

Efficacy of MitoTracker GreenTM and CMXRosamine to Measure Changes in Mitochondrial Membrane Potentials in Living Cells and Tissues

W. Pendergrass,* N. Wolf, and M. Poot

Department of Pathology, University of Washington, Seattle, Washington

Received 24 June 2003; Revision Received 31 January 2004; Accepted 4 February 2004

Background: Chloromethyl-X-rosamine (CMXRos) and MitoTracker Green (MTG) have proved to be useful dyes with which to measure mitochondrial function. CMXRos is a lipophilic cationic fluorescent dye that is concentrated inside mitochondria by their negative mitochondrial membrane potential (MMP). MTG fluorescence has been used as a measure of mitochondrial mass independent of MMP. The fluorescence ratio of the two dyes is a relative measure of the MMP independent of mitochondrial mass. Because MTG was recently reported to be sensitive to MMP, we have reevaluated the effects of loss of MMP on MTG and CMXRos fluorescence, using both flow cytometry and laser scanning confocal microscopy (LSCM).

Methods: Using flow cytometry, the relative fluorescence of CMXRos, R123, and MTG was determined in human lymphoblastoid cell lines (LCLs) with or without carbonyl cyanide *p*-trifluoromethoxyphenyl-hydrazone (FCCP), used to collapse the MMP. LSCM analysis was also used to evaluate the effect of FCCP on MTG and CMXRos fluorescence of mouse cells and viable lenses in culture. The cytotoxicity of the dyes was determined using flow anal-

ysis of endogenous NADH fluorescence. The sensitivity of MTG fluorescence to H₂O₂ was also evaluated using flow cytometry.

Results: CMXRos fluorescence was dependent on MMP, whereas MTG fluorescence was not affected by MMP, using either flow or LSCM. Specific staining of mitochondria was seen with both dyes in all cell types tested, without evidence of cytotoxicity, as determined by NADH levels. H₂O₂ damage slightly increased MTG staining of cells.

Conclusions: Our results indicate that CMXRos is a non-toxic sensitive indicator of relative changes in MMP, whereas MTG is relatively insensitive to MMP and oxidative stress, using both flow and LSCM analyses, provided optimal staining conditions are used. In addition, these dyes can be useful for the study of mitochondrial morphology and function in whole tissues, using LSCM.

© 2004 Wiley-Liss, Inc.

Key terms: mitochondrial function; MTG; CMXRos

Mitochondrial functions such as mitochondrial membrane potential (MMP) can be studied in live cells, using vital fluorescent lipophilic cationic dyes. These dyes are concentrated inside the mitochondrion and show proportionality between fluorescence emission and the mitochondrial parameter of interest (1–7). Because chloromethyl-X-rosamine (CMXRos) fluorescence in the cell is increased by both increased MMP and increased mitochondrial mass density per unit area, a method of controlling for changes in mitochondrial mass is required to test for specific changes in MMP.

Two approaches have generally been followed: procedures that use ratiometric dyes, i.e., dyes that exhibit different emission spectra depending on their intracellular concentration, as well as MMP, such as JC-1 (5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolylcarbocyanine iodide) (3,8); or procedures that use a combination of two mitochondrial dyes, one sensitive to MMP, and a

second dye that is not sensitive to MMP, to control for mitochondrial mass.

Fluorescent dyes that specifically stain mitochondria independent of MMP may include MitoTracker Green FM (MTG), MitoFluor Green (MFG), and nonyl acridine orange (7). The notion that these dyes stain mitochondria independently of the mitochondrial membrane potential has been challenged recently (9,10). Specifically, GM130, HL-60, and U937 cells pretreated with ion-channel perturbing drugs, including valinomycin or nigericin, showed

Grant sponsor: NIH; Grant numbers: RO1-EY-11733, P30 EYO1730, 99-01, P01-AG01751-18, P30ES07033.

*Correspondence to: W.R. Pendergrass, Department of Pathology 357470, University of Washington Medical School, Seattle, WA 98195. E-mail: pendergr@u.washington.edu

Published online 26 August 2004 in Wiley InterScience (www.interscience.wiley.com).

DOI: 10.1002/cyto.a.20033

clearly increased levels of MTG and MFG fluorescence (9). Cultures of neurons and astrocytes stained with MTG and CMXRos showed increased fluorescence after treatment with carbonyl cyanide *p*-trifluoromethoxyphenyl-hydrazone (FCCP). In a second study, FCCP treatment did not affect MTG fluorescence in neurons, but it did lead to strongly diminished MTG fluorescence in astrocytes (10). Since MTG and MFG are carbocyanine dyes, their sensitivity to changes in potassium levels may be similar to that of the prototype carbocyanine dye, DiOC6(3) (11). If so, the increased fluorescence of MTG and MFG caused by such inhibitors may relate to perturbation of potassium homeostasis, rather than to alterations in their MMP. Also, MTG at very high (micromolar) concentrations has been reported to uncouple mitochondrial respiration (10). Pre-treatment of neurons and astrocytes with hydrogen peroxide before cell staining affects MTG fluorescence, whereas the addition of peroxide to stained cells has no effect on MTG fluorescence (10). These mixed observations warrant a careful reexamination of the conditions for the use of MTG to evaluate mitochondrial mass under conditions where the MMP may be altered.

We have reexamined CMXRosamine (CMXRos, MitoTracker Red™) as a candidate dye as a relative measure of MMP, and MTG as an MMP-independent indicator of mitochondrial mass (5,6,12,13). CMXRos exhibits good photostability and responds to changes in MMP. In addition, it is retained after cell fixation (5,6), although CMXRos staining of live cells has been reported to be more quantitative than staining of fixed cells (14). In contrast, Minamikawa et al. (15) showed that human osteosarcoma cells stained with CMXRos exhibited clear photosensitizing effects and a concomitant decrease in cell survival after irradiation with high doses of ultraviolet (UV) light ($>1.2 \text{ J/m}^2$). Although another dye, rhodamine 123, has proved quite valuable in flow cytometric studies on mitochondria (2,16), its high sensitivity toward illumination with a microscope lamp limited the use of this dye in applications involving microscopy (6). Another MitoTracker dye, chloromethyltetramethylrosamine (MitoTracker Orange™), appears to inhibit respiratory complex I and to induce the mitochondrial permeability transition (17). This dye toxicity precludes its use as a tool to measure changes in MMP (17).

The above-outlined observations clearly indicate the need for a reexamination of the properties of certain dyes used to measure mitochondrial membrane potential (MMP) and function. We have used flow cytometry and laser scanning confocal microscopy (LSCM) to monitor any fluorescence changes in MTG, CMXRos, and rhodamine 123-stained cells following treatment with the uncoupler of phosphorylation, FCCP, to collapse the MMP. The toxicity of these dyes was also determined to be minimal using flow cytometry analysis of endogenous NADH levels in stained lymphoblastoid cell lines (LCLs). LSCM was similarly used to examine specific staining of mitochondria and the effects of MMP loss on MTG and CMXRos staining of mouse cells (both lens epithelial cells and tail fibroblasts), as well as whole viable mouse lenses.

We found CMXRos staining of mitochondria to be quite sensitive to MMP, while MTG staining was relatively unaffected by changes in MMP in all the cells and tissues studied. However, when using LSCM, removal of unbound MTG from the media was necessary prior to LSCM analysis to avoid increased MTG fluorescence in FCCP-treated tissue culture cells.

MATERIALS AND METHODS

Drugs and Dyes

CMXRosamine (CMXRos, MitoTracker Red™), rhodamine 123 (R123), Hoechst 33342, and MitoTracker Green FM™ (MTG), were obtained from Molecular Probes (Eugene, OR), dissolved in dimethylsulfoxide (DMSO) (200 μM stock concentration), and stored at -20°C in the dark. All other chemicals (hydrogen peroxide) and FCCP were from Sigma (St. Louis, MO). Except for hydrogen peroxide, all drugs were diluted into phosphate-buffered saline (PBS) and stored at -20°C in the dark.

Flow Cytometry

For each sample, 40,000 particles were analyzed with a Coulter Epics Elite Flow Cytometer (Coulter, Hialeah, FL) equipped with 10-mW UV excitation and 15-mW, 488-nm excitation. The following fluorescence emissions were collected: UV excited-blue autofluorescence (NADH) with a 450/35-nm bandpass filter; 488-nm excited MTG with a 525/40-nm bandpass filter; and 488-nm excited CMXRos fluorescence with a 645-nm longpass filter. The data were analyzed using MultiPlus software (Phoenix Flow Systems, San Diego, CA). To determine cellular NADH levels, UV-excited blue autofluorescence (emission maximum at 450 nm) was measured according to Thorell (18). Cells for NADH analysis were washed once in PBS, cooled to 0°C , and analyzed immediately. A negative control was prepared by fixing a sample of LCLs with ice-cold 70% ethanol, followed by a single wash with PBS.

LCL Cell Staining for Flow Cytometry

Human LCLs were obtained by Epstein-Barr virus (EBV) transformation of a peripheral blood sample from a healthy individual, cultured in RPMI-1640 medium supplemented with 16% fetal bovine serum (FBS) and 2 mM L-glutamine and passaged by dilution in fresh cell culture medium as soon as cell density has reached 0.5×10^6 cells per ml. Cells from logarithmically growing cultures were collected by centrifugation and resuspended at a cell concentration of 1×10^6 per ml in fresh RPMI-1640 medium. Drugs were added at the indicated concentrations, and cells were incubated at 37°C for 30 min. Thereupon, 200 nM of MTG and CMXRos, or 1 μM R123 were added, and the cells were stained for 30 min at 37°C in the dark. Immediately after staining cell suspensions were placed in a melting ice bath, and analyzed by flow cytometry (see below).

Preparation of Viable Mouse Lenses for Analysis With LSCM

Female (C57Bl/6 $\times$ C3H)F1 mice of various ages were used for all studies. The mice were obtained from the National Institute of Aging colony (NIA, Bethesda, MD), and maintained under specific pathogen free conditions until sacrifice. The mice were sacrificed and lenses removed as described by Pendergrass et al. (19). The lenses were washed in Dulbecco's minimal essential medium (DMEM) medium containing 25 mM HEPES and 10% FBS to avoid changes in pH during manipulation, cell isolation and staining.

Primary Mouse Lens Epithelial Cells and Tail Fibroblast Cells for LSCM Analysis

Following lens extraction, the basement membrane, which encapsulates the lens and onto which the lens epithelial cells (LECs) are attached, was removed from the crystalline component. Lens epithelial cells were dissociated from lens membranes with a solution of 0.1% collagenase/dispase (Roche, Indianapolis, IN) in PBS containing 20% serum (for 45 min at 37°C with agitation). The enzymes were inactivated by washing with Versene buffer (Gibco, Grand Island, NY) containing 0.1% EDTA, supplemented with 10% FBS buffer for 5 min. The cell suspension was centrifuged and the cells were plated into 35-mm collagen IV-coated dishes (Becton Dickinson, Bedford, MA) in 16% FBS in DMEM for 5 days in a 37°C incubator with 7% CO₂ and 20% O₂ prior to being stained and analyzed.

For preparation of tail fibroblasts from sacrificed mice, the skin was stripped from each tail sample, minced finely with a scalpel in a square plastic petri dish, while submerged in a solution of 3% bovine serum albumin containing 1 mg/ml (digestion solution) collagenase/dispase (Roche Diagnostics, IN). The minced tail skin from each sample was placed in a 15 ml tube containing 10 ml of digestion solution and warmed at 37°C for 30 min. Each tube was then vortexed and placed on a rotary mixer for an additional 30 min; 5 ml of 0.05% trypsin (Invitrogen, Grand Island, NY) was then added to the digestion solution. This was followed by incubation at 37°C for an additional 15 min. The tubes were then spun down, the supernatant decanted, and 10 ml of Versene added (1:5,000) (Invitrogen) for 10 min, to neutralize the collagenase/dispase. Samples were then spun down, the Versene decanted, followed by the addition of 2 ml of culture medium (DMEM + 16% fetal calf serum [FCS]), and the cells plated into Corning (Corning, NY) 25 cm² tissue culture flasks with DMEM media and 16% FBS for 1 week. The cells were then transferred to 8-well Lab-Tek (Nalge Nunc International, Naperville, IL) chambered glass coverslips with DMEM media and 16% FBS for an additional 3–4 days as above for the LECs. The cells were then stained and analyzed by LSCM.

Staining of Cultured Mouse Cells and Lenses With CMXRos and MTG for LSCM

The cultured mouse cells (either mouse LECs or mouse tail fibroblasts) or whole lenses were resuspended in Dulbecco's modified Eagle's medium supplemented with 10% FBS, 25 mM HEPES media, and stained with 40 nM MTG and 40 nM CMXRos for 60 min. at 37°C. In some experiments, the cells were pretreated for 60 min with FCCP and stains added to the FCCP containing solution for an additional 60 min at 37°C. The unbound dyes were then removed with three washes of the cultures with fresh DMEM with 10% FBS at 37°C. The fluorescence of the bound dyes was analyzed using a Bio-Rad MRC-1024 LSCM as described below. The fluorescence of the cells was stable for ≥ 3 h on ice after removal of unbound stain, and remained stable for ≥ 30 min after warming to room temperature for analysis.

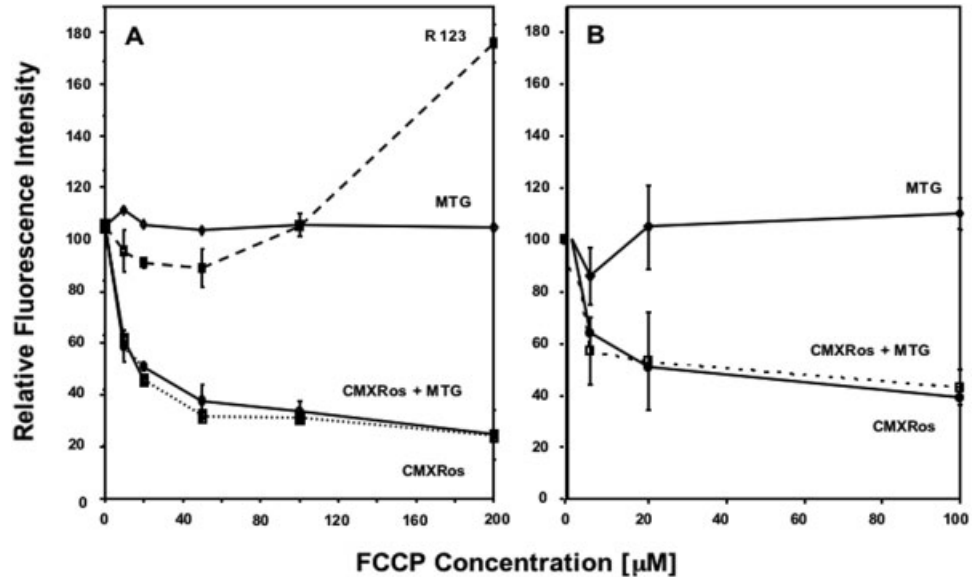
LSCM Analysis of Tissue Culture Cells

For analysis of tissue culture cells, a Bio-Rad MRC-1024 (Hercules, CA) laser scanning confocal microscope package was used. This package included a computer assisted Zeiss inverted microscope and a 15-mW krypton/argon laser (ILT laser, Aachen, Germany) emitting three excitation lines at 488, 568, and 647 nm. The 488-nm line was used to excite MTG. The emitted green fluorescence was filtered through a 522-nm DF 32 bandpass filter. CMXRos was excited by the 568-nm line of the same krypton/argon laser, and the emitted fluorescence filtered through a 605-DF 32 bandpass filter. The power setting for the laser was never used at $>10\%$, so that the maximum power of each band was 0.3 mW. The z -step was set at 0.75 μ m, and the laser pinholes, and gains, were adjusted into the mid-range of the sensitivity of the photomultiplier. Repeated analyses on the same field of cells gave identical results without significant bleaching or alteration in fluorescence intensities. The background fluorescence of the medium between cells was subtracted from each measurement. Samples within an experiment were all analyzed using the same settings and differences compared using a paired Student's *t*-test. Lasersharp 2.3 acquisition software was used for acquisition and retrieval of the confocal images, which were stored and analyzed using a Power Macintosh computer with NIH Image1.62 software (National Institutes of Health, Bethesda, MD).

LSCM Analysis of Whole Lenses

A 2-photon Zeiss LSM 510 laser scanning microscope was used for whole lens analyses so that the DNA fluorochrome Hoechst 33342 (20 μ M) could be co-visualized concurrently with the other dyes. When using this instrument, the MTG stain was analyzed using the 488 line of an argon laser (Lasertechnik, GmbH, 5 mW, run at 3–10% of full power). Emitted light was selected with a primary dichroic that blocked the 488-nm exciting light, a secondary optic that passed light <635 nm, a tertiary optic that passed light longer than 545 nm, and a bandpass filter selecting light of 565–615 nm.

FIG. 1. **A:** Flow cytometry analysis of the effect of FCCP treatment on staining of lymphoblastoid cell lines (LCLs) with 200 nM chloromethyl-X-rosamine (CMXRos) + 200 nM Mito-Tracker Green (MTG) (solid lines), 1 μ M R123 alone (dashed line), or 200 nM CMXRos alone (dotted line). **B:** Similar analysis done using laser scanning confocal microscopy (LSCM) analysis of mouse LECs stained with: 40 nM (CMXRos + 40 nM MTG), (solid lines) or 40 nM CMXRos alone $\pm$ FCCP. Details of staining methodology and analysis by flow cytometry or LSCM are presented in Materials and Methods. All points represent mean values and standard errors of a typical experiment performed in triplicate out of a series of three independent experiments. For further explanation, see text.



CMXRos illumination was with the 543 band of a helium/neon laser (Lasertechnik, Gmgh, 1 mW, run at 3–10% power). Emitted light was selected with a primary dichroic that blocks 543-nm exciting light, a secondary optic that passes light <635 nm, a tertiary optic that passes light >545 nm, and a bandpass filter that passes light 565–615 nm.

For Hoechst 33342 a 2-photon laser (a Mira Titanium Sapphire IR femtosecond (fs) pulse laser 50 mW from Coherent, Santa Clara, CA) was tuned to 758 nm. Emitted light was passed through a primary dichroic mirror passing light <650 nm, a secondary optic passing light <490 nm, and a bandpass filter passing light 435–485 nm. Only one fluorochrome was excited at a time per frame scan to ensure that only the desired probe was visualized.

RESULTS

Using flow cytometry, we determined the effects of the uncoupler of phosphorylation, FCCP, on the fluorescence of human LCL cells stained with CMXRos, MTG, or R123. As shown in Figure 1A, green MTG fluorescence emission did not change significantly in response to pretreatment with FCCP, whereas the red fluorescence emission of CMXRos, used alone or in combination with MTG, decreased markedly in response to increasing FCCP concentrations. R123, also used to follow MMP changes, showed only a modest decrease in fluorescence at low FCCP concentrations, and a contrasting increase in fluorescence at high FCCP concentrations indicating that it was not a good indicator for MMP changes. Vander Heijden et al. (16) made a similar observation, which they attributed to mitochondrial damage secondary to lowered MMP.

In Figure 1B, we used LSCM to examine mouse LECs stained with CMXRos and MTG, $\pm$ FCCP (for details, see Materials and Methods). A large and progressive loss in CMXRos fluorescence occurred with increasing FCCP

concentration, but no significant change in MTG staining was seen at even 100 μ M FCCP (Fig. 1B). A nearly identical result was obtained using mouse-tail fibroblasts instead of mouse LECs (not shown). In conducting LSCM studies, we found it necessary to wash out unbound MTG and CMXRos dyes from the cells prior to LSCM analysis. In unwashed cultures of stained mouse LECs or fibroblasts, the MTG fluorescence increased with FCCP concentration (data not shown). This wash step was not necessary using flow cytometry, probably because the unbound dyes were diluted over a 1,000-fold by the sheath fluid.

A typical LSCM image of mouse-tail fibroblasts stained with CMXRos and MTG is shown in Figure 2A. As expected, the nuclei were devoid of mitochondrial staining (seen as blank oval spaces within the stained cells). Individual mitochondria stained with MTG and CMXRos can clearly be seen. Cells treated with 40 μ M FCCP (Fig. 2B) demonstrated a dramatic loss of CMXRos staining without loss of MTG staining. Some retraction and rounding of fibroblasts can be seen in the FCCP-treated cells in Figure 2B. This was typical for cell cultures treated with higher doses (>10 μ M) of FCCP and probably represents secondary damage to the cells.

LSCM also provided an opportunity to examine mitochondrial function in LECs in whole viable mouse lenses in situ, as well as cells in culture. Figure 3A depicts a typical low-power (20 $\times$ objective) LSCM reconstruction of 13 confocal frames (each 1.5 μ m) from the apical portion of the central region of a viable mouse lens. This reconstruction displays the differential fluorescence of MTG, CMXRos, and Hoechst 33342 (specific for DNA) stained LECs on the surface of this part (front side) of the intact mouse lens. Although this reconstruction goes to a depth of 19.5 μ m into the lens, only the LECs on the surface of this portion of the lens are visible since they are the only cells in this part of the lens that contain nuclei or

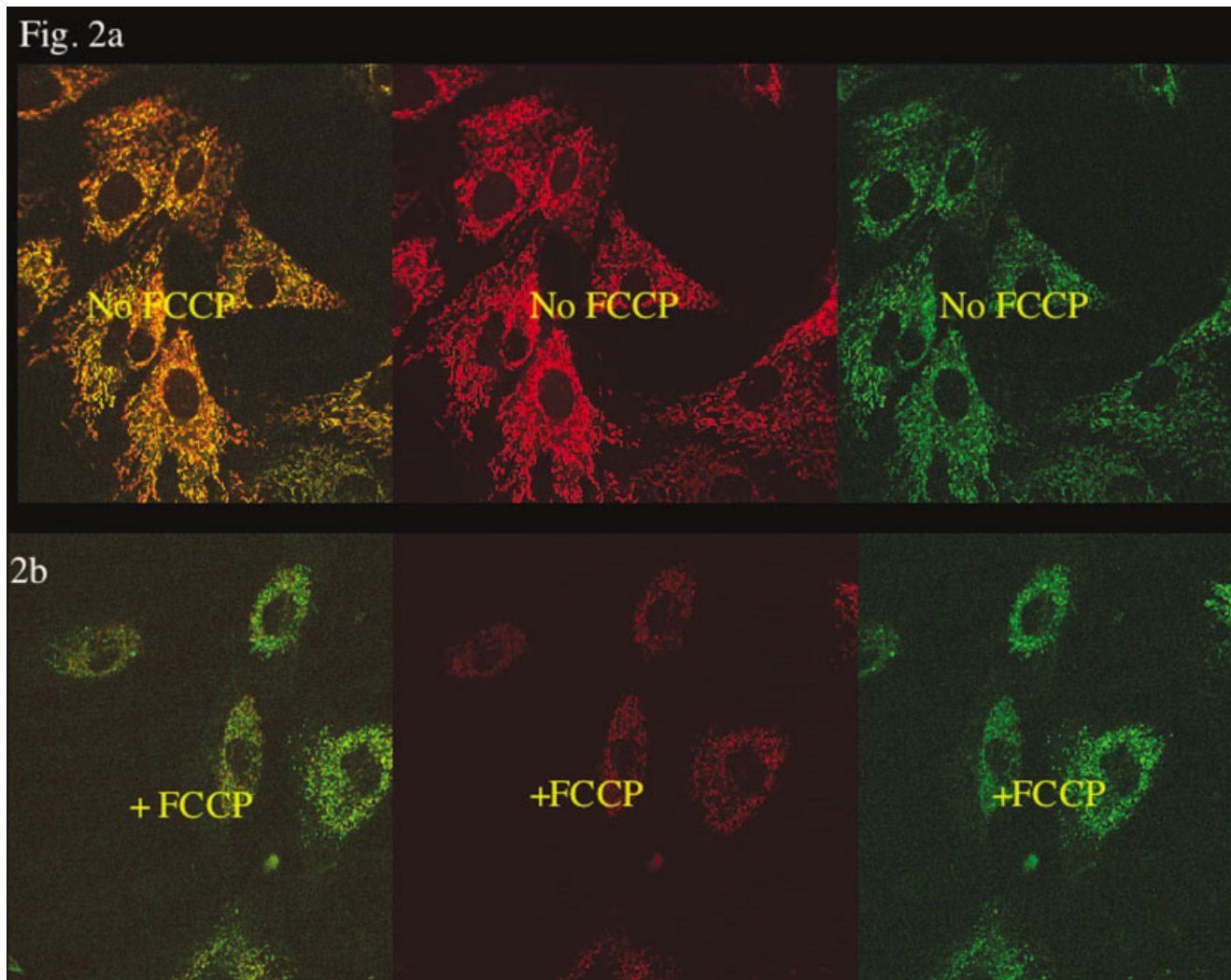


FIG. 2. Single laser scanning confocal microscopy (LSCM) images (60 $\times$ objective lens) of mouse tail fibroblasts stained with chloromethyl-X-rosamine (CMXRos) and MitoTracker Green (MTG). CMXRos is depicted as red and MTG as green. Dark spaces are nuclei in the cells. **A:** Untreated control cells. **B:** Cells pretreated with 40 μ M FCCP for 60 min at 37°C. Note specific mitochondrial staining with CMXRos and MTG, and loss of CMXRos fluorescence in cells treated with FCCP.

significantly stained mitochondria. The differentiated lens fiber cells lying below this part of the surface lack nuclei and have few mitochondria.

Figure 3B is a typical LSCM reconstruction of a similar mouse lens treated with 50 μ M FCCP for 60 min prior to staining with MTG and CMXRos. As with the cultured cells, the CMXRos staining was almost completely suppressed by pretreatment with the FCCP without altering MTG or Hoechst 33342 staining. Figure 3C is a high-power (40 $\times$ objective) single LSCM image of the same region of a control lens stained as in Figure 3A. Interestingly, Hoechst 33342 staining is also seen to co-localize with the MTG and CMXRos staining of the mitochondria in Figure 3C. The image has been enlarged electronically to demonstrate that the MTG and CMXRos stains are co-localized over the same mitochondrial structures. DNase I treatment of paraformaldehyde-fixed cells abolished the mito-

chondrial Hoechst staining, but not CMXRos or MTG staining (results not shown). This finding suggests that the Hoechst staining of mitochondria is due to staining of mitochondrial DNA.

It is conceivable that cell staining with CMXRos and MTG, as reported for CMTMR by Scorrano et al. (17), may induce mitochondrial toxicity. To investigate potential mitochondrial toxicity we monitored mitochondrial NADH levels in LCL cells by measuring UV-excited cellular blue autofluorescence (18). This was done using flow cytometry after cells were stained with JC-1, MTG, and CMXRos. As shown in Table 1, LCLs stained with MTG, CMXRos, or with both MTG and CMXRos, showed no significant decrease in NADH level, while JC-1 stained LCLs exhibited a slightly diminished NADH level. These data indicate that, at least in the absence of deliberate high-intensity illumination, as used by Minamikawa et al.

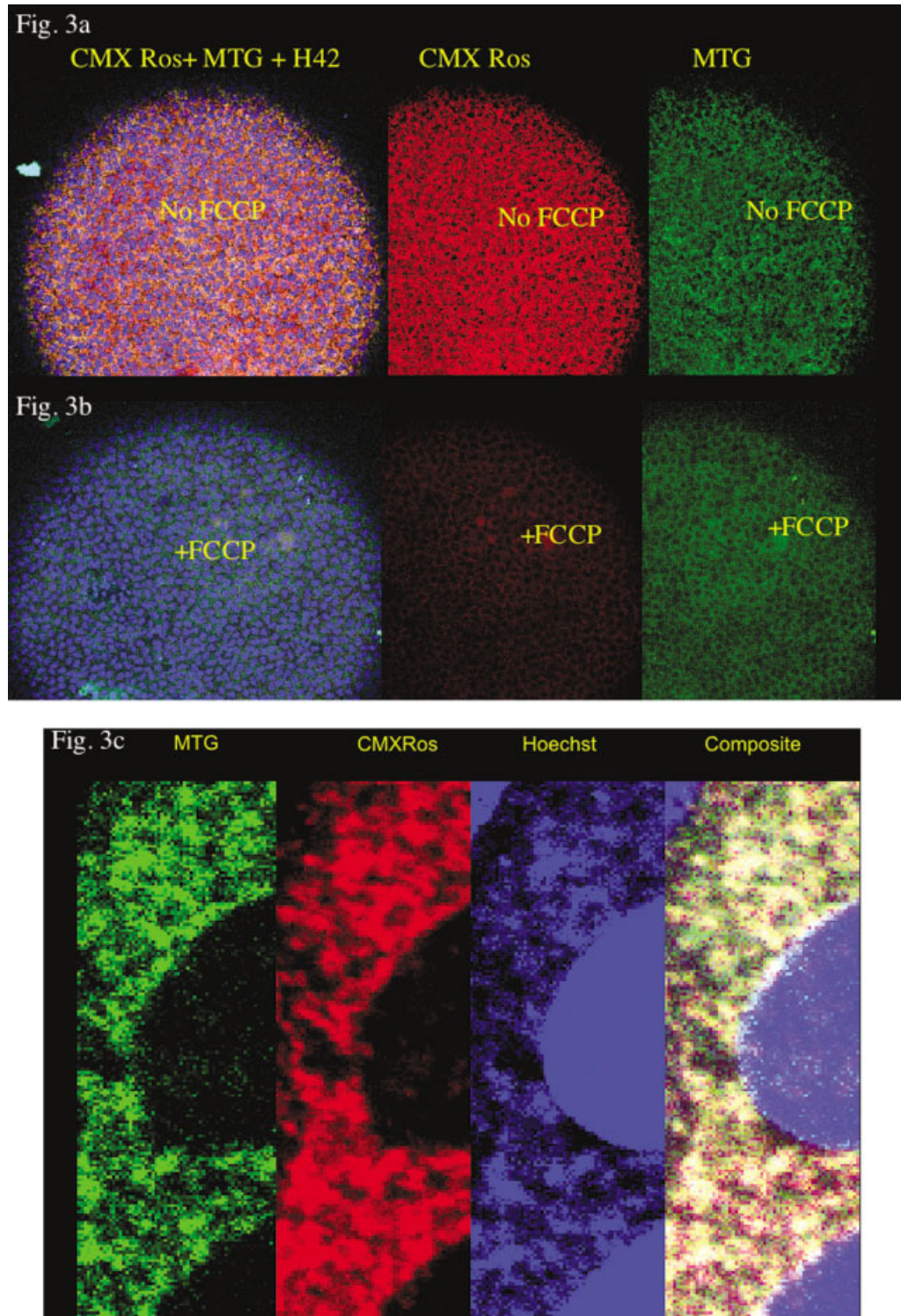


FIG. 3. **A:** Low-power (20 $\times$ objective) laser scanning confocal microscopy (LSCM) reconstruction of 13 individual frames (each frame was 1.5 μ m deeper than the last) of a freshly isolated whole viable mouse lens stained with 40 nM MitoTracker Green (MTG), chloromethyl-X-rosamine (CMXRos), and 10 μ M Hoechst 33342 (blue). These lens images (A–C) were prepared using a Zeiss 2 photon LSCM microscope to permit visualization of Hoechst 33342 stained DNA. Only the LECs on the surface of the apical portion of the Central Region of the lens are visible because the underlying lens fiber cells lack nuclei and mitochondria. **B:** Lenses preincubated with 50 μ M FCCP for 60 min prior to staining with CMXRos and MTG. Note loss of CMXRos, but not of MitoTracker Green (MTG) or Hoechst 33342 staining. **C:** Closeup of CMXRos, MTG, and Hoechst 33342 stained whole lens using 40 $\times$ objective with further electronic magnification. Note staining of mitochondrial structures with all three stains, including the DNA stain.

Table 1
Cytotoxicity in LCLs Treated With Mitochondrial Dyes From NADH levels

Dye	Relative NADH ^a levels as % untreated cells (SD)
JC-1 (10 μ M)	88.5 (7.3)
MTG (0.2 μ M)	96.4 (10.4)
CMXRos (0.2 μ M)	98.8 (10.8)
(MTG + CMXRos) (0.2 μ M)	94.5 (7.1)

LCL, lymphoblastoid cell line; MTG, MitoTracker Green; CMXRos, chloromethyl-X-Rosamine.

^aDetermined from UV-excited blue autofluorescence of treated LCLs as described in Materials and Methods.

(15), MTG and CMXRos did not induce an acutely toxic effect in LCLs.

Buckman et al. (10) demonstrated that hydrogen peroxide pretreatment affected MTG fluorescence in cultured neurons and astrocytes. MTG contains two chloromethyl residues and two chloride atoms attached to its benzimidazole moiety. Therefore, it is conceivable that exposure of MTG to a strong oxidant, such as hydrogen peroxide, may lead to removal of the chloride atoms and thus changes in the fluorescence properties of the dye. To investigate whether a potential chemical oxidation of MTG would lead to changes in its fluorescence intensity, we incubated MTG with a series of hydrogen peroxide concentrations in PBS and subsequently measured green fluorescence emission. As shown in Figure 4A, incubation of free MTG with a series of hydrogen peroxide concentrations led to a slight decrease in MTG fluorescence, which reached a plateau of >200 μ M hydrogen peroxide. This small effect is the opposite of our expectation of increasing MTG fluorescence with peroxide treatment of the dye. Since perturbation of mitochondrial function during apoptosis

and cellular senescence may lead to oxidative stress, we also tested whether MTG fluorescence was affected by pretreatment of the cells with H_2O_2 prior to staining with MTG (Fig. 4B). As shown in Figure 4B, MTG fluorescence was increased slightly by hydrogen peroxide treatment at >100 μ M hydrogen peroxide. Concomitant with this the LCL cells exhibited a clear decrease in viability by NADH fluorescence (Fig. 4C). These results indicate that hydrogen peroxide-induced oxidative stress and/or toxicity increases MTG staining slightly, probably secondary to cell toxicity.

DISCUSSION

Recently published observations (9,10,15) called into question the premises for use of certain fluorescent mitochondrial dyes (including CMXRos and MTG) for flow cytometric and confocal microscopy studies of mitochondrial structure and function. Therefore, we reexamined the usefulness of CMXRos and MTG to monitor changes in the MMP, as well as cellular content and morphology, of mitochondria. In both flow cytometric experiments with human LCLs and LSCM analyses of mouse LECs and fibroblasts, we found MTG staining of mitochondria to be unaffected by lowered mitochondrial membrane potential, whereas, CMXRos fluorescence clearly diminished. A similar phenomenon was seen in whole viable lenses.

We have noted that unless tissue culture cells were washed free of unbound MTG prior to LSCM analysis, damaged cells and FCCP treated cells demonstrated an increased MTG fluorescence. This was not necessary using flow cytometry, probably because the dyes were diluted over 1,000 times by the sheath fluid prior to laser illumination during flow analysis. Whole lenses did not demonstrate increased MTG fluorescence following FCCP even without washing out unbound MTG. Possibly mito-

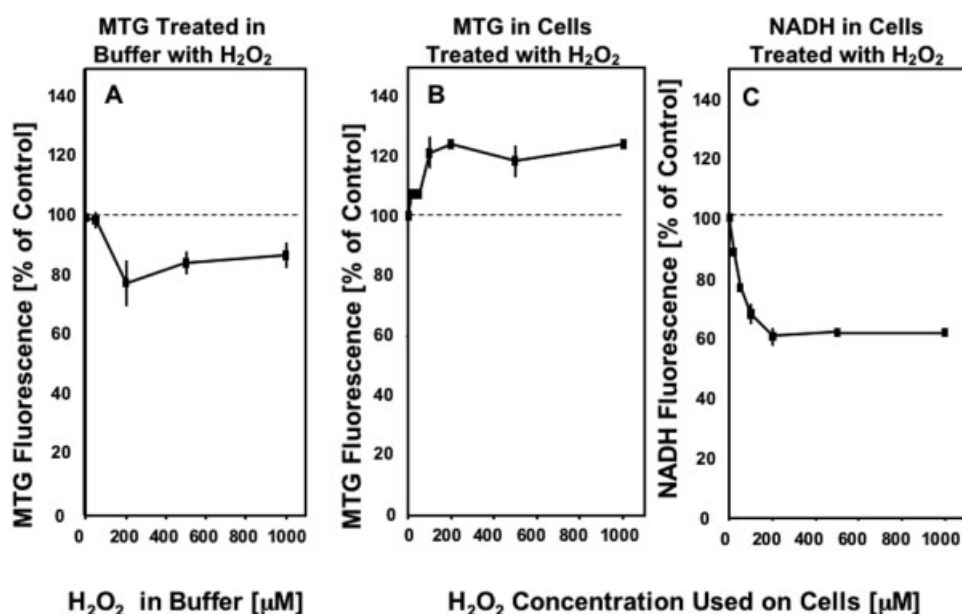


FIG. 4. A: Sensitivity of MTG fluorescence to H_2O_2 , while in solution in phosphate-buffered saline (PBS), as assayed with a fluorescent plate reader. B: MTG fluorescence in lymphoblastoid cell lines (LCLs) pretreated with hydrogen peroxide, and subsequently stained with MTG, and analyzed by flow cytometry. C: NADH levels of the LCL cells treated with H_2O_2 and analyzed by flow cytometry. All points represent mean values and standard errors of a typical experiment performed in triplicate out of a series of three independent experiments. For further explanation, see text.

chondria in the whole lenses were more resistant to secondary damage by FCCP than cells in culture. Following trypsinization, a few cells were always damaged, and remained partially rounded, such cells always demonstrated increased MTG fluorescence when the unbound dye was not removed. Such cellular damage either by drugs or other stresses may create or expose low-affinity binding sites for MTG that are not observed at low MTG concentrations present after the washing procedure.

Our results with FCCP pretreated human LCLs and mouse LECs are somewhat in contrast with those reported by Keij et al. (9). A major difference between the two studies is the kind of inhibitory drugs used; valinomycin and nigericin are known to affect cellular cation homeostasis. MTG is part of the family of carbocyanine dyes, some of which were shown to be sensitive to changes in cellular K^+ levels (11). Therefore, it is conceivable that the changes in MTG fluorescence found by Keij and co-workers primarily relate to drug effects on cellular cation levels. In addition, Keij et al. (9) used different cell types (GM130, HL-60, and U937) than we did. In our hands, HL-60 cells, which contain a very narrow rim of cytoplasm, and stain poorly with MTG and CMXRos, gave erratic results (not shown). Comparison of our data with those from Keij and coworkers should therefore caution against transposition of results from one cell type to another.

In experiments with R123 staining we found that low levels of FCCP pretreatment gave rise to a decrease in R123 fluorescence, and the reverse at higher FCCP levels. This result is in agreement with what has been reported by Vander Heiden et al. (16), and indicates that R123 is not reliably sensitive to changes in MMP.

MTG fluorescence of stained LCL cells did not change significantly in response to hydrogen peroxide at concentrations less than 100 μ M, but did increase approximately 20% at higher peroxide concentrations. We did not observe a loss of MMP or mitochondrial swelling during illumination of CMXRos stained cells with intensities or wavelengths used. This contrasts with the results reported by Minamikawa et al. (15) with stained cells exposed to strong UV light. Neither did we observe photobleaching of cells stained under these conditions. This result is consistent with the findings of Poot et al. (6). In summary, our data indicate that MTG fluorescence is relatively unresponsive to changes in MMP, while CMXRos staining is quite sensitive to changes in MMP provided unbound MTG stain is removed prior to LSCM. This means that, except for experimental situations in which higher levels of oxidative stress or serious cell damage can be suspected, the ratio of CMXRos/MTG fluorescence may very well reflect MMP independent of mitochondrial mass changes. We also observed that the Hoechst 33342 staining of mitochondria was sensitive to DNase 1, consistent with Hoechst visualization of mitochondrial DNA. This technique may prove useful for the study of the effects of aging, degenerative disease and genetic manipulation on mitochondrial function.

Although the results reported herein have improved our confidence in the use of CMXRos and MTG fluorescent

dyes for the study of mitochondrial morphology and function we would like to caution future investigators to not only determine whether the theoretical considerations regarding the use of fluorescent dyes in general (13) apply, but also to perform experiments, as detailed in the literature (9,10,15) and in this report, to determine whether the observations made, relate to the experimental system and not to untoward dye effects.

LITERATURE CITED

1. Johnson LV, Walsh ML, Chen LB. Localization of mitochondria in living cells with rhodamine 123. *Proc Natl Acad Sci USA* 1980;77:990-994.
2. Darzynkiewicz Z, Staiano-Coico L, Melamed MR. Increased mitochondrial uptake of rhodamine 123 during lymphocyte stimulation. *Proc Natl Acad Sci USA* 1981;78:2383-2387.
3. Smiley ST, Reers M, Mottola-Hartshorn C, Lin M, Chen A, Smith TW, Steele GD Jr, Chen LB. Intracellular heterogeneity in mitochondrial membrane potentials revealed by a J-aggregate-forming lipophilic cation JC-1. *Proc Natl Acad Sci USA* 1991;88:3671-3675.
4. Backway KL, McCulloch EA, Chow S, Hedley DW. Relationships between the mitochondrial permeability transition and oxidative stress during ara-C toxicity. *Cancer Res* 1997;57:2446-2451.
5. Macho A, Decaudin D, Castedo M, Hirsch T, Susin SA, Zamzami N, Kroemer G. Chloromethyl-X-Rosamine is an aldehyde-fixable potential-sensitive fluorochrome for the detection of early apoptosis. *Cytometry* 1996;25:333-340.
6. Poot M, Zhang YZ, Krämer JA, Wells KS, Jones L, Hanzel DK, Lugade AG, Singer VL, Haugland RP. Analysis of mitochondrial morphology and function with novel fixable fluorescent stains. *J Histochem Cytochem* 1996;44:1363-1372.
7. Poot M, Pierce RH. Detection of changes in mitochondrial function during apoptosis by simultaneous staining with multiple fluorescent dyes and correlated multiparameter flow cytometry. *Cytometry* 1999;38:311-317.
8. Cossarizza A, Kalashnikova G, Grassilli E, Chiappelli F, Salvio S, Capri M, Barbieri D, Troiano L, Monti D, Franceschi C. Mitochondrial modifications during rat thymocyte apoptosis: a study at the single cell level. *Exp Cell Res* 1994;214:323-330.
9. Keij JF, Bell-Prince C, Steinkamp JA. Staining of mitochondrial membranes with 10-nonyl acridine orange, MitoFluor Green, and MitoTracker Green is affected by mitochondrial membrane potential altering drugs. *Cytometry* 2000;39:203-210.
10. Buckman JF, Hernandez H, Kress GJ, Votyakova TV, Pal S, Reynolds JJ. MitoTracker labeling in primary neuronal and astrocytic cultures: influence of mitochondrial membrane potential and oxidants. *J Neurosci Methods* 2001;104:165-176.
11. Salvio S, Ardizzone A, Franceschi C, Cossarizza A. JC-1, but not DiOC6(3) or rhodamine 123, is a reliable fluorescent probe to assess $\Delta\psi$ changes in intact cells: implications for studies on mitochondrial functionality during apoptosis. *FEBS Lett* 1997;411:77-82.
12. Bossy-Wetzel E, Newmeyer DD, Green DR. Mitochondrial cytochrome c release in apoptosis occurs upstream of DEVD-specific caspase activation and independently of mitochondrial transmembrane depolarization. *EMBO J* 1998;17:37-49.
13. Poot M, Pierce RH. Detection of apoptosis and changes in mitochondrial membrane potential with chloromethyl-X-rosamine. *Cytometry* 1999;36:359-360.
14. Gilmore K, Wilson M. The use of chloromethyl-X-rosamine (MitoTracker red) to measure loss of mitochondrial membrane potential in apoptotic cells is incompatible with cell fixation. *Cytometry* 1999;36:355-358.
15. Minamikawa T, Sriratan A, Williams DA, Bowser DN, Hill JS, Nagley P. Chloromethyl-X-rosamine (MitoTracker Red) photosensitizes mitochondria and induces apoptosis in intact human cells. *J Cell Sci* 1999;112:2419-2430.
16. Vander Heiden MG, Chandel NS, Williamson EK, Schumacker PT, Thompson CB. Bcl-xL regulates the membrane potential and volume homeostasis of mitochondria. *Cell* 1997;91:627-637.
17. Scorrano L, Petronilli V, Colonna R, Di Lisa F, Bernardi P. Chloromethyltetramethylrosamine (MitoTracker Orange) induces the mitochondrial permeability transition and inhibits respiratory complex I. Implications for the mechanism of cytochrome c release. *J Biol Chem* 1999;274:24657-24663.
18. Thorell B. Flow-cytometric monitoring of intracellular flavins simultaneously with NAD(P)H levels. *Cytometry* 1983;4:61-65.
19. Pendergrass WR, Penn PE, Li J, Wolf NS. Age-related telomere shortening occurs in lens epithelium from old rats and is slowed by caloric restriction. *Exp Eye Res* 2001;73:221-228.