



A cross-platform public domain PC image-analysis program for the comet assay

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Abstract

The single-cell gel electrophoresis, also known as the comet assay, has gained wide-spread popularity as a simple and reliable method to measure genotoxic and cytotoxic effects of physical and chemical agents as well as kinetics of DNA repair. Cells are generally stained with fluorescent dyes. The analysis of comets—damaged cells which form a typical comet-shaped pattern—is greatly facilitated by the use of a computer image-analysis program. Although several image-analysis programs are available commercially, they are expensive and their source codes are not provided. For Macintosh computers a cost-free public domain macro is available on the Internet. No ready for use, cost-free program exists for the PC platform. We have, therefore, developed such a public domain program under the GNU license for PC computers. The program is called CASP and can be run on a variety of hardware and software platforms. Its practical merit was tested on human lymphocytes exposed to γ -rays and found to yield reproducible results. The binaries for Windows 95 and Linux, together with the source code can be obtained from: <http://www.casp.of.pl>.

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1. Introduction

The single-cell gel electrophoresis (SCGE), also known as the comet assay because damaged cells form a comet-shaped pattern after electrophoresis, is a sensitive method to measure genotoxicity and cytotoxicity of chemical and physical agents [1–3]. The comet assay has also been used to analyse the capacity of cellular DNA repair [4–6].

Although the methodology of the comet assay is straightforward and does not require sophisticated equipment, the analysis of comet images is not so simple. The visual classification of comets on the basis of their morphology and degree of damage is possible, but is not very precise because it relies on subjective decisions made by the investigator [3]. A better sensitivity is achieved by computer analysis of comet images. Several commercial applications are available, however, they are generally sold as combined software/hardware packages and are rather expensive. In addition they cannot be modified because their source codes are not supplied. Given the rapid

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development of computer technique, obtaining of comet images through a video camera or by scanning of photographs does not seem to be problematic for most research laboratories. A bigger obstacle is the availability of specialized software for the analysis of comet images.

A public domain macro is available on the Internet for NIH Image, a graphic program running under Macintosh computers (<http://rsb.info.nih.gov/nih-image/>) [7]. No corresponding, freeware programs exist for the PC platform. We have, therefore, developed a simple and user-friendly public domain program for the analysis of comet images. Its graphic user interface is based on a cross-platform library FOX (<http://www.fox-toolkit.org>), and so the whole application can be compiled and run under a variety of operating systems,

including MS Windows and Linux. The program, together with the source code is publicly available under the GNU License at <http://www.casp.of.pl>.

2. Description of the program

The program works with either color, or gray-scale images of fluorescence-stained comets saved in TIF format. Comets stained with silver (dark cells on white background) must be converted into negative images. An unlimited number of images can be marked, and the program will load them successively into a “image view” window (Fig. 1). Only comets oriented from left-hand (head) to right-hand side (tail) can be analysed correctly. The user can adjust various thresholds

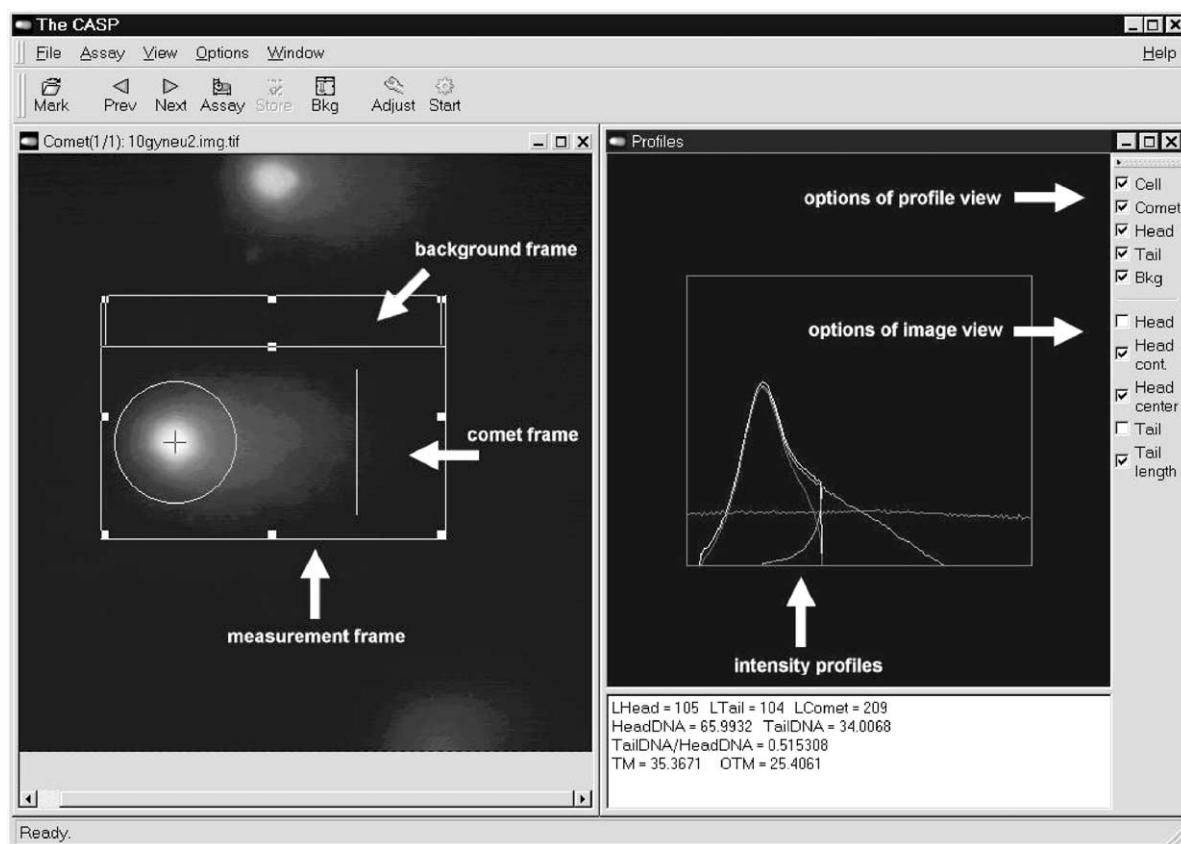


Fig. 1. View of the program windows. In the left-hand window the comet image with the measurement frame is visible, with tail and head marked. The measurement frame is split into two frames: a background and a comet frame. In the right-hand window the intensity profiles are plotted and the checkbox bar is shown with options of profile and image view. Below the profile window selected measurement results are printed.

of sensitivity and save the adjustments for future use. A measurement frame is drawn on the screen and its size adjusted. The adjustments are frozen to prevent accidental modification. The frame is moved onto a cell and measurement is activated. An intensity profile shows up on a “profile” window together with selected result values (Fig. 1) and the result can be saved. In addition to such parameters as head radius, tail length etc, the program calculates the tail moment (TM) and the Olive tail moment (OTM). If several cells are present in the same picture, the user can proceed with the measurement of another cell in the same picture or can load a new picture.

The saved results can be visualized during the working session. When measurements are terminated, the results are exported into a text file and they can be imported into a commercial spreadsheet calculation program.

Once all adjustments are set, 25–50 measurements can be performed in less than 10 min.

3. Hardware requirements and picture quality

The minimal hardware requirement for CASP is a PC with Windows 95 and a graphic card that supports at least 256 colors and with a screen resolution of 600×800 pixels. We have successfully tested the program on a 486 processor with 133 MHz and 16 MB RAM.

CASP analyses TIF images (LZW compressed or non-compressed). These must be either collected directly from the microscope through a CCD camera and an image-acquisition program or by scanning of photographs. Numerous image-acquisition programs are available commercially, which are sold together with appropriate CCD cameras (e.g. ISIS, Metasystems, Germany or MultiScanBase, Microsystems, Poland). CASP will load and analyse 8-bit greyscale images or color images of 8, 16, 24, 32 or 48 bits. Reliable measurements require that the comet images have sufficient resolution. In case of images collected through a CCD camera, the resolution will depend on the camera type and the microscope objective. In case of scanned photographs—on the scanner settings. The optimal head radius of a measured comet is between 40 and 80 pixels. Pictures of a total size of 512×512 pixels (75×75 DPI) fit into the image view window of

CASP. The head radius of the comet shown in Fig. 1 has a diameter of about 70 pixels.

4. Description of the algorithm

Images are imported from TIF files. To recognize the comet head and tail we assume that the most intensive points are placed in the head and the comet is oriented from the left-hand side (head) to the right-hand side (tail) of the image. Heavily damaged or apoptotic cells, which are easily recognized when encountered, do not always have the most intensely stained points in the head and measuring them will not yield correct results.

The program starts by determining several basic values.

- The maximum image intensity (IMAX).
- The background level intensity (IBKG), defined as the mean background intensity taken from the background test window (Fig. 1).
- The comet threshold (TC), where TC lies between IBKG and IMAX. This threshold value is set by the user and is necessary for the algorithm to work correctly, as only pixels with intensity greater than TC can belong to a comet.

Next, the algorithm attempts to find:

- the head center (HC),
- the head radius (HR),
- head area (HA),
- tail length (TL),
- tail area (TA).

Having computed these parameters we can easily determine all other values of interest.

4.1. Detection of the head center location and the head radius

The main problem with distinguishing between comet head and tail is the asymmetry of the image. The light intensity drops abruptly on the left-hand side of the head to the values typical of the background, while it is very difficult to tell where the border between the head and the tail is. Therefore, the methods used for analysis of the left-hand side of the comet head differ from those employed for the right-hand side.

We start by looking for the brightest points with 80–100% of maximum intensity and then calculate the head center as their center of gravity. Note that the TC is assumed to be much smaller than 80%, so only a relatively small fraction of the comet head is used to determine its center. To find the head radius (HR) we first find all points belonging to the so-called left-hand edge of the head. To find these points the program scans the image leftwards from the head center until it finds the first point with intensity below TC. This point will be called the leftmost point (LMP). LMP is the first point identified by the program as belonging to the left-hand edge of the head. For each point on the line between LMP and the head center the program scans the image up and down until it finds a pixel of intensity less than TC. All these pixels constitute the left-hand edge of the head. We define the radius of the head (HR) as a mean distance between the head center and all points located on the left-hand edge of the head.

If one draws a circle centered at HC of radius HR one finds that the edge of the comet head usually slightly differs from it. To take this into account we translate the upper-left part of the circle upwards by 10% of HR and, similarly, its lower-left part by 10% downwards. We now assume that a pixel belongs to the head center if and only if its intensity exceeds the required threshold (TC) and lies inside the modified circle.

Identification of the comet tail is also nontrivial. The major problem comes from the fact that if we locate all pixels with required intensity (TC), we usually find that they form one big “continent” surrounded by many disconnected islands. We have therefore implemented two methods of determining the area and length of the comet tail. In the first method the tail area is recognized simply as a compact region adjacent to the head on its right-hand side. In the second method we identify the tail with all pixel whose intensity exceeds TC. In both methods the tail length is calculated as the distance between the x -coordinates of the right-most point of the tail and the right-most point of the head. Now the program can proceed to calculate a few more parameters relevant to the comet assay [7].

- DNA head (DNAH), sum of intensities of all points of the head.

- DNA tail (DNAT), sum of intensities of all points of the tail.
- Percent tail DNA (%DNAT), $\%DNAT = 100DNAT / (DNAH + DNAT)$.
- Percent head DNA (%DNAH), $\%DNAH = 100 - \%DNAT$.
- Tail moment (TM), the product of the tail length and percent tail DNA, $TM = TL \times (\%DNAT)$.
- Olive tail moment (OTM), the product of the distance (in x direction) between the center of gravity of the head (CGH) and the center of gravity of the tail (CGT) and percent tail DNA, $OTM = (CGTx - CGHx) / \%DNAT$.

5. Application of the program

The program was tested by analyzing comets of human lymphocytes irradiated with γ -rays.

5.1. Cells and irradiation

Human peripheral blood lymphocytes were obtained from an adult male, separated from whole blood using Histopaque-1077, washed in RPMI-1640 and suspended in phosphate buffered saline. The cells were irradiated on ice, through a candle-wax bolus to allow charged particle equilibrium, with 1, 2 and 3 Gy (1.25 MeV γ -rays, ^{60}Co Siemens Theratron Elite 80).

5.2. Comet assay

The comet assay was carried out according to [1]. In brief, 100 μl of cell suspension was mixed with 100 μl of 2% low-melting temperature agarose (Sigma) and spread on a slide pre-coated with 500 μl of 0.5 % agarose. After 10 min of gelling at 4 °C, the cells were lysed for 1 h in a solution of 2.5 M NaCl, 100 mM EDTA, 10 mM Tris, 1% Triton X-100 at pH 10 and 4 °C. The gels were washed 3 times in re-distilled water for 5 min and placed on a horizontal gel electrophoresis unit (Kucharczyk, Poland). The unit was filled with electrophoresis buffer (300 mM NaOH, 1 mM EDTA, pH 13) and after 40 min of ‘unwinding’ at 4 °C, electrophoresis was carried out for 25 min (1 V/cm). The slides were washed 3 times in 400 mM Tris buffer, pH 7.5 and stained with 50 μl of 1 μM DAPI for 24 h.

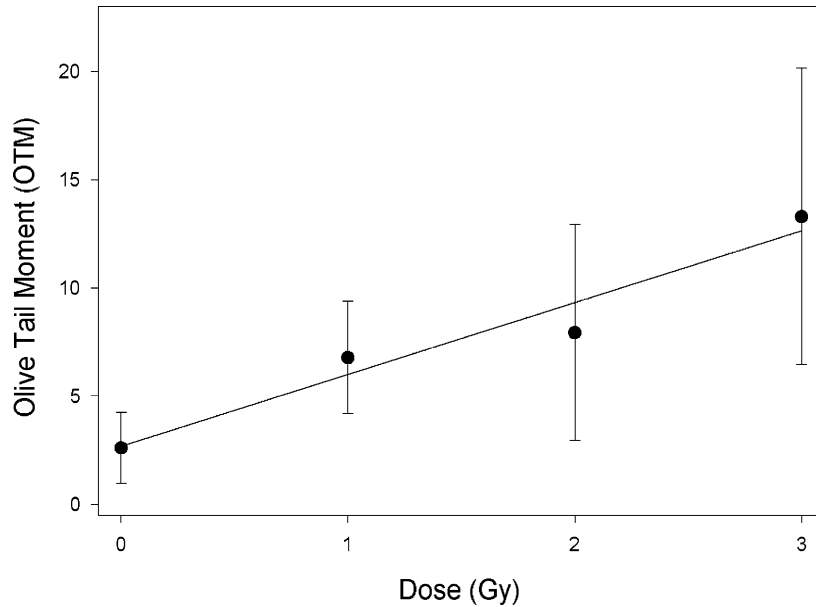


Fig. 2. The dose–response curve for Olive tail moment in cells exposed to γ -rays and analysed with the help of the described program. Error bars represent standard deviations of single cell measurements. Linear regression performed by the least square method. At least 20 cells were measured per dose.

All steps were conducted in dark to prevent additional DNA damage. Comet images were obtained using a NIKON fluorescence microscope coupled with a Pulmix TM 765 video camera and commercial software for image acquisition. At least 20 images per dose were analysed. The obtained dose–response curve is presented in Fig. 2.

Table 1

Results (expressed as Olive tail moments) obtained after repeated measurements of two series of comet images of lymphocytes irradiated with 2 Gy

	Series 1		Series 2	
	Scorer 1	Scorer 2	Scorer 1	Scorer 2
Mean value	7.98	7.98	9.02	9.00
Std	2.79	2.79	2.93	2.92
95% CI	1.05	1.05	1.08	1.08
Min. value	3.76	3.76	2.63	2.81
Max. value	13.34	13.34	16.74	16.68

Each series of images was analysed by two independent scorers. Images of series A and B were obtained from two, parallel microscopic slides. Thirty images were analysed per series. Std: standard deviation, CI: confidence interval.

In order to test the reproducibility of the image analysis, repeated measurements of comets were performed on lymphocytes irradiated with a dose of 2 Gy. Independently of each other, two scorers measured two sets of pictures obtained from two different microscopic slides. Measurements were performed with the same threshold settings, but each scorer independently adjusted the size of the measurement frame before the measurement. The results are presented in Table 1 and show identical results for series 1 and very close results in series 2. The mean Olive tail moment values of both series lie within the 95% confidence intervals. Thus, both results are indicative of a very good reproducibility of measurements.

6. Conclusions

The program presented here is a cost-free and reliable tool for measuring of comets on a PC computer. According to the suggestion of Helma and Uhl [7] we have developed the program as a multi-platform application under the GNU License. This means that the source code is available on the Internet or directly

from the authors. The program can thus be modified by the user and new measurement methods can be implemented. Windows and Linux executables can also be downloaded in a version ready for use without any modifications.

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