Fluorescent detection of senescence for imaging and flow cytometry applications

Multiplex with the CellEvent Senescence Green Probe for a more complete picture.

Aging is considered a significant risk factor for developing many chronic diseases, including cardiovascular diseases, cancers, and neurodegenerative diseases, and research indicates that many of these diseases are associated with cellular senescence. Senescent cells are cells that remain metabolically active but have stopped dividing due to telomere shortening, stress, or damage [1-4]. Although senescence appears to be an important pathway for controlling unlimited cell division, senescent cells that are not removed (e.g., as a result of cancer chemotherapy) contribute to a chronic, pro-inflammatory environment, increasing the risk of many age-related diseases. Recent work has shown that specific targeting of senescent cells results in increased life expectancy in a progeroid Ercc1$^{-/\Delta}$ mouse model, which has a premature aging phenotype [1,2]. As such there is great interest in identifying, characterizing, and modifying senescent cells.

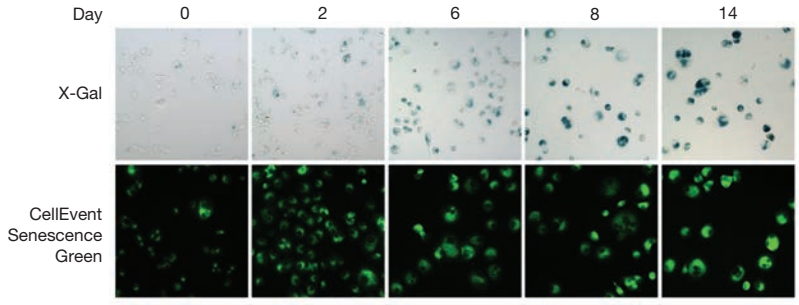

Figure 1. Comparison of senescent and nonsenescent cells labeled with CellEvent Senescence Green Probe or X-Gal. T47D human epithelial cells were untreated (on day 0) and then treated with 5 µM palbociclib every other day for 15 days to induce senescence. Cells were stained using the Invitrogen™ CellEvent™ Senescence Green Detection Kit (Cat. No. C10850) for 90 min or with X-Gal (5-bromo-4-chloro-3-indolyl β-D-galactopyranoside) overnight, and imaged using the Thermo Scientific™ CellInsight™ CX5 High-Content Screening (HCS) Platform and the FITC filter for CellEvent Senescence Green fluorescence, or brightfield for colorimetric X-Gal detection.

Senescence biomarkers

As the mechanisms of senescence are examined, several biomarkers have been identified, including those related to the release of pro-inflammatory cytokines and chemokines, as well as to an increase in beta-galactosidase activity (called senescence-associated β-gal, or SA-β-gal) and senescence-associated heterochromatin foci (SAHF). Of the currently available methods for identifying senescent cells, the detection of SA-β-gal is considered the gold standard for both cells in culture and vertebrate tissue. SA-β-gal activity is the result of increased acidic lysosomal β-galactosidase, which can be detected at near-neutral pH because it is so highly overexpressed. It is typically detected by adding a buffered X-Gal (5-bromo-4-chloro-3-indolyl β-D-galactopyranoside) solution to fixed and permeabilized cells, where the substrate is cleaved by lysosomal SA-β-gal, producing a blue-green precipitate. This colorimetric assay has several drawbacks, including inconsistent signal production, a lengthy protocol, and incompatibility with other cell function probes and flow cytometry.

In order to identify and quantify senescent cells by flow cytometry, researchers either detect other senescence biomarkers using the limited number of specific antibodies available, or detect SA-β-gal using the fluorescein-based β-gal substrate C_{12}FDG. Once inside the cell, the lipophilic, nonfluorescent C_{12}FDG is cleaved by intracellular β-gal, including SA-β-gal, producing a green-fluorescent product. However, even though it has been used since the mid-1990s, C_{12}FDG has limited utility because it tends to leak out of cells and is sensitive to fixation.

Introducing a fluorescent SA-β-gal probe

We have developed a sensitive substrate for β-gal that can be used for the fluorescent detection of senescent cells in both imaging and flow cytometry applications. The Invitrogen™ CellEvent™ Senescence Green Probe is a fluorescence-based β-gal substrate that contains two galactoside moieties, as well as an additional moiety that reacts with several functional groups found in proteins. This nonfluorescent substrate is cleaved by intracellular β-gal to produce a green-fluorescent product (excitation/emission maxima = 490/514 nm) that is well retained in cells due to its covalent binding to intracellular proteins. In addition, the CellEvent Senescence Green Probe is easy to use: simply fix the cells, add the reagent, incubate, and detect the fluorescence by imaging or flow cytometry.

To demonstrate the reliable and consistent detection of senescence, T47D human epithelial cells were induced with palbociclib (a specific CDK4/6 inhibitor that induces cell cycle arrest) for up to 15 days. Cell samples were removed throughout this time course and assayed using either CellEvent Senescence Green Probe or X-Gal, according to standard protocols. Figure 1 shows that the CellEvent Senescence reagent detected the same relative number of senescent cells as X-Gal, and that both substrates exhibited increased signal over time as senescence progresses. Furthermore, the CellEvent Senescence reagent only required a 90-minute incubation, whereas the X-Gal reagent required an overnight incubation to generate an equivalently sensitive signal.

Multiplexing with CellEvent Senescence Green Probe

Because the CellEvent Senescence reagent covalently binds to intracellular proteins upon enzymatic cleavage with β-gal, this reagent is better retained in cells compared with the traditional fluorescence-based β-gal substrate C_{12}FDG. In addition, the CellEvent Senescence reagent is compatible with cell fixation protocols, as well as with multiplex analysis with antibodies and other fixable cell health indicator reagents. Figure 2 demonstrates a multiparameter flow cytometry experiment in which cells were labeled with CellEvent Senescence reagent and antibodies specific for cyclins, which are differentially expressed throughout the cell cycle and downregulated in senescent cells.

Its compatibility with fixation means that the CellEvent Senescence Green reagent can be multiplexed with a variety of other cell health indicator reagents, including the Invitrogen™ Click-iT™ EdU cell proliferation assay (Figure 3) or the fixable Invitrogen™ FxCycle™ DNA stains for cell cycle analysis (data not shown).

Learn more about CellEvent Senescence Green Probe

In summary, we have developed a sensitive fluorescent substrate for β-gal that can be used for the detection of senescent cells using either fluorescence microscopy or flow cytometry. The CellEvent Senescence Green Probe is compatible with cell fixation and can therefore be multiplexed with antibodies or other fixable cell health indicator reagents for more complete immunophenotyping studies. Learn more at **thermofisher.com/cellevent-senescence**. ■

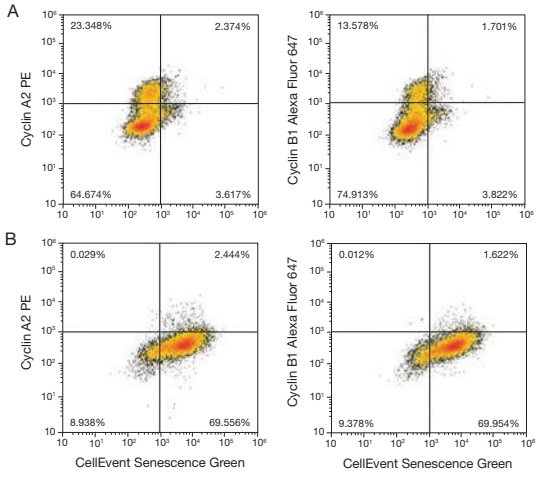

Figure 2. Decreased cyclin A2 and B1 with the onset of senescence. T47D human epithelial cells were **(A)** left untreated or **(B)** treated with palbociclib in media every other day for 15 days to induce senescence. Cells were stained using the Invitrogen™ CellEvent™ Senescence Green Flow Cytometry Assay Kit (Cat. No. C10840), in addition to anti–cyclin A2, PE conjugate, and anti–cyclin B1, Alexa Fluor™ 647 conjugate (Invitrogen™ Cyclin B1 Monoclonal Antibody (clone GNS1, Cat. No. MA5-14319) labeled using the Invitrogen™ Zenon™ Alexa Fluor™ 647 Mouse IgG1 Labeling Kit (Cat. No. Z25008)). Cells were analyzed on the Invitrogen™ Attune™ NxT Flow Cytometer. Untreated T47D cells progressed through the various cell cycle stages, as indicated by the expression of cyclin A2 and B1. The lack of cyclin expression in the treated T47D cells is consistent with cell cycle arrest in senescent cells.

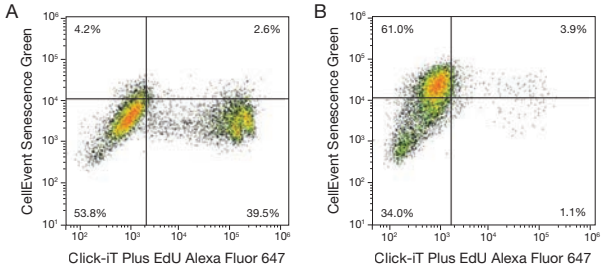

Figure 3. Decreased DNA synthesis in senescent cells. (A) Cycling, nonconfluent control WI-38 human lung fibroblast cells and **(B)** confluent cells that were passaged for 5 weeks to induce nonreplicative senescence were incubated with 10 µM EdU for 2 hr to allow incorporation of the thymidine analog into synthesizing DNA. Cells were subsequently trypsinized, resuspended in 1X PBS, fixed with 4% formaldehyde, and stained using the Invitrogen™ CellEvent™ Senescence Green Flow Cytometry Assay Kit (Cat. No. C10840). Following 1X PBS wash and permeabilization steps, EdU incorporation was detected using the Invitrogen™ Click-iT™ Plus EdU Alexa Fluor™ 647 Flow Cytometry Assay Kit (Cat. No. C10634). Cells were analyzed on the Invitrogen™ Attune™ NxT Flow Cytometer using 488 nm and 637 nm lasers and 530/30 nm and 670/14 nm bandpass filters. As confluency increases over time, cells stop dividing and DNA synthesis ceases, as indicated by the increase in CellEvent Senescence Green staining and the decrease in EdU-positive cells.

References

1. Childs BG, Durik M, Baker DJ et al. (2015) *Nat Med* 21:1424–1435.
2. Campisi J (2013) *Annu Rev Physiol* 75:685–705.
3. Althubiti M, Lezina L, Carrera S et al. (2014) *Cell Death Dis* 5:e1528.
4. Xu M, Pirtskhalava T, Farr JN et al. (2018) *Nat Med* 24:1246–1256.

Product	Quantity	Cat. No.
CellEvent™ Senescence Green Flow Cytometry Assay Kit	50 assays 200 assays	C10840 C10841
CellEvent™ Senescence Green Detection Kit	25 µL 100 µL	C10850 C10851