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New Generation Sensors for Caspase Activation and Mitochondrial Superoxide in Live Cell Microscopy

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Abstract and introduction

Neural cell health and stress readouts are critical indicators of altered or impaired function in normal and diseased states of excitable 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.

Here, we describe the testing and functional characterization of CellEvent™ Caspase 3/7 Red, a novel, red shifted sensor for measuring apoptosis in living cells. Our dye is comprised of a cell-permeant, fluorogenic DNA binding reporter dye that is liberated from a DEVD peptide substrate by caspase activation, permitting direct observation of apoptotic cells in situ. CellEvent™ Red operates in the Texas Red imaging band, with a 590nm excitation peak and emission peak near 610nm, permitting easy multiplex with Green Fluorescent Protein (GFP) or calcein stained cells in both traditional and High Content Analysis (HCA) microscopy configurations. This tool is benchmarked below for signal strength and specificity against CellEvent™ Caspase Green Dye in standard models of apoptosis. GFP compatibility and models of Caspase inhibition are also demonstrated, highlighting new, DMSO-free formulations of both red and green dyes.

Similarly, superoxide production in cells is traditionally probed in microscopy with the MitoSOX™ Red Mitochondrial Superoxide Indicator dye, which localizes to mitochondria and reports superoxide generation with a fluorogenic response. This dye has an unusually long Stokes' shift, requiring specialized 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 developed the MitoSOX™ Green Mitochondrial Superoxide Indicator, which localizes to mitochondria of live cells, where it selectively reports superoxide generation in live cells. With an Excitation/Emission profile in the GFP/FITC microscopy channel, a series of comparative studies are shown highlighting selectivity of the probe, localization and signal amplitude from the dye.

Materials and methods

Induction of Caspase 3/7 and mitochondrial superoxide in cells

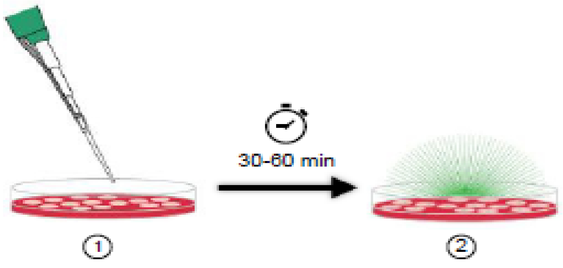
U-2 OS, A673 or primary hippocampal neurons were cultured in standard conditions and apoptosis was induced with either 1μM Staurosporine or 2 μM Camptothecin for the indicated time before dye was added to cells. Mitochondrial superoxide production was induced in cells by the addition of 30 μM MitoPQ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



Results

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

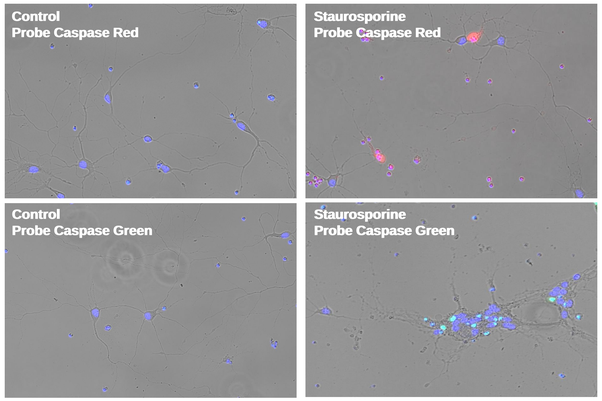


Figure 1. Neonatal rat hippocampal neurons were cultured four days on Poly D Lysine coated glass and treated with 0.1% DMSO carrier (Control) or 1 uM Staurosporine for three hours before staining with Hoechst nuclear dye (above, in blue) and CellEvent™ Red (top panels, red pseudocolor) or Green (bottom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

GFP Multiplex Imaging with CellEvent™ Red

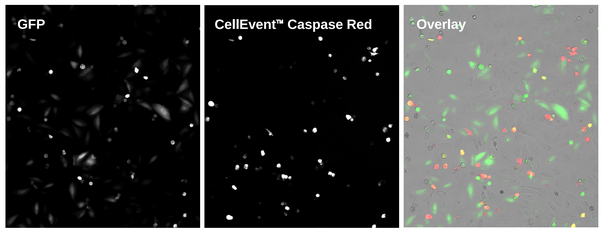


Figure 2. U-2 OS cells were plated and transduced with BacMam GFP Transduction Control according to product recommendations and then treated overnight with 2 uM camptothecin to induce apoptosis. CellEvent™ Caspase Red dye was prepared as directed and added to the cultures for 60 minutes in the cell culture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

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

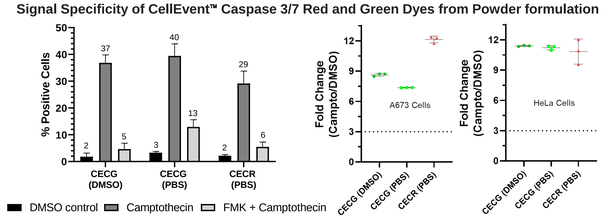


Figure 3. Camptothecin induction of apoptosis on A673 cells (left and middle panels) and HeLa cells (right panel) was measured in control or in FMK Caspase 3/7 inhibitor conditions and images captured for HCA quantification of percent positive and fold increase on the CellInsight™ CX-5 platform.

Mitochondrial Localization of MitoSOX™ Green in Live Cells

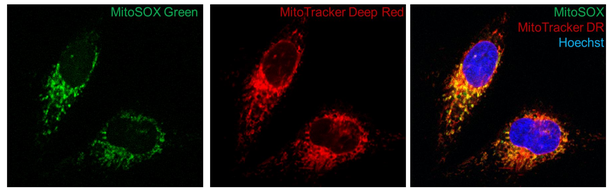


Figure 4. To test the spatial specificity of MitoSOX™ Green staining, MitoTracker Deep Red was co-stained on live cells, showing a clear co-localization of MitoSOX™ Green in the structures labeled by the MitoTracker dye. Above shows live cell microscopy images of MitoSOX™ Green in U2OS cells co-stained 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

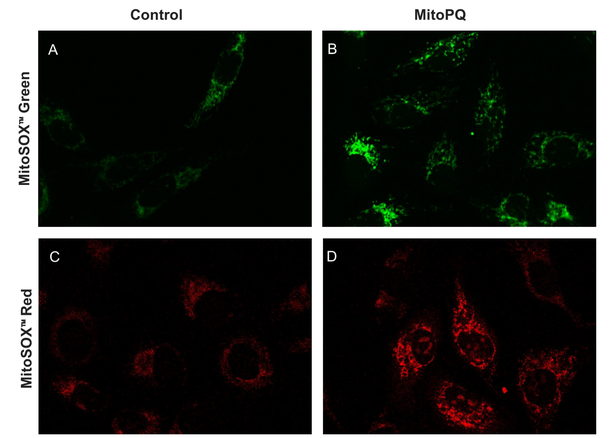


Figure 5. U2OS cells were treated with 30 μM MitoPQ or vehicle control overnight in low glucose media to induce mitochondrial superoxide production. Cells were stained with 1 μM MitoSOX Green **(A, B)** or 500 nM MitoSOX Red **(C, D)** for 30 minutes and then washed before imaging in HBSS on a confocal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

Fluorescence detection of superoxide in Neural Cultures

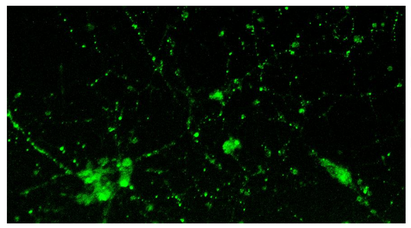


Figure 6. Neonatal rat hippocampal neurons were cultured four days on Poly D Lysine coated glass and treated overnight with MitoPQ before staining with MitoSOX Green and collecting widefield microscopic imagery on the EVOS™ M7000 Imaging System.

Fluorescent Response to Superoxide tested in Vitro

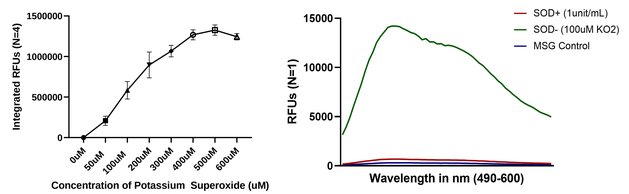


Figure 7. Left panel: MitoSOX™ Green showed low blank fluorescence, while additions of different concentrations of potassium superoxide triggered fluorescent increases (λ_{max}=516nm). Right panel shows that the fluorescent changes of the probe were caused specifically by superoxide. Superoxide dismutase (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

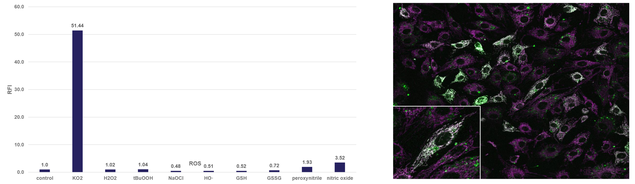


Figure 8. Fluorescent response of various ROS and reductants in vitro. The solution response of MitoSOX Green (10 μm, in water) with superoxide generated by KO2 at 460 nm with excitation at 490 nm after incubation at 25C for 30 min was compared with those of reactions with other ROS and reductants. RFUs 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 pseudocolor) 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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