New generation of fluorescent probes for cell-based measurements of caspase activation and mitochondrial superoxide

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Abstract and introduction

Cell health and stress readouts are critical indicators of altered or impaired function in normal and diseased states of cells, and work has been underway to develop improved small molecule sensor dyes compatible with traditional imaging and High Content Analysis (HCA) interrogation of apoptotic and mitochondrial stress pathways. The CellEvent™ Caspase Green dye effectively reports caspase activation, but suffers complications in assay configuration when attempting to multiplex with the Green Fluorescent Protein (GFP), calcein, or other 488 laser line tools in fluorescence microscopy. Here, we describe the testing and functional characterization of a new candidate molecule for measuring apoptosis in living cells. Our sensor is comprised of a fluorogenic reporter dye that is liberated from a DEVD peptide substrate by caspase activation, but operates in the Texas Red, 590nm excitation band, with an emission peak near 610 nm, permitting easy multiplex with GFP or calcein stained neurons in both traditional and HCA microscopy configurations. Similarly, mitochondrial superoxide accompanying cell stress is probed in microscopy with the MitoSOX™ Red Mitochondrial Superoxide Indicator dye, which localizes to mitochondria and reports superoxide generation, ignoring other Reactive Oxygen Species (ROS) and Reactive Nitrogen Species (RNS). This dye has an unusually long Stokes' shift, requiring specialized microscopy and HCA filters that excite at 405nm, and capture emission at 610nm for specific superoxide detection. This unconventional spectroscopic profile prevents the dye's use on many imaging platforms and promotes phototoxicity. To this end, our team has produced a dye with the same level of specificity for superoxide that will operate in one of the traditional fluorescence microscopy channels. Our candidate dye, here named MitoSOX™ Green Mitochondrial Superoxide Indicator also localizes to mitochondria of live cells and selectively reports superoxide generation, while ignoring other ROS and RNS species in ex vivo testing. With an Excitation/Emission profile in the GFP/FITC microscopy channel, a series of comparative studies in immortalized and neural cells are shown, highlighting photostability, specificity and signal amplitude from the dye. These reagents are research use only, not for diagnostic purposes

Materials and methods

Induction of Caspase 3/7 and mitochondrial superoxide in cells

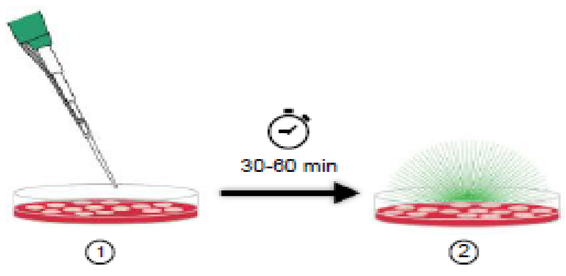
U-2 OS, A673 or primary hippocampal neurons were cultured in standard conditions and apoptosis was induced with either 1μM Staurosporine or 2 μM Camptothecin for the indicated time before dye was added to cells. Mitochondrial superoxide production was induced in cells by the addition of 30 μM MitoPQ overnight in low glucose cell culture medium.

Fluorescence analysis and imaging

Confocal fluorescence imaging was performed with a Zeiss LSM980 laser scanning microscope, and widefield fluorescence imaging was performed on an EVOS™ M7000 Imaging System. In separate studies, High Content Analysis (HCA) quantification was carried out on the CellInsight™ CX-5 High Content Screening platform. In vitro fluorescent response of MitoSOX™ green dye was measured on TECAN (excitation: 460 nm; emission scan: 490-600 nm). RFUs were calculated by summing wavelengths from 490-600 nm then subtracting the background (control).

Dye preparation and loading

CellEvent™ Caspase Sensor Dyes and MitoSOX™ Green dyes were prepared according to their product sheets and added to cells 30 to 60 minutes before detection. CellEvent™ Caspase 3/7 dyes may be imaged directly on cells in complete media without wash, while MitoSOX™ dyes are recommended to be removed and washed with buffer prior to image acquisition.



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Results

Induction of Caspase 3/7 and imaging neural cultures with CellEvent™ Red and Green

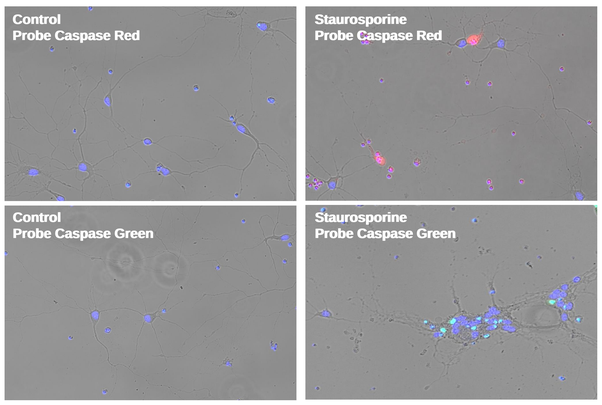

Figure 1. Neonatal rat hippocampal neurons were cultured four days on Poly D Lysine coated glass and treated with 0.1% DMSO carrier (Control) or 1 uM Staurosporine for three hours before staining with Hoechst nuclear dye (above, in blue) and CellEvent™ Red (top panels, red pseudocolor) or Green (bottom panels, green pseudocolor) and imaging on the EVOS™ M7000.

GFP Multiplex Imaging with CellEvent™ Red

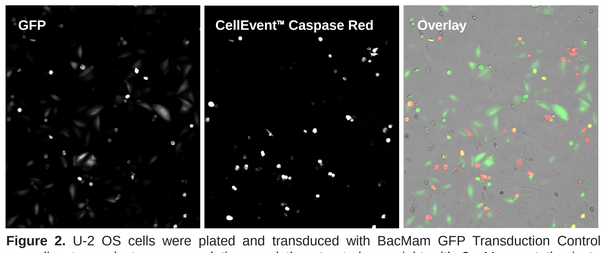

Figure 2. U-2 OS cells were plated and transduced with BacMam GFP Transduction Control according to product recommendations and then treated overnight with 2 uM camptothecin to induce apoptosis. CellEvent™ Caspase Red dye was prepared as directed and added to the cultures for 60 minutes in the cell culture incubator before imaging on the EVOS™ M7000.

Signal Specificity of CellEvent™ Caspase 3/7 Red and Green Dyes from Powder formulation

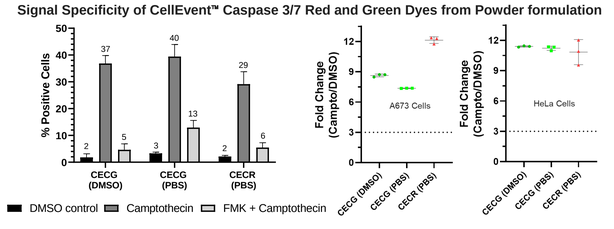

Figure 3. Camptothecin induction of apoptosis on A673 cells (left and middle panels) and HeLa cells (right panel) was measured in control or in FMK Caspase 3/7 inhibitor conditions and images captured for HCA quantification of percent positive and fold increase on the CellInsight™ CX-5 platform.

Mitochondrial Localization of MitoSOX™ Green in Live Cells

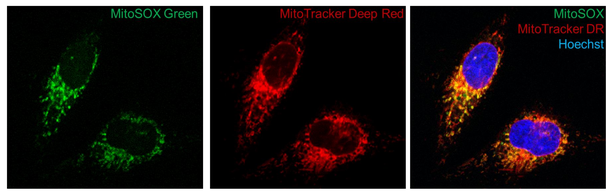

Figure 4. To test the spatial specificity of MitoSOX™ Green staining, MitoTracker Deep Red was co-stained on live cells, showing a clear co-localization of MitoSOX™ Green in the structures labeled by the MitoTracker dye. Above shows live cell microscopy images of MitoSOX™ Green in U2OS cells co-stained with MitoTracker Deep Red and Hoechst (blue). Cells were washed before imaging in HBSS on a confocal microscope.

Fluorescence Detection of Superoxide in Live Cells

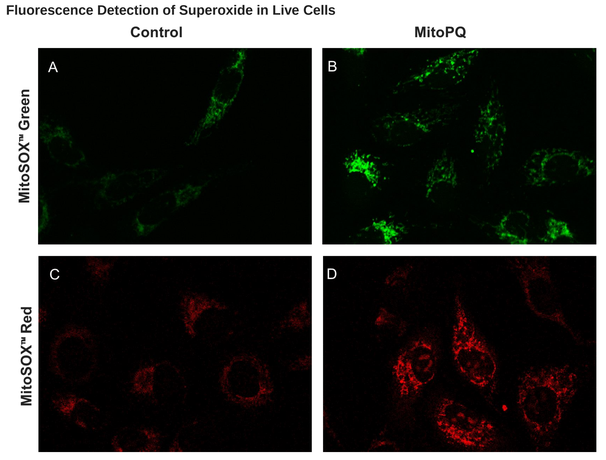

Figure 5. U2OS cells were treated with 30 μM MitoPQ or vehicle control overnight in low glucose media to induce mitochondrial superoxide production. Cells were stained with 1 μM MitoSOX Green **(A, B)** or 500 nM MitoSOX Red (**C, D**) for 30 minutes and then washed before imaging in HBSS on a confocal microscope.

Fluorescence spectra of MitoSOX™ Green and Red

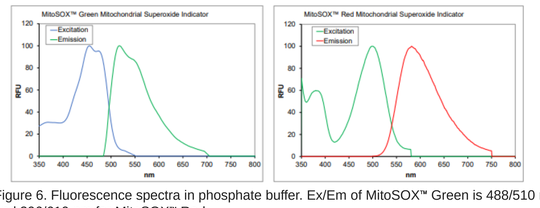

Figure 6. Fluorescence spectra in phosphate buffer. Ex/Em of MitoSOX™ Green is 488/510 nm and 396/610 nm for MitoSOX™ Red

Fluorescent Response to Superoxide tested in Vitro

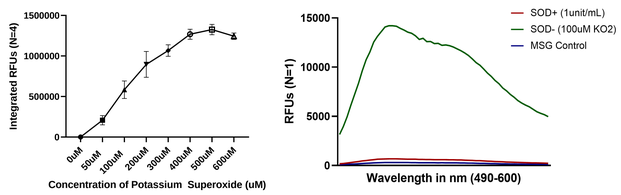

Figure 7. Left panel: MitoSOX™ Green showed low blank fluorescence, while additions of different concentrations of potassium superoxide triggered fluorescent increases (λ_{max}=516nm). Right panel shows that the fluorescent changes of the probe were caused specifically by superoxide. Superoxide dismutase (SOD), a scavenger of superoxide was used in the reactive system. After the reaction of SOD with KO2 in DMSO was carried out for 15 min, MitoSOX Green was added. MitoSOX Green (MSG) shows 95% signal inhibition using 1unit/mL of SOD, while a control addition with no SOD showed no signal increase above baseline.

Specificity of Fluorescence Response to Superoxide Over Other Reactive Oxygen Species

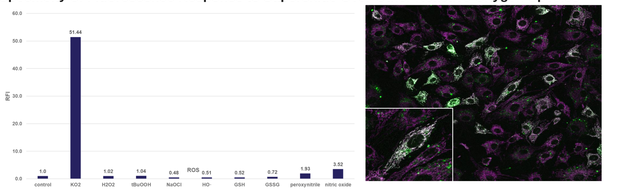

Figure 8. Fluorescent response of various ROS and reductants in vitro. The solution response of MitoSOX Green (10 μm, in water) with superoxide generated by KO2 at 460 nm with excitation at 490 nm after incubation at 25C for 30 min was compared with those of reactions with other ROS and reductants. RFUs were obtained by summing emission values from 490-600 nm For ROS, we used 100 μM final concentration except NaOCl and peroxynitrite (3 μM). 500μM final concentration for GSH and GSSG. Also shown (right panel) is co-localization of MitoSOX™ Green (green pseudo color) with TMRM (white pseudocolor) in Bovine Pulmonary Artery Epithelial (BPAE) cells.

Catalog information, trademarks and licensing

CellEvent™ Caspase 3/7 Green catalog number- C10432

CellEvent™ Caspase 3/7 Red catalog number- C10430

MitoSOX™ Green catalog number- M36005, M36006

MitoSOX™ Red catalog number - M36007, M36008

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