33-cm column of G25–150 (Sigma) equilibrated in 10 mM Tris, 50 mM KCl, pH 8.0. The void volume was pooled, dialyzed against one change of Tris-KCl buffer and two changes of distilled water, and lyophilized. For double-labeling experiments, the labeled Ficoll was resuspended in carbonate-bicarbonate buffer and relabeled with a second fluorophore at this point. Otherwise, the conjugate was resuspended at 25 mg/ml in Tris-KCl buffer and size-fractionated on Sepharose CL-6B as previously described (16). This step was necessary to eliminate the appearance of fluorescence in vesicles after microinjection into the cytoplasm of living cells. Included fractions having an optical density greater than or equal to half maximal were pooled, dialyzed against distilled water, filtered through a 0.2-μm filter (Millipore, Bedford, MA), and lyophilized for storage. The dye-to-Ficoll ratio was determined from the optical density of a known concentration of Ficoll, using a molar extinction coefficient of 118,000 for Cy3.18 at 555 nm, 200,000 for Cy5.18 at 650 nm, or 72,000 at 495 nm for FITC at pH 8.0.

Fluorometry

A stock solution of Cy3.18 and Cy5.18 was prepared by dissolving 7.5 mg of each in 1 ml of distilled water. This stock was diluted 1:100 for all fluorometric measurements. A stock solution of Cy3F70 and Cy5F70 was prepared by dissolving 6 mg of each together in 1 ml of distilled water. This stock was diluted 1:400 for all fluorometric measurements. The optical densities at the absorption maxima for each sample ranged from 0.1 to 0.2. The emission spectrum for each sample was scanned using a Spex Fluorolog 2 dual-beam fluorometer with a temperature-controlled sample chamber (Spex Industries, Metuchon, NJ) at an excitation wavelength of 520 nm for Cy3F70 and 600 nm for Cy5F70. The excitation slits were set at 0.5 mm and the emission slit width was 1.8 nm/mm. The emission intensity maxima were used to calculate the fluorescence intensity ratio (Cy3/Cy5). Ratios were corrected for the measured optical density of the sample and for the refractive index of the solvent when necessary. Reported ratios are the mean values from triplicate samples.

Viscometry

Kinematic viscosities were measured with a Cannon-Manning semimicro viscometer (No. 75, A834), which had a constant of 0.00896 cS/s at 40°C. Average viscosity was taken as the mean of three measurements. Kinematic viscosity was converted to intrinsic viscosity by multiplying by the measured density of the sample.

Cell culture

All cell lines were purchased from American Type Culture Collection, Rockville, MD. Cell culture reagents were purchased from GIBCO-Bethesda Research Laboratories, Grand Island, NY. CV1 cells were grown in Dulbecco's modified Eagle's medium, 10% fetal calf serum, and 1% penicillin-streptomycin. PtK_1 cells were grown in minimal essential medium, 10% fetal calf serum, 1 mg/ml sodium pyruvate, and 1% penicillin-streptomycin. Cell cultures were maintained at 37°C in a humidified 5% CO_2 atmosphere.

Microinjection

Lyophilized Cy3F70 and Cy5F70 were resuspended in injection buffer (2.5 mM 1,4-piperazinediethanesulfonic acid, pH 7.0, 0.05 mM $MgCl_2$, 0.05 mM KCl) at a concentration of 6 mg/ml, sonicated for 30 s at room temperature and clarified for 5 min at 23 psi in a Beckman Airfuge (Beckman, Fullerton, CA). Microneedles were prepared from Kwik-Fil Borosilicate Glass Capillaries (1B120F-4; World Precision Instruments, Inc., Sarasota, FL) on a Model 720 vertical pipette puller (David Kopf Instruments, Tujunga, CA). Microinjection was carried out using an Eppendorf Microinjector 5242 (Carl Zeiss Instruments, Inc., Thornwood, NY) with an MO-202 three-axis micromanipulator with hydraulic fine control (Narishige Co., Ltd., Tokyo, Japan) on an Axiovert 35 microscope (Carl Zeiss Instruments, Inc.). Cells were maintained at ~37°C during the injections using a KT Model 5000 thermoelectric stage (Micro Devices, Inc., Jenkintown, PA). After injection,

cells were rinsed well three times in complete Dulbecco's minimal essential medium without phenol red and allowed to recover 1–3 h prior to imaging. We estimate that the amount injected was less than 10% of the cell volume.

Digital fluorescence microscopy and ratio imaging

Coverslips containing injected cells were assembled into a 60-mm-diameter modified Sykes-Moore perfusion chamber (Custom Scientific Inc., Dallas, TX) with a 0.5-mm-thick sapphire window with 0001 crystal orientation and epitaxial polish as the upper window of the chamber (Crystal Systems, Inc., Salem, MA). The window is optically clear and conducts heat in the plane of window six times more efficiently than glass. When used in conjunction with the Microdevices thermoelectric stage, this chamber maintains the cultures at 37°C with a temperature differential between the edge and the center of the chamber < 0.3°C.

Fluorescence was viewed with a 40× Plan-Neofluar lens on an Axiovert 35 microscope, and images were acquired using a Photometrics Series 200 Cooled CCD camera (Photometrics Ltd., Tucson, AZ) input to a Perceptics 9200 image processor with μVax host (Perceptics, Knoxville, TN). Fluorescein fluorescence was viewed with a 485/20-nm bandpass excitation filter and 542/45-nm bandpass emission filter. Cy3 fluorescence was viewed with a 546/12-nm bandpass excitation filter and a 590/40-nm bandpass emission filter. Cy5 fluorescence was viewed with a 646/19-nm bandpass excitation filter and a 682/22-nm bandpass emission filter (Omega Optical, Brattleboro, VT). All exposure times were 3 s. Background images were obtained by moving to a part of the coverslip containing uninjected cells. Background subtracted images were registered interactively, using the nucleus as a fiduciary marker, and were ratioed using the floating point accelerator board of the Perceptics 9200. The Perceptics automatically rescales the images for display between 0 and 255 gray levels, but retains the scaling factor and gives calibrated values. Although a shading correction was not performed, ratio values did not differ significantly at the center and near the edges of the field, and only cells in the center 50% of the field were analyzed. To obtain the mean cytoplasmic value of Cy3/Cy5, regions of interest were outlined interactively, taking care to avoid regions where the probes were obviously excluded by groups of membrane-bound organelles and stress fibers. The mean ratio value and variance within the region of interest were calculated using Perceptics BioVision Software.

A microscope standard curve was obtained by diluting the injection mixture 1:100 in mixtures of glycerol:phosphate-buffered saline (PBS) ranging from 0 to 70% glycerol. Samples were placed in the cell chamber and imaged under exactly the same conditions as cells. Background images were obtained from samples that contained no fluorescent Ficolls. Background-subtracted fluorescence images were ratioed as above and the mean value of the ratio for a rectangular region of interest covering the center 50% of the field was obtained. Reported ratios are the mean of triplicate or quadruplicate measurements.

Fluorescence recovery after photobleaching

Fluorescence recovery after photobleaching (FRAP) was performed as previously described (17). Briefly, the 488-nm line of a SpectraPhysics argon ion laser operated at 200 mW was used to bleach fluorescein, and the 514-nm line was used to bleach Cy3.18. Relative fluorescence intensities were measured with a photomultiplier (Hamamatsu Corp., Middlesex, NJ) and analyzed according to Yguerabide (18) on an IBM-PC/AT using a program written with ASYST software (Macmillan Software Co., New York, NY). For solution measurements, the $1/e^2$ radius of the bleached spot was 19 μm, and for cells, the radius was 5.4 μm.

RESULTS

Cy3/Cy5 fluorescence intensity ratio is a function of solvent viscosity

The fluorescence intensity ratio (Cy3/Cy5) for a mixture of free Cy3.18 and Cy5.18 in solution increased more than fourfold as viscosity was varied from 0.99 to 20.8 cP by varying the concentration of glycerol from 0 to 70% in PBS at 25°C (Fig. 1 *A*). Values of Cy3/Cy5 measured in glycerol:PBS at 37°C and in 0–40% ethanol in PBS at 25°C fell on the same curve of ratio versus viscosity, suggesting that Cy3/Cy5 is sensitive to solvent viscosity rather than dielectric constant or temperature. This conclusion was borne out by the more detailed characterization of Cy3/Cy5 that was carried out for mixtures of Cy3F70 and Cy5F70 (see below).

For a mixture of Cy3F70 and Cy5F70 in solution, Cy3/Cy5 increases 2.3-fold as glycerol is varied from 0 to 70% (Fig. 2). The smaller change in ratio was due primarily to a higher ratio value in PBS, suggesting that Cy3.18 becomes partially immobilized on binding to F70. Values for ratio versus viscosity for the mixture of Cy3F70 and Cy5F70 fall on a single master curve when viscosity is varied using glycerol, sorbitol, or sucrose or by changing the temperature of the solvent (Fig. 2). The quantum yield of Cy5F70 increased slightly as viscosity was elevated using glycerol, sucrose, or sorbitol. The increase was comparable for all three solutes (~14%). No similar increase was apparent in the data for the free dye.

There was no significant change in the extinction coefficient of either Cy3F70 or Cy5F70 as they were transferred from PBS to 70% glycerol, although there was a slight red shift of a few nanometers for both dyes. A similar red shift was evident in the emission spectra of both dyes. This red shift was also evident in 40% ethanol, suggesting it is due to a very modest sensitivity to the dielectric constant of the solvent. Since increasing glycerol concentration and decreasing temperature have opposite effects on the dielectric constant of the solvent, these results indicate that Cy3/Cy5 is insensitive to dielectric constant despite the small red shift in the absorption and emission spectra of both dyes. This was

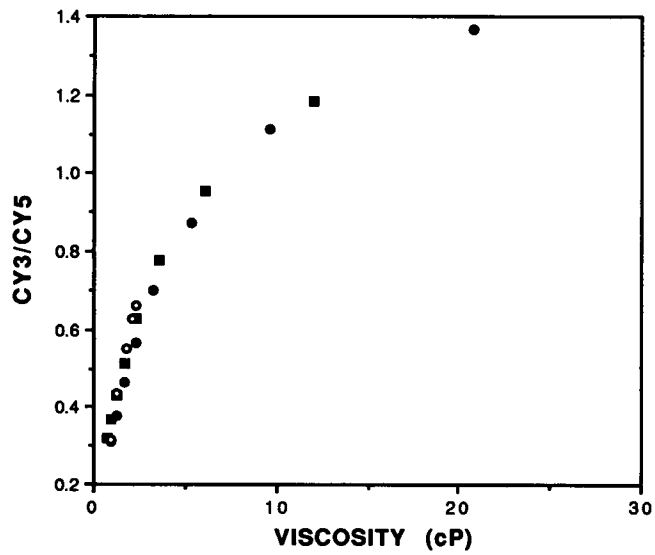

FIGURE 1 Viscosity dependence of Cy3/Cy5 fluorescence intensity ratio for Cy3.18 and Cy5.18 in solution. ●, 0–70% glycerol/PBS at 25°C; ■, 0–70% glycerol/PBS at 37°C; ○, 0–40% ethanol at 25°C.

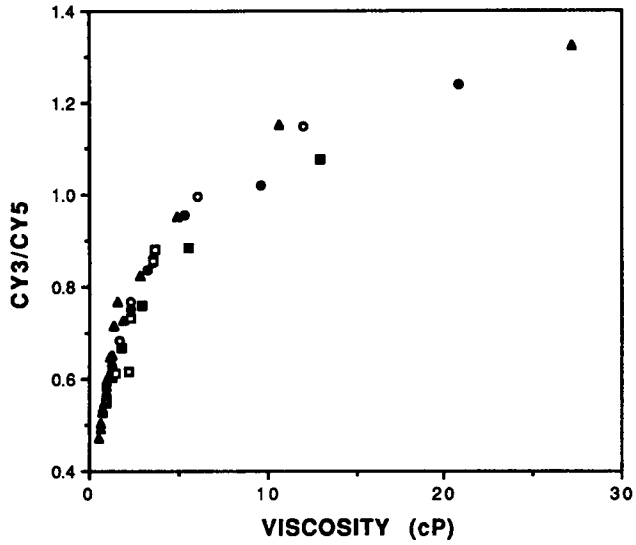

FIGURE 2 Viscosity dependence of Cy3/Cy5 fluorescence intensity ratio for Cy3F70 and Cy5F70 in solution. Cy3/Cy5 versus viscosity. ●, 0–70% glycerol/PBS at 25°C; ○, 0–70% glycerol/PBS at 37°C; ■, 0–50% sucrose/PBS at 25°C; ▲, 0–60% sorbitol/PBS at 25°C; △, distilled water 5–50°C; □, dimethyl sulfoxide/distilled water at 25°C.

confirmed by the result that Cy3/Cy5 ratio was only slightly increased in 40% ethanol, which has nearly the same dielectric constant as 70% glycerol but about 8-fold lower viscosity (Table 1).

Data from samples dissolved in distilled water fell on the same curve as data from samples in PBS, indicating that Cy3/Cy5 is insensitive to the ionic strength of the solvent over this range. This was confirmed by the insensitivity of Cy3/Cy5 to varying the concentration of KCl from 0 to 500 mM (Fig. 3 *A*). In fact, the quantum yields of both Cy3F70 and Cy5F70 were completely insensitive to ionic strength over this range. Within the limits possible, we also varied viscosity by various combinations of dimethyl sulfoxide and PBS. These data also fell on the master curve of ratio versus viscosity, indicating that ratio is independent of the ability of the solvent to hydrogen bond to the dye (Fig. 2). Additionally,

TABLE 1 Fluorescence intensity ratios for a mixture of Cy3F70 and Cy5F70 in different solvent systems as % increase over the value in PBS

	Viscosity	Dielectric constant	Cy3/Cy5
30% glycerol	2.26 cP	71.44	+53%
70% glycerol	20.8 cP	58.52	+268%
40% ethanol	2.34 cP	63.3	+55%

Since the increase in Cy3/Cy5 ratio in 40% ethanol is similar to that in 30% glycerol which has about the same viscosity, Cy3/Cy5 ratio is not sensitive to dielectric constant. Furthermore, 70% glycerol and 40% ethanol have similar dielectric constants but very different viscosities and very different Cy3/Cy5 ratios. All measurements were made at 25°C. Samples were excited at peak wavelengths and fluorescence intensities were recorded at the peak wavelengths for emission. Viscosities were measured as described under Materials and Methods. Dielectric constants were taken from Timmermans, "Physico-Chemical Constants of Binary Systems," Vol. 4, Interscience.

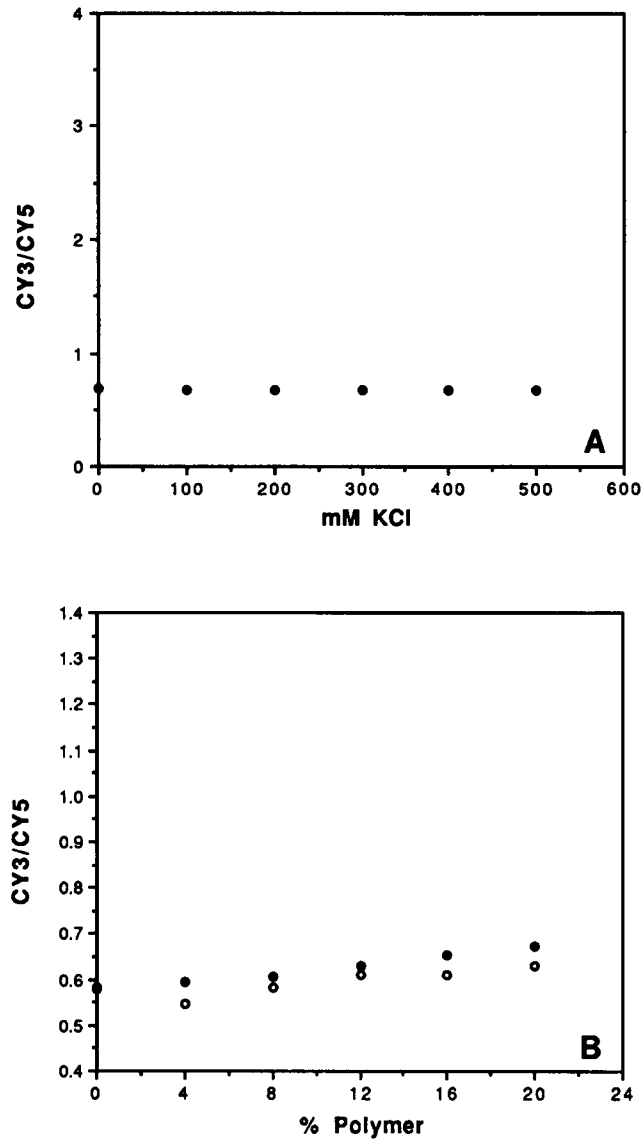

FIGURE 3 (*A*) Cy3/Cy5 ratio is insensitive to changes in ionic strength over the range 0–0.5. Cy3/Cy5 for Cy3F70 and Cy5F70 in solution is plotted versus mM KCl. (*B*) Cy3/Cy5 ratio is insensitive to high concentrations of macromolecules. Cy3/Cy5 for Cy3F70 and Cy5F70 in solution is plotted versus % polymer by weight. ●, Ficoll 400; ○, ovalbumin. The bulk shear viscosity of 20% Ficoll 400 was 23 cP. The bulk shear viscosity of 20% ovalbumin was 3 cP.

Cy3/Cy5 was essentially insensitive to high concentrations of polymers such as ovalbumin and Ficoll 400 (Fig. 3 *B*), which increase the shear viscosity of the bulk, but should not change solvent viscosity appreciably. The quantum yields of Cy3F70 and of Cy5F70 were unaffected by concentrations of ovalbumin as high as 20%, suggesting that Cy3F70 and Cy5F70 do not bind to proteins even at protein concentrations comparable to that of cytoplasm.

No binding of Cy3F70 and Cy5F70 is detectable by FRAP

We designed FRAP experiments to see whether Cy3F70 and Cy5F70 interact with intracellular components. Because our

laser had no line at a wavelength suitable to bleach CY5.18, we double-labeled F70 with FITC along with Cy5.18 and used the 488 line to bleach the fluorescein moiety. The recovery curves for FTC-F70, Cy3F70 and FTC, Cy5F70 could be fit to one component. Both Cy3F70 and the double-labeled FTC, Cy5F70 gave the same diffusion coefficient as F70 labeled with FITC alone and all three labeled Ficolls exhibited complete fluorescence recovery (Table 2). Since we have previously shown that FTC-Ficoll does not exhibit high affinity interactions with immobile cellular components (see Discussion for details), this result suggests that high affinity binding of the probes to immobile intracellular components can be neglected when using Cy3/Cy5 ratio to map solvent viscosity in the cytoplasm of living cells.

Microscope standard curve and error analysis

Mean ratio values from triplicate or quadruplicate pairs of images of Cy3F70 and Cy5F70 in homogeneous solutions of PBS:glycerol are plotted in Fig. 4 *A*. For each data point, the standard error of the mean was <0.8%. For a single ratio image of a given field, the square root of the intrascene variance gave a standard deviation on the order of ±5% of the image mean value. For measurements in living cells, differences in ratio between two subcellular regions of interest were taken to be significant only if they differed by > 5%.

Average solvent viscosity of the cytoplasm in living cells

Because F70 is too big to fit through nuclear pores, the cytoplasmic and nuclear compartments can be studied separately by microinjection into one or the other. We observed no difference between the subcellular distributions of Cy3F70 and Cy5F70 following injection into the cytoplasm. The solvent viscosity of the cytoplasm was mapped by ratio imaging in subconfluent interphase tissue culture cells. Repeated imaging of the same field returned the same ratio values, indicating that differential photobleaching of the two fluorophores had a negligible effect on ratio. CV1 cells had a mean cytoplasmic ratio value of 0.94 ± 0.05 (mean ± SE; n = 28 cells). PtK_1 cells had a mean ratio value of 0.96 ± 0.07 (mean ± SE; n = 22 cells). These values are not significantly different from PBS at 37°C, which had a mean ratio value of 0.97 ± 0.02 (mean ± SE) (Fig. 4 *B*). The intracellular variance of ratio values was comparable to that of the homogeneous solutions used to obtain a standard curve, suggesting that no significant spatial variation of solvent viscosity exists under these conditions. Visual inspection of the ratio image in Fig. 4 *B* gives the impression that some domains of the cytoplasm are darker than others. These regions contain numerous vesicles or stress fibers, which exclude Cy3F70 and Cy5F70, lowering the mean ratio value in these regions. These regions were not included in the analysis. The extreme margins of the cells were also omitted from the analysis to avoid motion artifacts due to membrane ruffling.

TABLE 2 Diffusion coefficients (*D*) and percent fluorescence recovery of fluorescent Ficoll 70 in the cytoplasm of living cells

Probe	$D \times 10^7$ cm²/s	% Recovery
FTC-F70 (n = 22)*	2.00 ± 0.5	96.5 ± 3.0
Cy3F70 (n = 32)	2.27 ± 0.42	97.0 ± 3.5
FTC, Cy5F70 (n = 24)	2.03 ± 0.78	102.4 ± 5.78

* Numbers in parentheses are the number of measurements used to find the mean values. Errors are given as mean ± SE.

DISCUSSION

In principle, the ratio of Cy3/Cy5 fluorescence intensity should be insensitive to solvent effects other than viscosity, since the only chemical difference between the two dyes is the length of the polymethine chain interconnecting the indole rings. Our measurements of Cy3/Cy5 ratio under a variety of solvent conditions show that in solution the ratio reports solvent viscosity and is insensitive to the dielectric constant, ionic strength, temperature, and hydrogen bonding ability of the solvent. The lack of any significant change in the extinction coefficient of Cy3.18 in going from PBS to 70% glycerol suggests that the radiative lifetime does not change, consistent with the hypothesis that the increase in quantum yield reflects a decrease in the rate of de-excitation by internal conversion.

Cy3/Cy5 ratio measurement of solvent viscosity is a simple method suited for a wide variety of applications. The free dyes or Ficoll adducts can be used for fluorometric measurements of solvent viscosity in complex solutions. The Ficoll adducts can be trapped in liposomes and resealed vesicles for fluorometric measurement of the solvent viscosity of their interior milieu. Similarly, endocytosed Cy3F70 and Cy5F70 can be used to measure the solvent viscosity of the endocytic compartment in living cells. Microinjected Cy3F70 and Cy5F70 can be used to map the solvent viscosity of the cytoplasm or nucleus of living cells. Although the solvent viscosity of cytoplasm in at least three cell types does not appear to differ significantly from water, ratio imaging of Cy3/Cy5 may still be informative under physiological conditions or in subcellular compartments where solvent viscosity is increased. The most serious potential artifact for the use of Cy3/Cy5 to map solvent viscosity in living cells is binding of the labeled Ficolls to intracellular components in such a way as to affect the quantum yield of one dye or the other. Ficoll itself should not exhibit any binding interactions with intracellular components because it is very hydrophilic and has a low density of ionizable groups. We have previously shown that FTC-Ficolls ≤ 18 nm in hydrodynamic radius exhibit 100% recovery after photobleaching in the cytoplasm of living cells (19). In addition, labeled Ficoll readily diffuses out of cells that have been lysed with non-ionic detergents such as Triton X-100 or during preparation for whole mount electron microscopy. These data suggest that FTC-Ficoll does not bind with high affinity to immobile intracellular components.

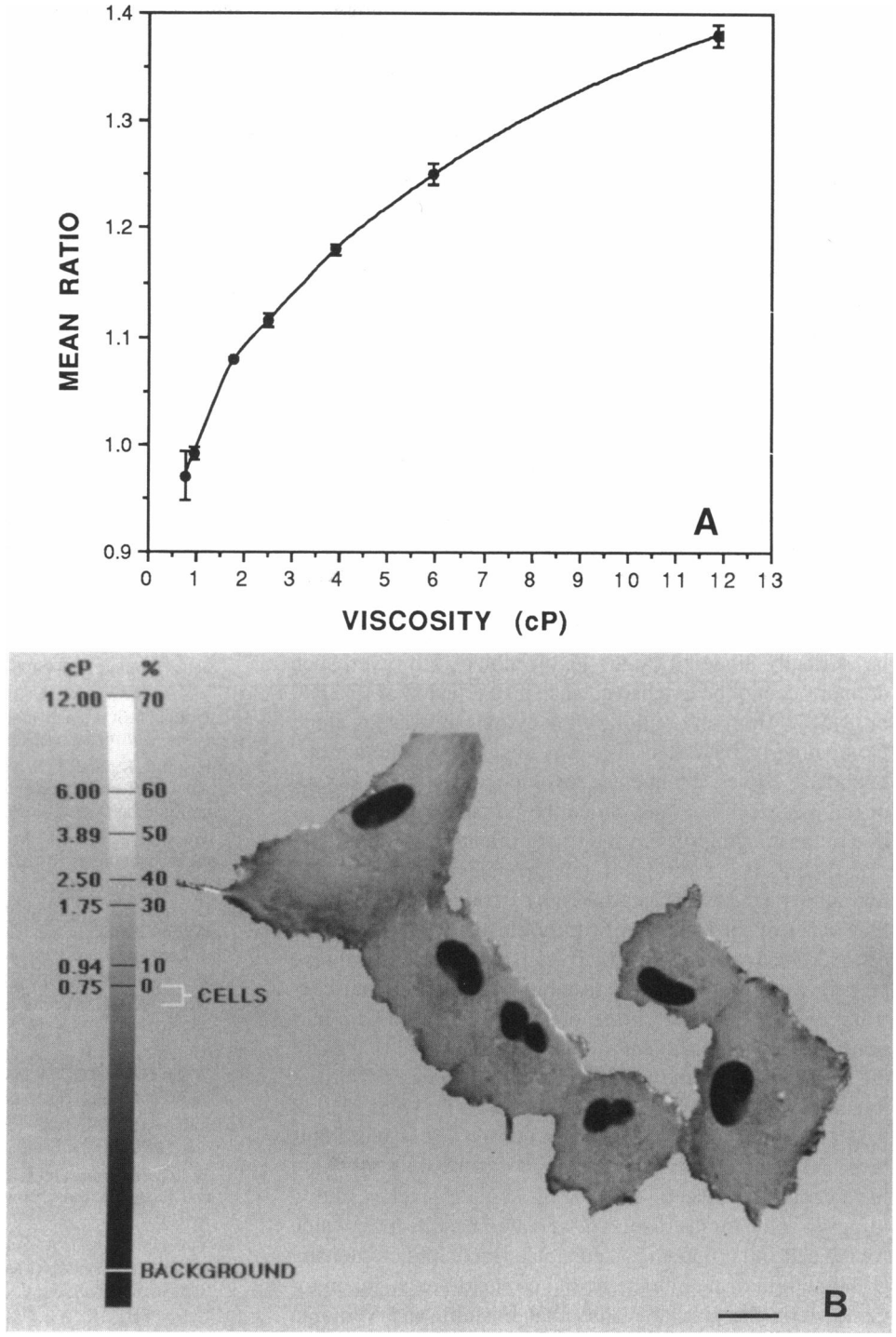

FIGURE 4 Ratio imaging of solvent viscosity in the cytoplasm of living cells. (*A*) Microscope standard curve of Cy3/Cy5 ratio versus viscosity. Mean ratio is the mean of quadruplicate samples. Viscosity is the mean of triplicate samples. Error bars represent mean ± SE. Homogeneous solutions of glycerol/PBS containing a 1:50 dilution of the Cy3F70 and Cy5F70 stock solution were placed in the temperature-controlled cell chamber and ratio imaged as described under Materials and Methods. This dilution resulted in camera exposure times comparable to those used for living cells. Background images were obtained from solutions without the fluorescent dyes. (*B*) Cy3/Cy5 ratio maps of cytoplasmic solvent viscosity in subconfluent CV1 cells at 37°C. The gray wedge is calibrated in cP on the left and percent glycerol on the right. The background ratio value, taken from the value over the nuclei, which do not contain any probe, is indicated on the gray wedge. The mean ratio ± SE for the cells in this field also is indicated to the right of the gray wedge. Overall mean ratio values for cytoplasmic solvent viscosity in CV1 cells and PtK1 cells were 0.94 and 0.96, respectively. These values are not significantly different from the value in PBS at 37°C, which has a viscosity of 0.75 cP. In general, no significant spatial variation was observed. Regions containing many vesicles or stress fibers appear darker due to exclusion of F70 from these structures. These regions were omitted from the calculation of mean ratio. Bar = 20 μm.

Modeling of the diffusion of FTC-Ficoll in cells also indicates that the behavior of FTC-Ficoll in the cytoplasm can be explained solely by hydrodynamic interactions with cytoplasmic components (17). FRAP measurements of the intracellular mobility of F70 labeled with Cy3.18 or Cy5.18 did not differ from F70 labeled with FITC, suggesting that Cy3.18 and Cy5.18 also do not bind with high affinity to large or stationary intracellular components. We note that this result does not rule out transient binding interactions or binding to highly mobile components such as soluble proteins. However, the fact that Cy3/Cy5 ratio is insensitive to concentrations of ovalbumin in the range reported for cytoplasmic protein concentration supports the idea that, even in living cells, this ratio is a reliable reporter of cytoplasmic solvent viscosity. Recently, Kao et al. (20) reported success in clamping cytoplasmic solvent viscosity by bathing cells in glycerol-containing media. We have attempted to apply this approach to our cells. In all cases, Cy3/Cy5 increased when cultures were perfused with media containing 20, 30, or 40% glycerol. On average and in two out of four experiments, the

increase in Cy3/Cy5 was commensurate with the increase in viscosity of the bathing medium. However, many cells exhibited mean Cy3/Cy5 ratios considerably higher than predicted. We noted that the cells underwent rapid and marked morphological changes upon exposure to glycerol, even at the lowest percentage. We interpreted these changes as shrinkage due to the extreme hyperosmolality of the glycerol-containing medium. In our hands, these changes were not reversed even after 10–20-min incubation in glycerol. In summary, the ability of intracytoplasmic Cy3F70 to respond to environmental changes is not blunted by binding to intracellular components, but it is likely that the cellular response to glycerol is more complex than a simple elevation of solvent viscosity.

The low viscosity of cytoplasm and the lack of significant spatial variation in ratio maps of solvent viscosity, suggests that most cytoplasmic water has properties indistinguishable from bulk water. Naturally, our probes can only sample the viscosity of compartments to which they have access, and we have not ruled out the possibility that they will be excluded from domains with highly immobile water and greatly altered solvent properties or that their close approach to surfaces will be sterically hindered by the Ficoll moiety. However, such domains cannot be extensive, since no exclusion of Cy3F70 or Cy5F70 from any region of the cytoplasm was apparent. No coating of increased ratio was observed on membrane-bounded vesicles, the nuclear envelope, or the inner surface of the plasma membrane. Since the nominal spatial resolution of the imaging system at the magnification used for these experiments is 0.5 μm/pixel, this indicates that the layer of hydration on these surfaces does not extend outward for more than 0.5 μm. In fact, significantly elevated ratio values at distances considerably less than 0.5 μm from the membrane surface would have been detectable if their fractional contribution to the intensity of fluorescence recorded by a particular pixel was sufficient to raise the gray value to $\geq$5% of the mean value for the cell. For example, a layer of solvent having a viscosity of 12 cP would have a ratio value of 1.38 based on our microscope standard curve (Fig. 4 *A*). If this layer extended 60 nm from the surface of vesicles, it would have covered $\sim$12% of the area of a single pixel, resulting in a gray value for the pixel $\sim$5% greater than the mean value for the cell and producing a detectable rim of higher intensity at the margin of membrane bound compartments. Taken together with the similar results that Fushimi and Verkman obtained by an independent method (11), our results strongly suggest that the short proton NMR relaxation times recorded from cells and tissues are due to a small fraction of highly immobile water of hydration and that extensive ordering of intracellular water is highly unlikely. Differences in relaxation times in normal and neoplastic tissues may have more to do with water content than water mobility (21, 22).

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