New-generation, functional probes for caspase 3/7 and mitochondrial superoxide and ready-use phenotypic cell painting tools

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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 3/7 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, red shifted 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 cells in both traditional and HCA microscopy configurations. Further, we demonstrate data with DMSO-free lyophilized forms of the CellEvent Caspase Green and Red dyes. 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 t k s' 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 y ' 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. Additional work details the development and validation testing of the Image-IT™ Cell Painting Kit from Thermo Fisher Scientific, which packages the six canonical CellPaint dyes into a stable and ready-use format for assay development or higher throughput applications in phenotypic profiling.

These reagents are research use only and not for diagnostic purposes.

Materials and methods

Induction of Caspase 3/7 and mitochondrial superoxide in cells

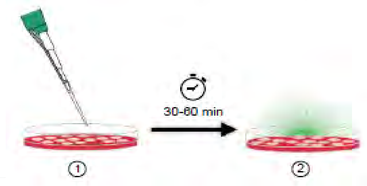
U-2 OS, A673 or primary hippocampal neurons were cultured in standard conditions and apoptosis was induced with either 1μM Staurosporine or 2 μM Camptothecin for the indicated time before dye was added to cells. Mitochondrial superoxide production was induced in cells by the addition of 30 μM MitoPQ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



Results

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

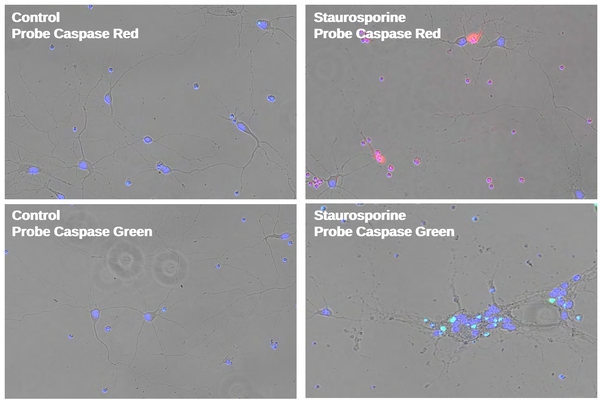


Figure 1. Neonatal rat hippocampal neurons were cultured four days on Poly D Lysine coated glass and treated with 0.1% DMSO carrier (Control) or 1 uM Staurosporine for three hours before staining with Hoechst nuclear dye (above, in blue) and CellEvent™ Red (top panels, red pseudocolor) or Green (bottom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

GFP Multiplex Imaging with CellEvent™ Red

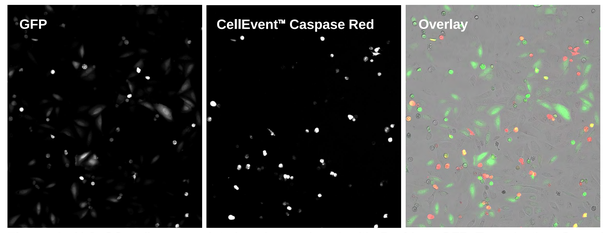


Figure 2. U-2 OS cells were plated and transduced with BacMam GFP Transduction Control according to product recommendations and then treated overnight with 2 uM camptothecin to induce apoptosis. CellEvent™ Caspase Red dye was prepared as directed and added to the cultures for 60 minutes in the cell culture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

Specificity of CellEvent™ Caspase 3/7 Red and Green Dyes from DMSO-free Formulation

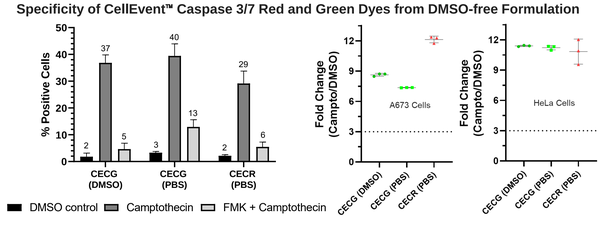


Figure 3. Camptothecin induction of apoptosis on A673 cells (left and middle panels) and HeLa cells (right panel) was measured in control or in FMK Caspase 3/7 inhibitor conditions and images captured for HCA quantification of percent positive and fold increase on the CellInsight™ CX-5 platform.

Mitochondrial Localization of MitoSOX™ Green in Live Cells

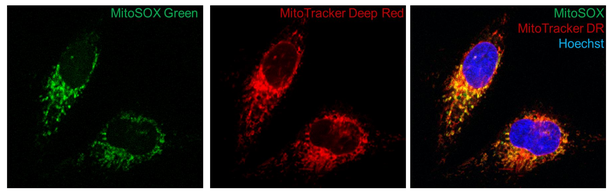


Figure 4. To test the spatial specificity of MitoSOX™ Green staining, MitoTracker Deep Red was co-stained on live cells, showing a clear co-localization of MitoSOX™ Green in the structures labeled by the MitoTracker dye. Above shows live cell microscopy images of MitoSOX™ Green in U2OS cells co-stained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

Live Cell Detection of Superoxide with MitoSOX™ Green

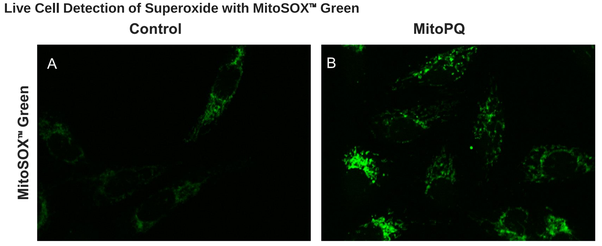


Figure 5. U2OS cells were treated overnight with control (A) or 30 μM MitoPQ (B) in low glucose media to induce mitochondrial superoxide production. Cells were stained with 1 μM MitoSOX Green

Fluorescent Response to Superoxide tested in Vitro

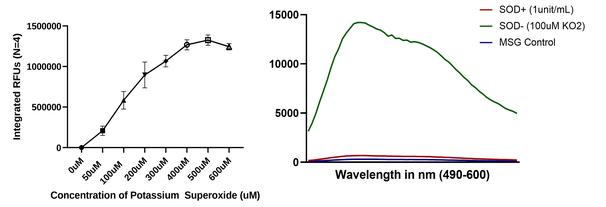


Figure 6. Left panel: Potassium superoxide triggers a dose-dependent increase (λ_{max}=516nm) in signal from MitoSOX™ Green. Right panel shows fluorescent signal in control and superoxide dismutase (SOD) conditions. After the reaction of SOD with KO2 in DMSO was carried out for 15 min, MitoSOX™ Green 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

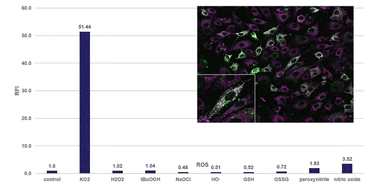


Figure 7. Fluorescent response of various ROS and reductants in vitro. Also shown (Inset) is co-localization of MitoSOX™ Green (green pseudocolor) with TMRM (white pseudocolor) in Bovine Pulmonary Artery Epithelial (BPAE) cells.

Morphological cellular profiling with the Image-IT™ Cell Painting kit

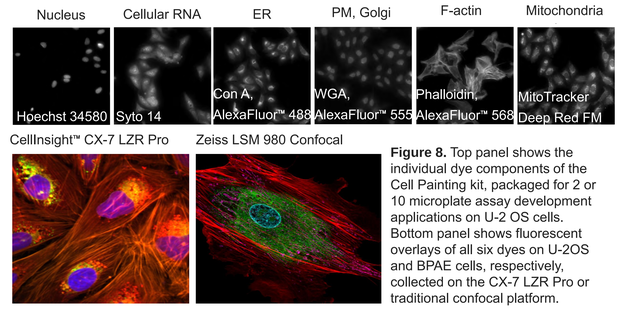


Figure 8. Top panel shows the individual dye components of the Cell Painting kit, packaged for 2 or 10 microplate assay development applications on U-2 OS cells. Bottom panel shows fluorescent overlays of all six dyes on U-2OS and BPAE cells, respectively, collected on the CX-7 LZR Pro or traditional confocal platform.

CellPaint Spheroids Maximum Intensity Projection from Z-Stack

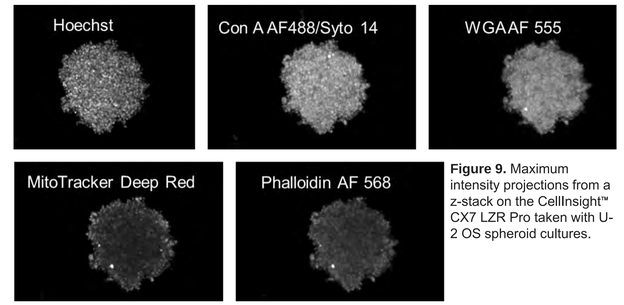


Figure 9. Maximum intensity projections from a z-stack on the CellInsight™ CX7 LZR Pro taken with U-2 OS spheroid cultures.

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

Image-iT™ Cell Painting Kit catalog number – I65000, I65500

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