bio-protocol

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Whole-mount Senescence-Associated Beta-Galactosidase (SA-β-GAL) Activity Detection Protocol for Adult Zebrafish

Marta Marzullo[1, 2, *], Mounir El Maï[2, 3, *] and Miguel Godinho Ferreira[2, 3, *]

[1]IBPM CNR c/o Department of Biology and Biotechnology, Sapienza University of Rome, Rome, Italy
[2]Instituto Gulbenkian de Ciência, 2780-156 Oeiras, Portugal
[3]Institute for Research on Cancer and Aging of Nice (IRCAN), CNRS UMR7284 INSERM U1081 Université Côte d'Azur, 06107 Nice, France
*For correspondence: marta.marzullo@uniroma1.it; Mounir.EL-MAI@unice.fr; Miguel-Godinho.FERREIRA@unice.fr

Abstract

Senescence-associated beta-galactosidase (SA-β-GAL) is an enzyme that accumulates in the lysosomes of senescent cells, where it hydrolyses β-galactosides. With p16, it represents a well-recognized biomarker used to assess senescence both *in vivo* and in cell culture. The use of a chromogenic substrate, such as 5-bromo-4-chloro-3-indoyl-β-d-galactopyranoside (X-Gal), allows the detection of SA-β-GAL activity at pH 6.0 by the release of a visible blue product. Senescence occurs during aging and is part of the aging process itself. We have shown that prematurely aged zebrafish accumulate senescent cells detectable by SA-β-GAL staining in different tissues, including testis and gut. Here, we report a detailed protocol to perform an SA-β-GAL assay to detect senescent cell accumulation across the entire adult zebrafish organism (*Danio rerio*). We also identify previously unreported organs that show increased cell senescence in telomerase mutants, including the liver and the spinal cord.

Keywords: SA-β-GAL, Senescence, Aging, Whole organism, Zebrafish

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Background

Cellular senescence is defined as a permanent cell cycle arrest driven by different mechanisms (including telomere shortening, DNA damage, genotoxic stress, and inflammatory cytokines release) that culminate in the activation of p53 and the cyclin-dependent kinase inhibitor p16 (Collins and Sedivy, 2003; Coppé *et al.*, 2008; Li *et al.*, 2016). Alongside the activation of p53 and p16, senescent cells possess a panel of features, detected both in cell culture and in animal models, that can be used as biomarkers for cellular senescence (Gorgoulis *et al.*, 2019). One of the most widely used markers for detecting senescent cells is the Senescence-associated beta-galactosidase (SA-β-GAL; Dimri *et al.*, 1995), an enzyme that cleaves β-D-galactose residues in β-D-galactosides. This enzyme accumulates in the lysosomal compartment of senescent cells, where its activity is detectable at pH 6.0 (Dimri *et al.*, 1995). The number and size of lysosomes increase in senescent cells; therefore, SA-β-GAL activity increase is also related to increased lysosome content (Lee *et al.*, 2006).

Accordingly, a method has been developed to detect senescence cells based on the use of 5-bromo-4-chloro-3-indoyl-β-d-galactopyranoside (X-Gal), a colorless, soluble compound consisting of galactose linked to an indole. At pH 6.0, the SA-β-GAL enzyme hydrolyses the X-Gal, releasing a deep blue, insoluble product on the cell culture or in the tissue, allowing the detection of senescent cells (Dimri *et al.*, 1995).

Previous studies revealed how senescent cells accumulate with aging (Biran *et al.*, 2017). Consequently, SA-β-GAL activity increases with aging in different tissues. Using a zebrafish telomerase mutant (tert-/-), we previously showed that SA-β-GAL positive cells accumulate in the gut, testis, and kidney (Henriques *et al.*, 2013; El Maï *et al.*, 2020). We also discovered that progressive telomere shortening is associated with a cell fate transition from apoptosis to senescence during aging of telomerase deficient zebrafish. In young telomerase mutants, proliferative tissues exhibit DNA damage and p53-dependent apoptosis, but no senescence. However, the same tissues in older animals display loss of cellularity, increased pro-proliferation signaling, and cell senescence becomes predominant. We showed that prematurely aged fish accumulate senescent cells, detectable by SA-β-GAL staining, and correlate with increase in number of senescence-associated p16 positive cells (El Maï *et al.*, 2020).

Common protocols designed to identify senescent cells in aged tissues are performed on isolated organs. One of the advantages of zebrafish is its small size. This peculiarity allows us to observe, in the same longitudinal histological section, several organs from the same individual. Here, we report a whole-mount SA-β-GAL assay to detect the accumulation of cell senescence in the entire adult zebrafish (*Danio rerio*). Fixed and stained fish can be sectioned and the slides analyzed under bright-field microscopy, or a slide scanner, for blue staining evaluation in the organs of interest.

Materials and reagents

Animals

Adult zebrafish (*Danio rerio*) 9-month-old

Materials

1. Polypropylene centrifuge tube 15 mL (Corning, catalog number: 352096)
2. Glass microscope Superfrost plus slides 25 × 75 × 1 mm (Menzel Gläser, catalog number: J1800AMNZ)
3. Cover Glass 24 × 36 mm (Labelians, catalog number: LCO2436)

Reagents

1. Tricaine/MS-222 (Pharmaq)
2. Tris base (Sigma-Aldrich, catalog number: T-1503)
3. PFA 16% (Thermo Scientific™, catalog number: 28908)
4. Potassium hexacyanoferrate(II) trihydrate (Sigma-Aldrich, catalog number: P9387)
5. Potassium hexacyanoferrate(III) (Sigma-Aldrich, catalog number: 244023)
6. X-Gal (5-Bromo-4-Chloro-3-Indolyl-B-D-Galactopyranoside) (Sigma-Aldrich, catalog number: B4252)
7. N,N-Diméthylformamide (DMF) (Sigma-Aldrich, catalog number: 227056)
8. Magnesium Chloride Hexahydrate ($MgCl_2 \cdot 6H_2O$, Sigma-Aldrich, catalog number: 7791-18-6)

9. Sodium Chloride (NaCl, Sigma-Aldrich, catalog number: S7653)
10. Potassium Chloride (KCl, Sigma-Aldrich, catalog number: 7447-40-7)
11. Sodium phosphate dibasic (Na_2HPO_4, Sigma-Aldrich, catalog number: 71505)
12. Potassium phosphate dibasic (KH_2PO_4, Sigma-Aldrich, catalog number: 1.05104)
13. Ethylenediaminetetraacetic acid (EDTA, VWR, catalog number: A10713.0I)
14. Nuclear Fast red (Sigma-Aldrich, catalog number: 1001210500)
15. Histology cassettes (Simport, catalog number: M505)
16. Embedding Molds (Sakura Finetek, catalog number: 4133)
17. 10% buffered Formalin (VWR, catalog number: 9713.9010)
18. Xylene (VWR, catalog number: 28973.363)
19. Ethanol 100% (EtOH, VWR, catalog number: VWRC20821.330)
20. Paraffin (Merck Millipore, catalog number: 1.11609.9025)
21. 1 M Tris pH 9 (see Recipes)
22. Tricaine/MS-222 400 mg/L solution (see Recipes)
23. Tricaine/MS-222 200 mg/L solution (see Recipes)
24. 4% Paraformaldehyde (see Recipes)
25. 10× Phosphate Buffered Saline (PBS, pH 7.4) (see Recipes)
26. 1× Phosphate Buffered Saline (PBS, pH 7.4) (see Recipes)
27. 1× Phosphate Buffered Saline (PBS, pH 6) (see Recipes)
28. Ethylenediaminetetraacetic acid (EDTA, 0.5 M, pH 8) (see Recipes)
29. X-Gal stock solution (25 mg/mL) (see Recipes)
30. X-Gal staining solution (see Recipes)
31. Potassium hexacyanoferrate (II) trihydrate (0.5 M) (see Recipes)
32. Potassium hexacyanoferrate (III) (0.5 M) (see Recipes)
33. Magnesium Chloride ($MgCl_2$ 0.5 M) (see Recipes)
34. X-Gal staining solution (see Recipes)
35. Ethanol 70% (see Recipes)
36. Ethanol 95% (see Recipes)

Equipment

1. Roller mixer (CAT Ingenieurbuero, catalog number RM5)
2. Dry incubator at 37°C (Memmert, catalog number: UNB 100)
3. NanoZoomer Digital slide scanner (Hamamatsu, catalog number: C13140-01)
4. Tissue processor (Leica Biosystems, catalog number: HistoCore PEARL)
5. Tissue Embedding Console System (Sakura Finetek, catalog number: Tissue-Tek® TEC™ 5)
6. Automated Rotary Microtome (Leica Biosystems, catalog number: RM2255)

Software

1. NDP.view2 Image viewing software (U12388-01 Hamamatsu) to open and analyze NanoZoomer scans. Free download from https://www.hamamatsu.com/eu/en/product/life-science-and-medical-systems/digital-slide-scanner/U12388-01.html

Procedure

A. SA-β-GAL Staining procedure

Day 0
Starve the fish overnight for at least 17 h, leaving them in their aquarium.

Day 1
1. Sacrifice fish by placing them in a container with 50 mL of 200 mg/L Tricaine MS-222 for 30 min.
   Note: Death can be determined by checking cessation of breath and gill movements.
2. Proceed with fixation by individually incubating fish in 15 mL centrifugation tubes containing at least 10 mL of 4% PFA for 3 days, at 4°C on the roller mixer.
   Notes:
   a. *Differences in PFA quality might affect fixation of the entire fish and, therefore, the quality of β-galactosidase detection. It is then important to prepare fresh 4% PFA solution for each experiment.*
   b. *Incubation with gentle agitation helps the penetration of PFA and improves fixation of inner organs.*

Day 4
1. Wash each sample three times for 1 h in at least 10 mL of PBS pH 7.4, at 4°C on the roller mixer.
2. Wash each sample 1 h in at least 10 mL of **PBS pH 6.0,** at 4°C on the roller mixer.
   Note: Pre-incubating fish at pH 6.0 is crucial to ensure an optimal SA-β-GAL reaction in the next step of the protocol.
3. Incubate each fish with at least 10 mL of X-Gal staining solution for 24 h at 37°C in the dark.
   Note: It is crucial to ensure that SA-β-GAL activity detection is performed at pH 6.0. Lowering the pH below pH 5.9 results in false positives, while increasing the pH above pH 6.1 produces false negatives.

Day 5
1. Wash each sample three times for 5 min in at least 10 mL of PBS pH 7.4 at room temperature on the roller mixer.
2. Proceed with de-calcification for 48 h in 10 mL of 0.5 M EDTA pH 8 at room temperature.
   Note: To avoid loss of SA-β-GAL staining, fish should not be incubated for more than 48 h in EDTA.

B. Embedding procedure

1. After de-calcification, remove EDTA by incubating fish in water for at least 30 min at room temperature.
2. Insert each sample into a histology cassette.
3. Proceed with the embedding of the samples in a tissue processor using the following program:
   a. 10% buffered formalin for 1 h
   b. Distilled water for 2 min
   c. Ethanol 70% for 2 h
   d. Ethanol 95% for 1 h
   e. Ethanol 100% for 1 h
   f. Ethanol 100% for 1 h
   g. Ethanol 100% for 2 h
   h. Ethanol 100% for 1 h
   i. Xylene for 30 min
   j. Xylene for 1 h
   k. Xylene for 1 h
   l. Paraffin for 30 min at 62°C
   m. Paraffin for 1 h at 62°C
   n. Paraffin for 1 h at 62°C
4. Using the Tissue Embedding Console System, insert each sample into an embedding mold and pour paraffin into the mold. Allow for solidification of the paraffin block by placing the mold containing each sample and paraffin onto the cryomodule plate of the Tissue Embedding Console.
5. **Counterstaining procedure**
   a. Prepare 5 µm section slides from each paraffin-embedded sample using a microtome.

b. Counterstain the slides with Nuclear fast red, following the manufacturer's protocol. Briefly, after conventional deparaffinization and rehydration, wash slides for 1 min at room temperature in distilled water and stain them by incubating for 10 min at room temperature in Nuclear fast red-aluminum sulfate solution 0.1%. After 1 min, wash in distilled water and proceed with conventional dehydration and xylene incubation prior to slide mounting.

C. Image acquisition

1. Acquire images using a bright-field microscope or slide scanner.
   Note: The images of the different tissues shown in Figure 1 have been selected from a scan of a whole-body fish section. The slide scans were acquired with a NanoZoomer Digital slide scanner with a 40× source lens and analyzed with the NDPi software.

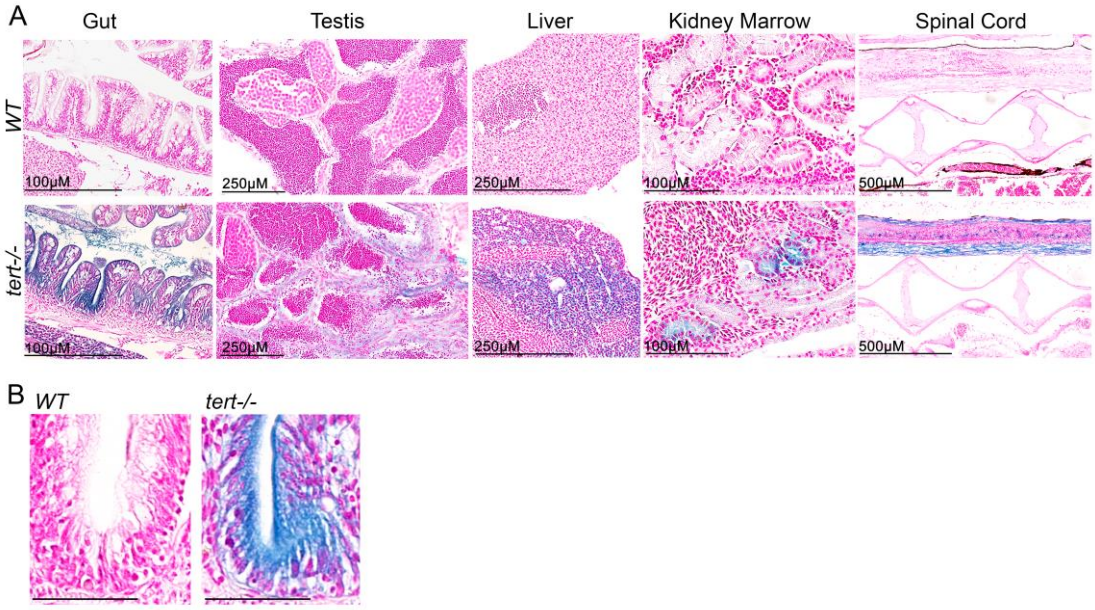

Figure 1. SA-β-GAL staining allows the detection of cell senescence in prematurely aged zebrafish tissues. A) Representative images of fish assayed for SA-β-GAL activity. Blue staining shows the presence of SA-β-GAL in different tissues of prematurely aged fish (*tert-/-*) compared to age-matched controls (wild type, *WT*). Corresponding scale bar represented in each panel **B)** Zoom-in of gut villi in A, allowing to precisely distinguish the cells stained in blue. Scale bar 50 µm.
Note: A significant increase in SA-β-gal positive cells is seen by 9 months of age in tert-/- tissues and by 24 months in WT tissues.

Concluding Note:
We analyzed different tissues of adult zebrafish for presence of SA-β-GAL blue staining. Intensity and localization may change due to biological or technical issues (i.e., age of the fish, health status, penetration of the fixative, longitudinal sections, etc.). Thus, it is crucial to have the proper number of technical and biological replicates for effective result interpretation. In our experiments, we performed a minimum of three biological replicates for each genotype and age.

Recipes

1. **1 M Tris pH 9**
   a. Dilute 121.4 g tris base in 850 mL of Milli-Q water.
   b. Adjust pH to 9.
   c. Bring volume to 1 L.

2. **Tricaine/MS-222 400 mg/L solution**
   a. Dissolve 2 g of tricaine/MS-222 in 489.5 mL of Milli-Q water.
   b. Add 10.5 mL of 1 M Tris (pH 9).
   c. Adjust pH to 7.

3. **Tricaine/MS-222 200 mg/L solution**
   Add 50 mL of tricaine/MS-222 to 50 mL of fish system water.

4. **4% Paraformaldehyde**
   Make fresh solution by adding 10 mL PFA 16% to 30 mL of PBS.

5. **10× Phosphate Buffered Saline (PBS, pH 7.4)**
   a. Add 80 g of NaCl, 2.0 g of KCl, 14.4g of Na_2HPO_4, and 2.4 g of KH_2PO_4 to 800 mL of Milli-Q water.
   b. Adjust pH to 7.4.
   c. Bring volume to 1 L.
   d. Autoclave and store at room temperature.

6. **1× Phosphate Buffered Saline (PBS, pH 7.4)**
   Dilute 100 mL of 10× PBS pH 7.4 in 900 mL of Milli-Q water and keep at 4°C.

7. **1× Phosphate Buffered Saline (PBS, pH 6)**
   a. Dilute 100 mL of 10× PBS pH 7.4 in 800 mL of Milli-Q water.
   b. Adjust the pH to 6.0.
   c. Bring up the volume to 1 L and keep at 4°C.

8. **Ethylenediaminetetraacetic acid (EDTA, 0.5 M, pH 8)**
   a. Add 146.12 g of EDTA to 850 mL of Milli-Q water.
   b. Adjust the pH to 8.0.
   c. Bring up the volume to 1 L.
   d. Filter and store at room temperature.

9. **X-Gal stock solution (25 mg/mL)**
   Dissolve 150 mg of X-Gal in 5mL of DMF.

10. **Potassium hexacyanoferrate (II) trihydrate (0.5 M)**
    a. Dissolve 2.11 g of Potassium hexacyanoferrate (II) in 10 mL of Milli-Q water.
    b. Store at 4°C in the dark.

11. **Potassium hexacyanoferrate (III) (0.5 M)**
    a. Dissolve 1.646 g of Potassium hexacyanoferrate (III) in 10 mL of Milli-Q water.
    b. Store at 4°C in the dark.

12. Magnesium Chloride ($MgCl_2$ 0.5 M)

Dissolve 5.1 g of Magnesium Chloride Hexahydrate in 50 mL of Milli-Q water.

13. X-Gal staining solution

a. To prepare the SA-β-GAL staining solution (1 mg/mL X-Gal, 5 mM potassium ferrocyanide, 5 mM potassium ferricyanide, 2 mM Magnesium chloride in PBS pH 6), add 1 mL of 0.5 M Potassium hexacyanoferrate (II) trihydrate, 1 mL of 0.5 M Potassium hexacyanoferrate (III), 400 µL of 0.5 M $MgCl_2$ and 4 mL of 25mg/mL X-Gal to 93.6 mL of 1× PBS pH 6.0.

b. Make a fresh solution for each experiment and keep it in the dark until use.

Note: X-Gal concentration can be adjusted up to 1.5% to increase the staining.

14. Ethanol 70%

Add 300 mL of Milli-Q water to 700 mL of ethanol 100%.

15. Ethanol 95%

Add 50 mL of Milli-Q water to 950 mL of ethanol 100%.

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The protocol described has been previously used and described in El Maï *et al.* (2020).
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Ethics

Animal experimentation: All Zebrafish work was conducted in Portugal according to National Guidelines and approved by the Ethical Committee of the Instituto Gulbenkian de Ciência and the Direção Geral de Alimentação e Veterinária (DGAV, Approval number: 010294) and in France by the Animal Care Committee of the IRCAN, the regional (CIEPAL Cote d'Azur 531 #697) and national (French Ministry of Research #27673-2020092817202619) authorities.

Competing interests

The authors declare that no competing interests exist.

References

Biran, A., Zada, L., Abou Karam, P., Vadai, E., Roitman, L., Ovadya, Y., Porat, Z. and Krizhanovsky, V. (2017). Quantitative identification of senescent cells in aging and disease. *Aging Cell* 16(4): 661-671.

Collins, C. J. and Sedivy, J. M. (2003). Involvement of the INK4a/Arf gene locus in senescence. *Aging Cell* 2(3): 145-150.

Coppé, J. P., Patil, C. K., Rodier, F., Sun, Y., Munoz, D. P., Goldstein, J., Nelson, P. S., Desprez, P. Y. and Campisi, J. (2008). Senescence-associated secretory phenotypes reveal cell-nonautonomous functions of oncogenic RAS and the p53 tumor suppressor. *PLoS Biol* 6(12): 2853-2868.

Dimri, G. P., Lee, X., Basile, G., Acosta, M., Scott, G., Roskelley, C., Medrano, E. E., Linskens, M., Rubelj, I., Pereira-Smith, O., *et al.* (1995). A biomarker that identifies senescent human cells in culture and in aging skin *in vivo*. *Proc Natl Acad Sci U S A* 92(20): 9363-9367.

El Maï, M., Marzullo, M., de Castro, I. P. and Ferreira, M. G. (2020). Opposing p53 and mTOR/AKT promote an *in vivo* switch from apoptosis to senescence upon telomere shortening in zebrafish. *Elife* 9: e54935.

Gorgoulis, V., Adams, P. D., Alimonti, A., Bennett, D. C., Bischof, O., Bishop, C., Campisi, J., Collado, M., Evangelou, K., Ferbeyre, G. *et al.*, (2019). Cellular Senescence: Defining a Path Forward. *Cell* 179(4): 813-827.

Henriques, C. M., Carneiro, M. C., Tenente, I. M., Jacinto, A. and Ferreira, M. G. (2013). Telomerase is required for zebrafish lifespan. *PLoS Genet* 9(1): e1003214.

Lee, B. Y., Han, J. A., Im, J. S., Morrone, A., Johung, K., Goodwin, E. C., Kleijer, W. J., DiMaio, D. and Hwang, E. S. (2006). Senescence-associated beta-galactosidase is lysosomal beta-galactosidase. *Aging Cell* 5(2): 187-195.

Li, T., Liu, X., Jiang, L., Manfredi, J., Zha, S. and Gu, W. (2016). Loss of p53-mediated cell-cycle arrest, senescence and apoptosis promotes genomic instability and premature aging. *Oncotarget* 7(11): 11838-11849.