

## Digital Image Analysis of Cells Stained with the Senescence-Associated $\beta$ -Galactosidase Assay

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### Abstract

Cellular senescence plays important roles in the aging process of complex organisms, in response to stress and in tumor suppression. Several markers can be used to identify senescent cells, of which the most widely used is the senescence-associated  $\beta$ -galactosidase (SABG) activity. Here we describe a procedure for digital image analysis of cells stained by the SABG staining technique at pH 6 or at pH 4. This analysis is highly reproducible and sensitive to subtle differences in staining intensities resulting from diverse cellular senescence pathways in culture.

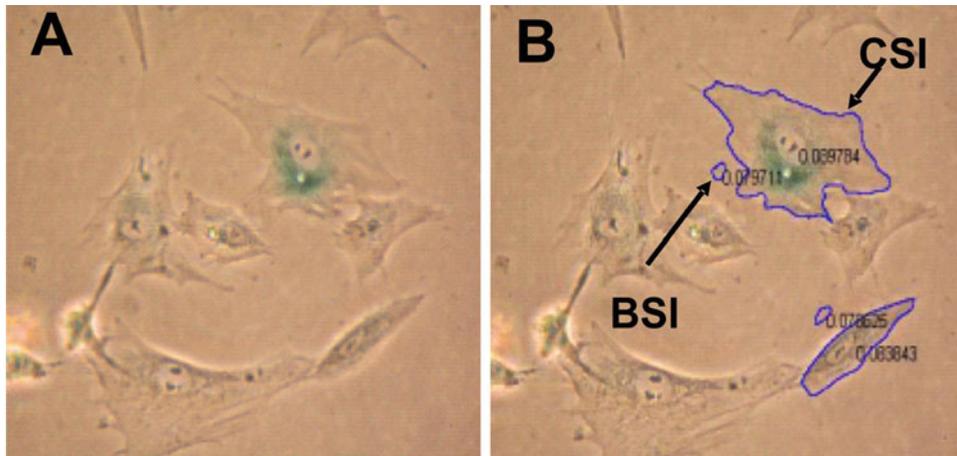
**Key words** Senescence,  $\beta$ -Galactosidase, pH 6, pH 4, Digital analysis, BGAV

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### 1 Introduction

Several markers of senescence have been described [1], among them senescence-associated  $\beta$ -galactosidase (SABG) activity. This marker has been the most extensively utilized biomarker for cellular senescence (CS) both in in vitro (reviewed in ref. 2) and in in situ [3–6] studies. The SABG method is a simple procedure, enables visualization of senescent cells in a heterogeneous population, and is specific for CS regardless of the initiating trigger [2]. The protein product of the lysosomal  $\beta$ -galactosidase 1 (GLB1) gene is the source of the SABG activity [7]. In senescent cells, both the mRNA and the protein levels of the GLB1 gene are significantly elevated, and the enzymatic activity increases concomitantly [7]. Consequently, the enhanced enzymatic activity in senescence can be detected not only at the optimal pH for activity, pH 4.5, but also at a suboptimal pH of 6.0. This finding constitutes the basis for the use of SBAG activity at pH 6.0 as a marker for cellular senescence [7].

Scoring of the SABG-stained cells has been based on experimentalist-dependent determination of a given cell staining as being positive and is not anchored in well-defined criteria. In addition, the blue staining in positive cells is impossible to quantify



**Fig. 1** Application of the digital image-processing tool to the standard SABG assay. (a) SABG staining at pH 6.0 of fibroblast cells at population doubling 34. (b) Image analysis of the field shown in Fig. 1a using the MATLAB tool described. The cell membrane borders and the background control area (*small circles*) are marked manually. The logarithmic ratio of the cytoplasmic staining intensity (CSI) divided by the background staining intensity (BSI) yields the BGAV. (Reproduced from Shlush et al. 2011 [9] with permission from BioMed Central)

objectively, such that cells with strong, moderate, or weak blue staining may all be recognized as equally positive. This renders the method insensitive to subtle effects of various stressors on CS and might contribute to the inconsistency in replicating SABG assay results in skin biopsies [8].

The limitation described above motivated us to develop a quantitative in situ SABG assay. We applied digital image processing to quantify the SABG staining, following the use of the previously described protocol of the in situ SABG assay [4]. This quantification method, based on color intensity analysis of photographed images of stained cells, generates values of cell staining intensity (CSI) measured in arbitrary intensity units. CSI values are the product of the ratio between the green plus blue values of each pixel in the manually marked cell cytoplasm and the total color (red, green, and blue) in the same cytoplasm, divided by the cell surface area. Standardization of each single CSI value is achieved by dividing the CSI value with the adjacent background staining intensity (BSI) value (Fig. 1a, b). In order to decrease the signal-to-noise ratio, we applied a logarithmic transformation to the ratio CSI/BSI and termed the value obtained  $\beta$ -galactosidase activity value (BGAV). The BGAVs are highly sensitive and reproducible [9]. In addition, we have also varied the pH of the assay to broaden the range of histochemically detectable activity and enable the detection of subtle changes in BGAVs, which are evident only when performing the SABG staining at the nonstandard pH 4.0 [9]. SABG staining can be applied also to OCT-frozen tissues [9, 10]; however, the quantitation of the resulting signals requires expertise in histological analysis of the relevant tissue.

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## 2 Materials

### 2.1 SABG Staining Solution Components

1.  $\text{MgCl}_2$ : 1 M solution in double distilled water (DDW). Weigh 9.5 g and dissolve in DDW to final volume of 100 ml. Store at room temperature (RT).
2. NaCl: 5 M solution in DDW. Weigh 29.2 g and dissolve in DDW to final volume of 100 ml. Store at RT.
3.  $\text{K}_3\text{Fe}(\text{CN})_6$ : 100 mM solution in DDW (*see Note 1*). Weigh 3.29 g and dissolve in DDW to final volume of 100 ml. Store at RT.
4.  $\text{K}_4\text{Fe}(\text{CN})_6 \cdot 3 \text{H}_2\text{O}$ : 100 mM solution in DDW (*see Note 1*). Weigh 4.22 g and dissolve in DDW to final volume of 100 ml. Store at RT.
5. Citric acid solution: 0.1 M solution in DDW. Weigh 1.92 g and dissolve in DDW to final volume of 100 ml. Store at RT.
6. Disodium phosphate ( $\text{Na}_2\text{HPO}_4$ ) solution: 0.2 M solution in DDW. Weigh 2.83 g and dissolve in DDW to final volume of 100 ml. Store at RT.
7. X-gal (5-bromo-4-chloro-3-indolyl- $\beta$ -D-galactoside) stock solution ( $\times 20$ ): 20 mg/ml dissolved in dimethyl formamide (DMF) (*see Note 1*). Aliquot 1 ml portions and freeze at  $-20^\circ\text{C}$ . We used X-gal from various sources and all worked well.

### 2.2 Preparation of the SABG Solution Mix

1. Components for the staining solution can be prepared in advance but should only be mixed together shortly before carrying out the staining.
2. Prepare a fresh mix of citric acid/phosphate buffer at the required pH. Staining at various pHs requires different ratios of the 0.1 M citric acid solution and the 0.2 M disodium phosphate solution:  
For a citric acid/phosphate buffer at pH 4.0, mix 38.55 ml of 0.2 M  $\text{Na}_2\text{HPO}_4$  with 61.45 ml of 0.1 M Citric acid.  
For a citric acid/phosphate buffer at pH 6.0, mix 63.15 ml of 0.2 M  $\text{Na}_2\text{HPO}_4$  with 36.85 ml of 0.1 M Citric acid.  
Verify the pH with pH indicator (litmus) paper.
3. Thaw 1 ml of frozen 20 mg/ml X-gal and place on ice.
4. Mix the following components to prepare a 20 ml volume of staining solution:  
12.4 ml of DDW, 40  $\mu\text{l}$  of 1 M  $\text{MgCl}_2$  (final concentration 2 mM), 600  $\mu\text{l}$  of 5 M NaCl (final concentration 150 mM), 1 ml of 100 mM  $\text{K}_3\text{Fe}(\text{CN})_6$  (final concentration 5 mM), 1 ml of 100 mM  $\text{K}_4\text{Fe}(\text{CN})_6$  (final concentration 5 mM), 4 ml of the citric acid/phosphate solution (prepared at chosen pH), and 1 ml of 20 mg/ml X-gal (final concentration 1 mg/ml).

### **2.3 Cell Fixation Components**

1. Cold phosphate-buffered saline (PBS). To prepare PBS dissolve 8 g of NaCl, 0.2 g of KCl, 1.44 g of Na<sub>2</sub>HPO<sub>4</sub>, and 0.24 g of KH<sub>2</sub>PO<sub>4</sub> in 800 ml of H<sub>2</sub>O. Adjust pH to 7.4 and add H<sub>2</sub>O to a final volume of 1 L.
2. 25 % glutaraldehyde solution (Sigma G6257) (*see Note 1*).
3. Prepare fixation solution immediately before fixation by diluting 1:50 the 25 % glutaraldehyde solution with cold PBS to a final concentration of 0.5 % glutaraldehyde (*see Note 2*).

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## **3 Methods**

### **3.1 Preparing Cells for SABG Staining**

1. Seed cells 48 h prior to staining at a density of  $2-4 \times 10^4$  cells/well in a six well plate (*see Note 3*).

### **3.2 Staining Procedure**

1. Remove media from cell culture.
2. Wash cells briefly with cold PBS.
3. Add 2 ml of 0.5 % glutaraldehyde to each well, and place on ice for 5 min.
4. Wash briefly twice with cold PBS.
5. Add 2 ml of freshly prepared SABG staining solution.
6. Incubate for 8 h at 37 °C. Do not incubate in a CO<sub>2</sub> incubator.
7. End staining procedure by washing 3 × 5 min with cold PBS.
8. Add 3 ml of PBS and store at 4 °C until images are collected (*see Notes 4 and 5*).

### **3.3 Microscopy and Image Acquisition**

1. Color images of the SABG-stained cells should be captured with a color digital camera, mounted on an inverted light microscope, using a 10× objective. Any high-quality color digital camera is adequate. Optimal exposure should be set for each individual experiment and should be constant for all images taken for a specific experiment.
2. Images must be saved as TIFF files. Other formats are not acceptable for analysis.
3. A minimum of 250 cells should be analyzed for each experiment or time point in order to reach statistically valid BGAVs (*see Note 6*).

### **3.4 Digital Analysis of the Acquired Images**

1. Use MATLAB package version 7.0 or above.
2. Download the MATLAB applications for cell marking (segmentGui.m) and for color analysis (postProcess3.m) from the following website:  
<http://md.technion.ac.il/pictures/storage/45/47.zip>

3. The files: `postprocess3.m`, `segmentGui.asv`, `segmentGui.fig`, `segmentGui.m`, and `batchscript.m` must be placed in the same folder with the figures. Therefore, for each series of images analyzed, the above four files should be copied into the figures' directory.
4. Point MATLAB to the working folder (the folder with the images and applications of the software). Press on the three dots (...) on the MATLAB toolbar to open the browser for the folder window.
5. In the command window type `segmentGui` and then press "Enter".
6. Double click on the figure you want to analyze (\*.tif).
7. Press the button "start segmentation."
8. Mark the button "whole cell segmentation."
9. Mark the cell borders (*see Note 7*) and press "Enter" when you finish marking each cell.
10. Mark a control region adjacent to the analyzed cell. The control region must be smaller than the smallest cell (in the figure) for the program to recognize it as a control region. Mark the control regions with three points, as near as possible to the cell itself (see example in Fig. 1) (*see Note 7*).
11. Press "stop segmentation" when you have finished analyzing all cells in a figure.
12. Press "save segmentation."
13. Close the `segmentGui` application (an error note will appear—ignore it).
14. For single-image analysis type in the MATLAB command window exactly the following:  
`postProcess3 ("Image Name.tif")`  
In the place of "Image Name" type the name of your specific image.
15. For multiple-image analysis type in the MATLAB command window the command "batch\_script" and all the marked and saved figures will be analyzed (it may take a few minutes for analysis of 20 figures).
16. The output of `postProcess3.m` (for a single image) and of `batch_script` (for multiple images) will be both a figure file of the results and a text file (both will be created in the same directory of the processed images) that may be easily copied to EXCEL or to any other text editor.
17. After copying the data to an EXCEL file, the columns will appear in the same order as in the example file (Fig. 2).

	A	B	C	D	E	F	G	H	I	J	K	L
1	Date + Hour	Figure Name	CSI	BSI	Total staining per cell	Total staining per control cell	Cell area	Control area	CSIBSI	BGAV	Note	
2	21/05/2007 10:46	Image00001.tif	0.134	0.073	1062.82	17.40	7937.00	239.00	1.84	263.78		
3	21/05/2007 10:46	Image00001.tif	0.151	0.075	1717.75	11.21	11365.00	150.00	2.01	303.92		
4	21/05/2007 10:46	Image00001.tif	0.086	0.077	1040.80	18.89	12054.00	218.00	1.12	48.01		
5	21/05/2007 10:46	Image00001.tif	0.083	0.075	1200.75	21.89	14541.00	290.00	1.11	44.02		
6	21/05/2007 10:46	Image00001.tif	0.084	0.073	2474.50	13.36	29411.00	183.00	1.15	60.96		
7	21/05/2007 10:46	Image00001.tif	0.099	0.077	3255.42	26.08	32968.00	340.00	1.29	109.14		
8												
9												

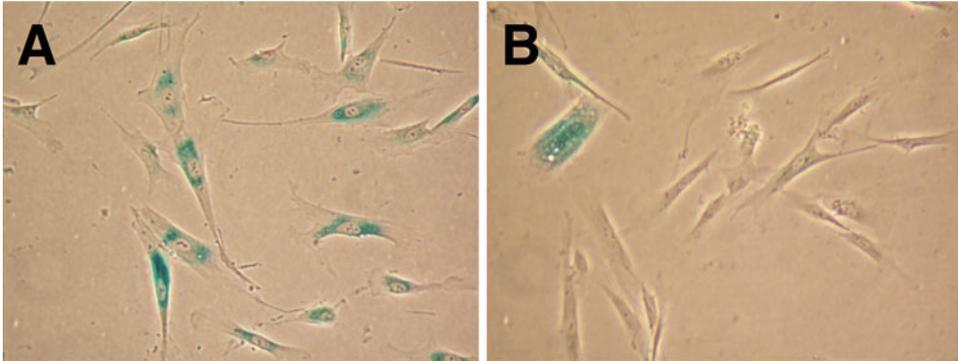
**Fig. 2** An example of an EXCEL file containing the results produced by the MATLAB analysis of the SABG staining

### 3.5 Statistical Analysis

In most instances the BGAVs did not fit a normal distribution according to either Shapiro-Wilk  $W$  or Kolmogorov-Smirnov normality tests [9]. Therefore, rather than using a student's  $t$ -test, which assumes normal distribution, we use the Kolmogorov-Smirnov test for analysis of the BGAV values.

## 4 Notes

1. Handle with caution. This material is a biohazard.
2. Alternatively, fixation may be carried out using freshly prepared 3 or 4 % formaldehyde, at RT for 5 min, as described in other SABG staining protocols [11, 12].
3. This cell density ensures that cells will not reach confluence before staining is carried out and is adjusted for human primary fibroblasts. If rapidly growing cells are plated for staining, adjust the number of cells seeded in order to avoid confluent cultures at the day of staining. Avoid seeding cell clumps. These conditions will enable better scoring of SABG-positive cells.
4. We found that staining peaks 16 h after the SABG solution is replaced with PBS, and following 48 h the staining reaches a plateau and remains stable for as long as 2 months [9]. We therefore recommend acquisition of the images for analysis at least 48 h after removing the SABG solution and preferably during the first 2 weeks after the staining was performed.
5. When staining is carried out at pH 4, strong staining is evident in all cells after 8 h (see example in Fig. 3). Varying staining intensities are not apparent by visual inspection alone after staining at pH 4 and however are detected by digital analysis.
6. The number of images captured should be adjusted based on the density of the cells viewed per microscope field.



**Fig. 3** SABG staining of primary fibroblasts (population doubling 38) at different pHs. **(a)** Staining at pH 4. **(b)** Staining at pH 6

7. A few tips for cell marking (segmentation):

- (a) The borders of adjacent cells must not overlap. If they do, analysis of the whole figure will fail. Therefore, mark cells that are far enough apart to avoid this problem.
- (b) Start marking at one point at the cytoplasmic border of the cell and continue by clicking with mouse left click along the border of the cytoplasm all around the cell until you reach the starting point. Once done press “Enter” and the line will close automatically.
- (c) When marking a cell – do not create any loops that will automatically be regarded as segments (cells) and will create an error.
- (d) You may mark one control area per figure to save time, although in most cases it will result in a much less accurate quantitation due to an unequal spread of background over the figure. Therefore, standardization to the figure background is best achieved by marking a background area adjacent to each analyzed cell.

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