

A public domain image-analysis program for the single-cell gel-electrophoresis (comet) assay

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Abstract

The single-cell gel electrophoresis (or comet) assay has gained widespread acceptance as a cheap and simple genotoxicity test, but it requires a computer-assisted image-analysis system. As commercial programs are expensive and inflexible, we decided to develop an image-analysis system based on public domain programs and make it publicly available for the scientific community. Our system is based on the scientific image-processing program NIH Image, and was written in its Pascal-like macro language. User interaction was kept as simple as possible, to enable the measurement of a large number of cells with a few keystrokes. Therefore, the time for image analysis is very low, even on slow computers. The comet macro can be obtained from <http://mailbox.univie.ac.at/christoph.helma/comet/>, NIH Image is available at <http://rsb.info.nih.gov/nih-image/>. Both programs are free of charge. © 2000 Elsevier Science B.V. All rights reserved.

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1. Introduction

The single-cell gel electrophoresis (or comet) assay is a rapid and sensitive fluorescence microscopic method for the detection of primary DNA damage at the individual cell level [4,5]. During the last 10

years, it has gained widespread acceptance for genotoxicity testing. Compared to other genotoxicity tests, the comet assay is a cheap and simple method, but it is advisable to use a computer-assisted image-analysis system to measure the comet parameters. It is possible to purchase commercial applications for this purpose, but they have several disadvantages:

1. They are expensive (> 10.000 Euro (US\$)/system without hardware).
2. Their source code and measurement algorithms are not available. This has several drawbacks, especially for scientific applications:
 - It is impossible to understand (and evaluate) the measurement algorithms;

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- It is impossible to improve the measurement methods and experiment with new parameters for genetic damage;
- It is impossible to adopt the program for other purposes in the lab (e.g., counting of Ames plates or analysis of electrophoretic gels).

3. The operation of some programs is complicated and time consuming (requirement of two monitors, many adjustments for one measurement).

The goal of the present work was to develop an image-analysis program for the comet assay, which is easy and efficient to use, and to make the source code publicly available. This work is based on the public-domain image processing and analysis program NIH Image. It was developed at the Research Services Branch (RSB) of the National Institute of Mental Health (NIMH), part of the National Institutes of Health (NIH) and is widely used in research areas ranging from astronomy to medical sciences. NIH Image was originally developed on Macintosh computers, but meanwhile a free PC version and a Java program are also available. NIH Image and its derivatives can be obtained from: <http://rsb.info.nih.gov/nih-image/>

NIH Image can be customized in three ways: via a built-in Pascal-like macro language, via externally compiled plug-in modules and on the Pascal source-code level. We decided to use the built-in macro language to facilitate adoptions and the portation to other operating systems. The following sections describe the operation from a user's point of view (Section 4) and the underlying definitions, methods and algorithms for the measurement of various comet parameters (Section 5). The macro is publicly available under the GNU License at <http://mailbox.univie.ac.at/christoph.helma/comet/>.

2. The comet assay

The comet assay is based on the principle that DNA damage reduces the size of DNA fragments. This effect is detected by applying an electrophoretic field to lysed cells. The stained DNA fragments form a typical comet-shaped migration pattern. A detailed description of the protocol employed in our experiments can be found in Ref. [6]. Briefly, cells were exposed to test compounds, spread on agarose-coated

slides, lysed, subjected to electrophoresis and stained with ethidium bromide.

3. Image analysis

The slides were evaluated under a fluorescence microscope (Nikon Optiphot-2 with a Nikon Plan Fluor 10/0.3 objective). The Images were acquired from the microscope with a JVC TK-C1360 1/2-in. CCD video camera. Experiments with several other camera models demonstrated that it is very important to use a highly sensitive camera with an on-chip integration, although expensive fluorescence cameras are not required. It is possible to integrate pictures with NIH Image, but this process is much slower than on-chip integration. Image analysis was performed on an Apple Power Macintosh 7500/100 (PPC601, 100 MHz, 16 MB RAM, MacOS 7.6.1.) without additional video hardware and on an Apple Power Macintosh G3 (PPC G3 233 MHz, 96 MB RAM, MacOS 8.1) with a Pinnacle miromOTION DC30 framegrabber card.

The images were analyzed with the public-domain NIH-Image program (developed at the U.S. National Institutes of Health and available on the Internet at <http://rsb.info.nih.gov/nih-image/>) and the comet analysis macro described below (available on the Internet at <http://mailbox.univie.ac.at/christoph.helma/comet/>). The macro is an extensively documented plain text file, thus it should be possible to make adjustments even without programming experience.

4. Using the comet macro

We tried to keep the users' interaction with the macro as simple as possible to reduce the time and keystrokes required for each measurement and to ensure uniform measurement conditions. For this reason, we did not implement an interactive mode, which allows the user to define head or tail areas, but it is possible to reject measurements that are obviously wrong. To compensate for different light intensities, the threshold between comet and background may be changed, the new settings are automatically memorized for the current measurement session.

Table 1
Summary of the measurement operations

| Step | Keystroke | Task |
|------|------------|---|
| 1 | C | Opens a camera window to choose a suitable area on the slide |
| 2 | A | Imports the picture into NIH Image, inverts the picture and distinguishes DNA from the background |
| 3 | mouseclick | Selects the cell for measurement |
| 4 | M | Measures the comet |
| 5 | K | Accepts the measurement and proceed with Step 3 (or 1) |
| 6 | D | Discards the measurement and proceed with Step 3 (or 1) |
| 7 | E | Enters a new slide code |
| 8 | Q | Quits the measurements |

Table 1 gives a quick overview about the steps needed for one measurement. First, it is necessary to load the Macro into NIH Image by using the Special-Load Macros' Menu Item. One keystroke

opens a camera window for adjustments on the microscope (Fig. 1, Step 1 in Table 1). After selecting and focusing a suitable area of the slide on the microscope, the picture, which may contain one or

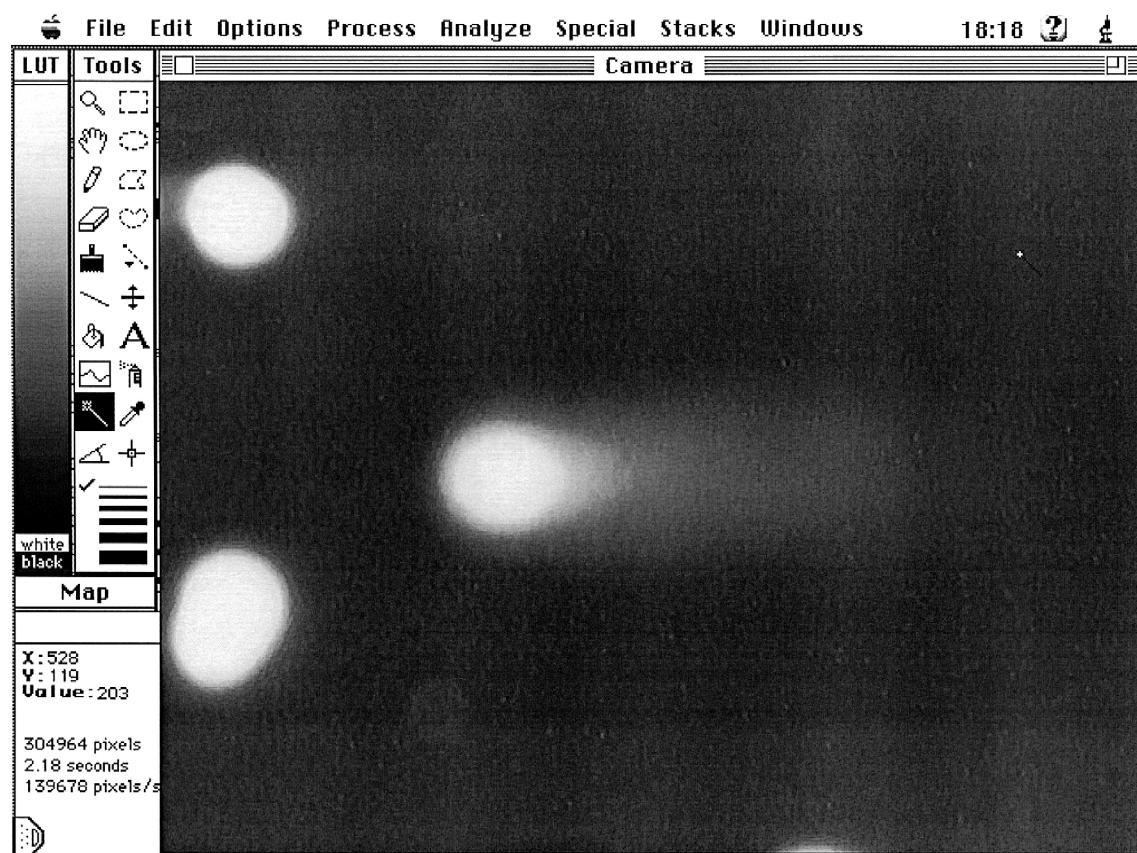


Fig. 1. Selection of a new area on the slide (Step 1 in Table 1).

more cells, is imported into NIH Image (Step 2 in Table 1). Each cell on this picture can be selected consecutively with the outlining (wand) tool for measurement (Fig. 2, Steps 3 and 4 in Table 1). A schematic outline, indicating the most important items of the comet (Head, Tail) and measured values, is presented to the user and he/she is asked to accept or discharge the measurement (Fig. 3, Steps 4 and 5 in Table 1). The user may then proceed with the measurement of another cell of the same picture (Step 3 in Table 1, already measured cells are marked to prevent accidental duplicate measurements) or with the acquisition of a new picture (Step 1 in Table 1).

The macro tries to enforce coded slides by asking for code numbers instead of dose/compound information. After 50 measurements, the user will be automatically reminded to change the slide. Mea-

surements for each slide are stored in text files which may be imported into spreadsheet or statistics packages for further analysis. In addition, we wrote a Perl script to facilitate the necessary data rearrangements for statistical analysis (also available at <http://mailbox.univie.ac.at/christoph.helma/comet/>).

5. Measurement of comet parameters

There is considerable ambiguity about the definition and relevance of parameters to be measured in the comet assay. In many cases, publications using the comet assay fail to provide information about the parameters used in their work. This section will explain the measurement methods used by the comet macro. Together with the comments contained in the macro itself, it will be easy to reconstruct and mod-

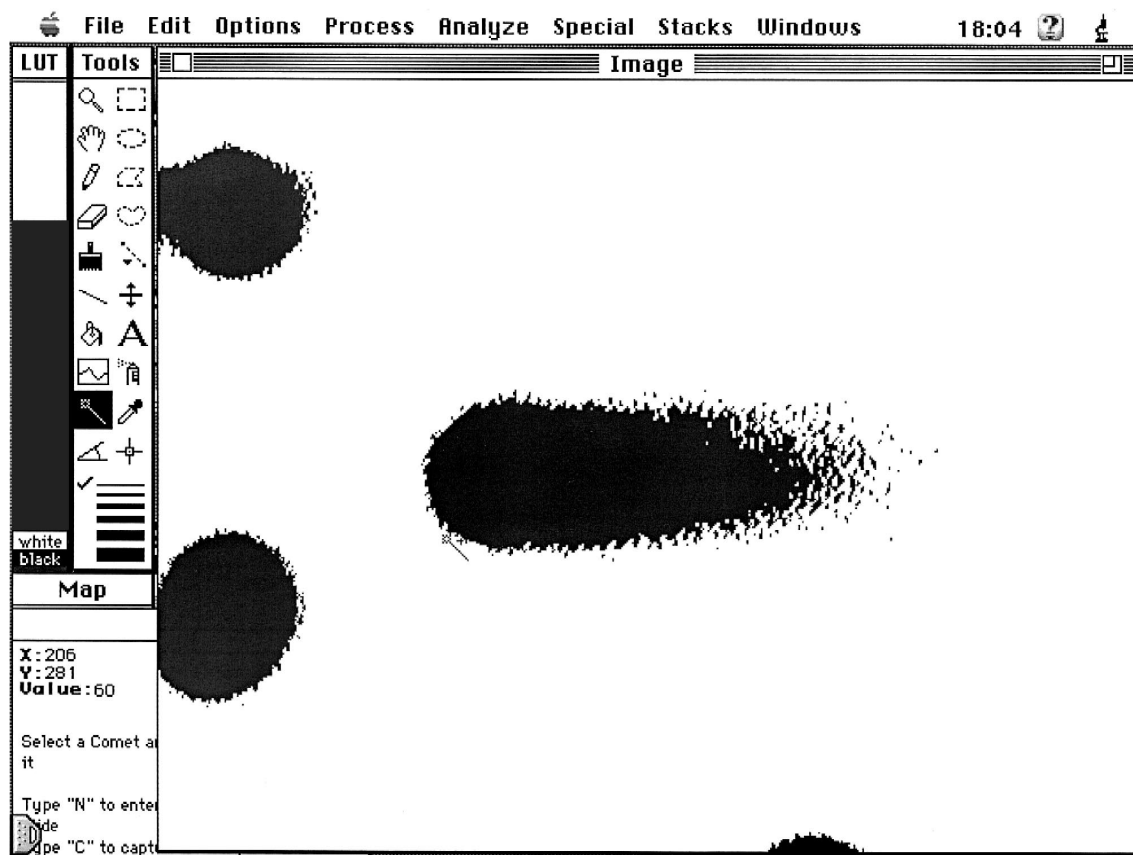


Fig. 2. Inverted and highlighted comets (Step 2 in Table 1). The comet in the center will be measured next.

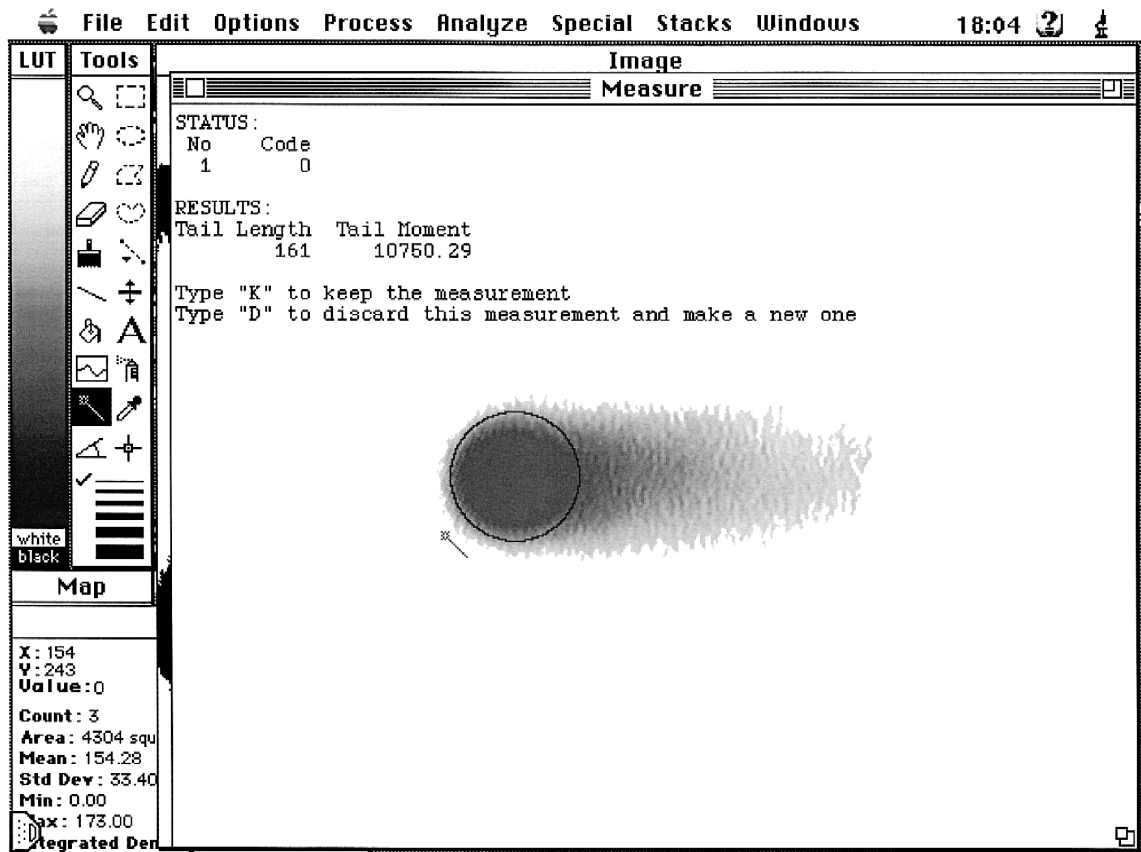


Fig. 3. Display of the measured comet and the most important parameters (Step 4 in Table 1). The user may accept or discard the measurement.

ify the measurement methods. Fig. 4 visualizes the basic properties and variables of a comet. The macro assumes that comets are oriented from left (head) to right (tail). A wrong orientation will result in measurement errors, which are easily detected by the information window, displayed after each measurement.

5.1. Detection of the border of the comet

The comet macro automatically sets a threshold value to distinguish the comet from the background. It is possible to adjust this value using the LUT tool of NIH Image. The manually adjusted values will be automatically recorded and used for further measurements to enable uniform conditions within an experiment. Individual cells are selected for measurement

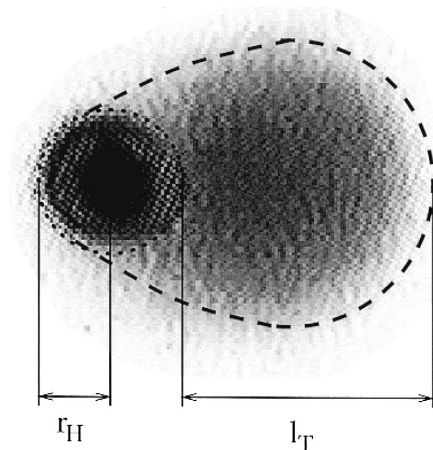


Fig. 4. Definition of basic comet properties. r_H , head radius; l_T , tail length; dotted lines, boarder of the comet head; dashed lines, boarder of the comet tail.

by the outline (wand) tool. The macro copies the selected cell to another window and starts the measurements with the calculation of the area and DNA content (intensity) of the whole comet (including head and tail) using the functions of NIH Image. Then the head is located and measured. Finally, the properties of the tail are computed as difference between the values for the complete comet and the head:

- Comet Area, A_C : The area covered by the whole comet.
- Mean Comet Intensity, \bar{I}_C : The mean intensity of the pixels located in the comet area.
- Comet DNA, DNA_C : The sum of the intensities located in the comet area — $DNA_C = A_C \cdot \bar{I}_C$.

5.2. Detection of the center of the head

Theoretically, the center of the head should be located at the point of maximum intensity. To avoid misplacements due to noise in the picture, we look for the area with 100–80% of the maximum intensity and locate the center of the head at the center of it. If several separated areas with high intensities are detected, the largest one is selected for the computation of the head coordinates.

Although this procedure is appropriate only if the maximum intensity occurs within the head region and not in the tail, it worked well in our lab during the last 3 years. For investigators using different cells or genotoxic agents, it may be necessary to make adjustments in the macro (e.g., to select the left-most area with high intensity for the estimation of the head coordinates). The distance between the left-most edge of the whole image and the center of the head is the radius of the head (Fig. 4). It is assumed that the shape of the head is circular. This enables the measurement of the following parameters:

- Tail length, l_T : Distance between the right end of the head and the end of DNA migration (according to Ref. [1]). There is no general agreement on this definition, other authors equal the total comet length to the tail length [5] or commence tail length from the center of the head [3].
- Head Area, A_H : The area covered by the cell nucleus.
- Mean Head Intensity, \bar{I}_H : The mean intensity of the pixels located in the head area.

- Head DNA, DNA_H : The sum of the intensities located in the head area — $DNA_H = A_H \cdot \bar{I}_H$.

5.3. Calculation of tail parameters

The subtraction of the head parameters from the corresponding properties of the whole comet yields the following tail parameters:

- Tail Area, A_T : The area covered by the comet tail — $A_T = A_C - A_H$.
- Tail DNA, DNA_T : The difference between total comet DNA and head DNA — $DNA_T = DNA_C - DNA_H$.
- Percent Tail DNA, % DNA_T : The percentage DNA in the tail — $\% DNA_T = 100DNA_T / (DNA_T + DNA_H)$.

For the tail moment, we use the simplified definition proposed by Ashby et al. [1] as follows.

- Tail Moment, M_T : The product of tail length and fraction of DNA in the tail — $M_T = l_T \cdot \% DNA_T$.

Discrepancies in the calculation of tail moments arise from the diverse definitions of comet length (see above) and from the presence or absence of denominators in the defining equation. Other definitions need the detection of the center of gravity of the tail [2]. We have implemented such an algorithm in previous versions of the macro (available on request from the authors), but the results were much noisier than with the simple definition above.

6. Conclusion

The program presented in this article provides a cost-efficient, fast and reliable method for the automated measurement of comet-assay parameters. In our lab and others, it was used during the last 3 years without any problems. Due to its simple design, it is very easy to use and runs very fast, although it is based on a rather slow interpreted macro language.

It is, in our opinion, especially in the scientific domain, very important to make measurement methods transparent and reproducible. The best way to achieve this goal is to publish the source code of measurement programs. This allows the critical examination of algorithms and the implementation of new measurement methods. The comet measurement program presented in this article is one step into this direction.

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