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# Evaluation of Cellular Senescence through Fluorescence Characterization

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## ABSTRACT AND INTRODUCTION

Aging is considered a risk factor for developing many chronic diseases which includes cardiovascular diseases, cancer, and neurodegenerative diseases. Researchers are now showing that many of these diseases are associated with cellular senescence. Cellular senescence is an important process for the removal of damaged cells, but if senescent cells are not removed a chronic pro-inflammatory environment ensues, increasing the risk of many age-related diseases. These cells accumulate as a result of cancer chemotherapy, are associated with age-related diseases, and are important for development. Recent work has shown that specific targeting of senescent cells results in increased life expectancy in a progeroid Ercc1-/Δ mouse model[1,2]. As such there is great interest in identifying, characterizing and targeting senescent cells. Senescent cells are identified by the release of pro-inflammatory cytokines and chemokines (SASP), increased beta galactosidase activity (b-gal), senescence associated heterochromatin foci (SAHF), and changes in morphology which are increased size and a flattened appearance.

There is no single good marker for determining senescence, so these cells are identified through the collective interpretation of multiple markers. Senescence associated b-gal (SA-β-gal) is considered the "gold standard" for identifying senescent cell, and it is detected with the blue-green precipitating colorimetric x-gal substrate. However, this substrate is limited since it cannot be used in flow cytometry or other fluorescence based platforms. Even though $C_{12}$FDG, a fluorescein based b-gal substrate, has been used since the mid-90's, it has limited utility restricted by its lack of fixability. A new b-gal substrate is demonstrated here which does not leak out of cells; enabling researchers to more easily identify and evaluate cellular senescence.

In this study we use a fluorescence approach to assess the senescence phenotype based on a combination of fluorescence based imaging and ICC, flow cytometry, cell sorting, and qPCR. We examine several cell based models including replicative senescence characterized by telomere shortening in primary fibroblasts, cellular senescence characterized through DNA damage pathway by doxorubicin, oxidative stress senescence induced by confluency in atmospheric oxygen environment (SIPS), and senescence induced by cyclin D blockade (palbociclib treatment). We found that a multiplexed fluorescence based approach to characterizing senescent cells can contribute to the understanding of cellular senescence in model systems.

## MATERIALS AND METHODS

T-47D (human breast cancer cell line) (ATCC, Manassas, VA) were maintained on RPMI 1640 + 10% FBS and treated with palbociclib (Selleck Chemicals, Houston, TX) to induce senescence through cyclin D blockade. Fluorescence based detection of β-gal activity used the CellEvent Senescence Green Flow Cytometry Assay kit β-gal substrate for flow or CellEvent Senescence Green Detection kit for imaging were performed at 37˚C for 1-2 hours in the absence of $CO_2$ following manufacture's instructions (Thermo Fisher Scientific). Detection of β-gal activity using colorimetric x-gal substrate performed overnight at 37˚C incubation in the absence of $CO_2$ following manufacture's instructions (Cell Signaling Technology, Danvers, MA). Lysosomal and mitochondrial staining with 50 nM LysoTracker Blue DND-22 and 100 nM MitoTracker Deep Red (respectively) diluted in complete medium and incubated on cells at 37˚C for 30 minutes prior to imaging or fixing and staining with green β-gal substrate. Cell proliferation was performed Click-iT EdU Alexa Fluor 647 Flow Cytometry Kit (Thermo Fisher Scientific) using a 2 hour pulse of 10 μM EdU and detected with click chemistry following manufacture's instructions. All imaging performed on EVOS FL Auto 2 Imaging System or CellInsight CX5 HCS Platform. Flow cytometry performed on Attune NXT Flow Cytometer (Thermo Fisher Scientific, Waltham, MA). Reagents from Thermo Fisher Scientific unless indicated otherwise.

## Figure 1. β-gal colorimetric vs fluorescent labeling

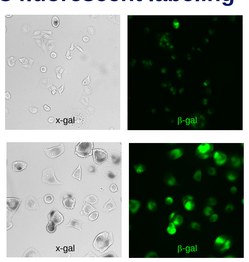
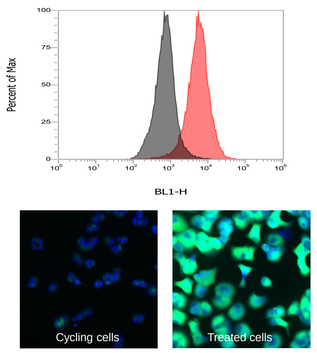



Figure 1. **Concordance of fluorescence and colorimetric β-gal staining.** Identical fields first stained and imaged with fluorescent CellEvent Senescence Green β-gal substrate then stained overnight with colorimetric x-gal substrate show concordance of signal. Upper panels are cycling T-47D cells and lower panels are palbociclib treated cells. CellEvent β-gal forms covalent fluorescent product in 1-2 hours. X-gal colorimetric requires overnight staining and is not multiplexable with other fluorescent markers.

## Figure 2. β-galactosidase labeling in treated cells

Figure 2. Flow analysis of T-47D cells treated ± 0.5 uM palbociclib for 15 days to induce senescence through CDK 4/6 checkpoint blockade For flow analysis, cells were typsinized, resuspended in 1X PBS, and fixed in 4% paraformaldehyde for 10 minutes at room temperature, prior to staining with CellEvent Green Senescence Probe for 90 minutes in a 37ºC incubator (no $CO_2$). Cells were washed once in PBS with 1% BSA and resuspended in 1X PBS before analyzing on the Attune NXT BL1 channel. The median fluorescence intensity of the senescent cell peak (ΔMFI) is approximately 1 log higher than the non-treated population. Images show upregulation of b-gal activity in treated condition. Gain and exposure matched. (Counterstained with Hoechst 33342)




## Figure 3. Cell sorting of senescent cells using covalent green β-gal substrate

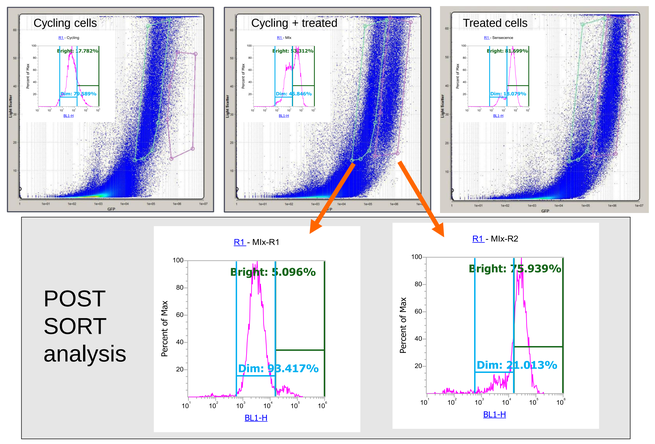



**Figure 3: Sorting of senescent cells**. T-47D cells treated ± palbociclib to induce a senescence phenotype characterized by up-regulation of b-gal activity were stained with green b-gal substrate (CellEvent Senescence Green Flow Cytometry Assay Kit) and sorted in the green channel using iSORT Automated Cell Sorter. Sorted samples were then analyzed on the NXT Attune flow cytometer for purity and show good separation.

## Figure 4. β-gal + mitochondrial and lysosomal staining

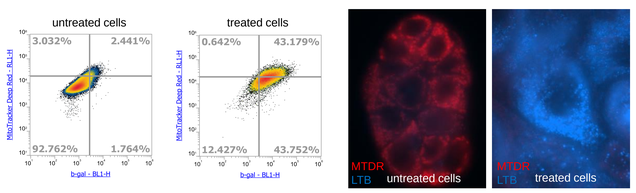



**Figure 4: Mitochondria and lysosomal stained T-47D cells ± palbociclib treatment.** MitoTracker Deep Red (MTDR) multiplexed with CellEvent Senescence Green β-gal Probe and analyzed by flow cytometry. Treated cells showing increased mitochondrial and β-gal activity over untreated control. LysoTracker Blue staining increases in lysosomes of treated cells and over untreated control cells (right images).

## Figure 5. Fluorescent β-gal substrate labeling of hippocampus section in Nrf2KO mouse model

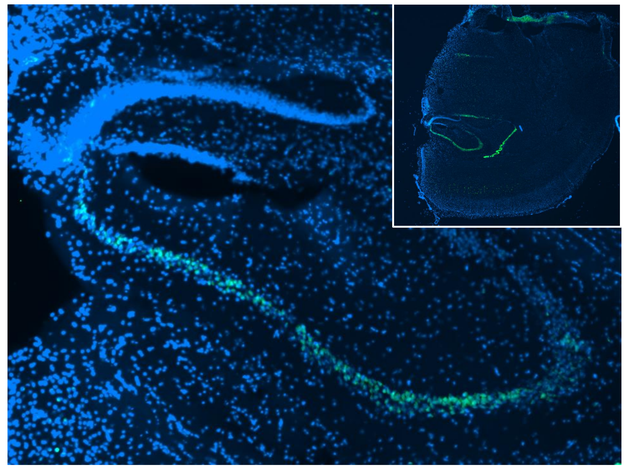


**Figure 5: Cleavage of β-gal substrate results in covalently attached green fluorescent signal.** Frozen brain tissue from 6-month-old Nrf2KO mouse[4] was cut to 10 um sections, fixed in 4% neutral buffered formaldehyde, rinsed in PBS and incubated with CellEvent Senescence Green β-gal substrate at pH 5 for 2 hours at 37˚C. Washed with PBS and mounted with Prolong Gold + DAPI. Imaged on Keyence fluorescence bz-9000 microscope. Band of β-gal positive cells visible in the dentate gyrus region.

## Figure 6. qRT-PRC assessment of SASP and other markers of senescence using Taqman probes

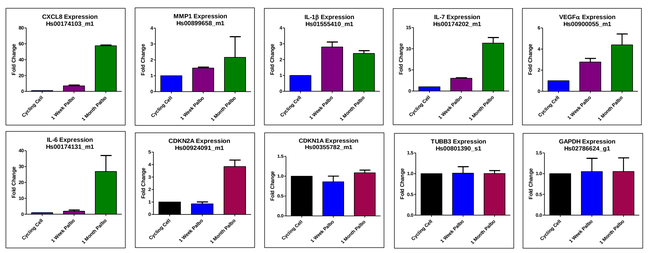
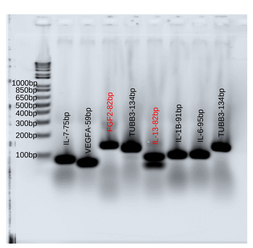



**Figure 6: RNA prepared from T-47D cells ± palbociclib treatment** $\Delta\Delta C_T$ determination of fold gene expression change compared to cycling cells indicated in bar graphs. *TUBB3 and GAPDH* as a control. Replicate samples extracted for RNA with PureLink RNA Mini Kit. 4 technical replicates analyzed by qRT-PCR with TaqMan primer-probes as indicated. Example of gel analysis using E-Gel EX 2% agarose to confirm amplicon size (below).

| Target | Probe | Size (bp) | Class | Change |
|---|---|---|---|---|
| CXCL8 | Hs00174103_m1 | 101 | chemokine | ↑↑ |
| MMP1 | Hs00899658_m1 | 64 | chemokine | ↑ |
| IL-1β | Hs01555410_m1 | 91 | cytokine | ↑ |
| IL-7 | Hs00174202_m1 | 75 | cytokine | ↑↑ |
| VEGFα | Hs00900055_m1 | 59 | growth factor | ↑ |
| IL-6 | Hs00174131_m1 | 95 | cytokine | ↑↑ |
| CDKN2A | Hs00924091_m1 | 84 | p16 | ↑ |
| CDKN1A | Hs00355782_m1 | 66 | p21 | no change |
| TUBB3 | Hs00801390_s1 | 134 | housekeeping | no change |
| GAPDH | Hs02786624_g1 | 157 | housekeeping | no change |




## Figure 7. Multiplexing proliferation (EdU) with senescence detection thru β-gal staining

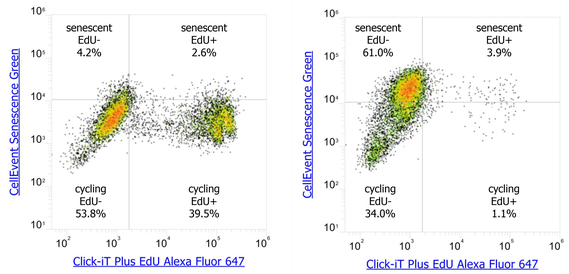



**Figure 7: Click-iT EdU combined with CellEvent Senescence Green β-gal substrate.** T-47D cells are pulsed with EdU then fixed and stained with CellEvent Senescence β-gal substrate prior to permeabilization and click reaction. Untreated T-47D cells are cycling and show a proliferating population as measured by EdU incorporation and basal levels of b-gal activity (left). Treatment with palbociclib results in CDK 4/6 blockade causing T-47D cells to enter early senescence and no longer proliferate along with an increase in β-gal activity (right).

## CONCLUSIONS

There is no single marker which entirely defines senescence. Senescence cells are often characterized by having an increased size, and flatter appearance, and increased β-galactosidase activity, and *p16*$^{Ink4a}$ expression. They can have heterochromatin foci (SAHF), in addition to increased secretion of cytokines and chemokines (SASP). Often they have increased mitochondrial load and dysfunctional lysosomes with increased ROS activity[5-7]. Use of β-gal substrates until now either as colorimetric (x-gal) or fluorescent but non-covalent ($C_{12}$FDG) has been limiting for its use with other markers. However, since the cleaved β-gal product of the CellEvent Senescence Green β-gal Reagent binds covalently to cellular components, multiplexing with other relevant markers that require permeabilization is possible. Combined with gene expression and protein expression profiles of cytokine and chemokine, a profile of the senescence phenotype can be better characterized in model systems of senescence.

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