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Method

OpenComet: An automated tool for comet assay image analysis

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ABSTRACT

Reactive species such as free radicals are constantly generated *in vivo* and DNA is the most important target of oxidative stress. Oxidative DNA damage is used as a predictive biomarker to monitor the risk of development of many diseases. The comet assay is widely used for measuring oxidative DNA damage at a single cell level. The analysis of comet assay output images, however, poses considerable challenges. Commercial software is costly and restrictive, while free software generally requires laborious manual tagging of cells. This paper presents *OpenComet*, an open-source software tool providing automated analysis of comet assay images. It uses a novel and robust method for finding comets based on geometric shape attributes and segmenting the comet heads through image intensity profile analysis. Due to automation, OpenComet is more accurate, less prone to human bias, and faster than manual analysis. A live analysis functionality also allows users to analyze images captured directly from a microscope. We have validated OpenComet on both alkaline and neutral comet assay images as well as sample images from existing software packages. Our results show that OpenComet achieves high accuracy with significantly reduced analysis time.

Introduction

Cellular DNA is constantly attacked by chemical agents including reactive oxygen and nitrogen species (ROS/RNS) and other environmental factors such as UV and radiation. DNA damage is associated with the etiology of many major diseases. In particular, oxidative DNA damage has been implicated in cardiovascular disease, neurodegenerative diseases and ageing, and its pathological relevance at various stages of carcinogenesis has been studied (reviewed in [1]). Oxidative DNA damage is considered to be one of the important parameters in biomonitoring the human health impact of dietary antioxidants, smoking, and other lifestyle and environmental factors such as carcinogens or UV rays [2,3]. Various experimental methods have been proposed for measuring oxidative DNA damage [3]. The comet assay, in specific, is widely used for measuring oxidative and other types of DNA damage [4].

The comet assay, also known as single-cell gel electrophoresis (SCGE), is a simple, sensitive and reliable method for studying DNA damage induced by physical and chemical agents [5–7]. The basic principle of the comet assay method is simple. Cells are embedded in agarose and lysed, followed by electrophoresis. Upon electrophoresis, undamaged DNA in a supercoiled state remains intact while damaged DNA strand breaks are revealed. These relaxed loops of damaged DNA extend to the anode to form a comet-shaped structure. Comets can then be visualized by staining with a DNA-binding dye using fluorescence microscopy. To assess the level of DNA damage, the comet size, shape and the amount of DNA within it needs to be measured. Ostling and Johanson introduced the microgel electrophoresis method to measure DNA strand breaks under neutral pH condition [8]. Later, the assay was modified and performed under alkaline pH conditions [5], which remains in its most commonly used form. Variants of the comet assay protocol have been proposed for measuring different forms of DNA damage such as single strand breaks, alkali-labile sites [5], double strand breaks [9] as well as DNA cross-links [10].

The comet assay has several features which make it an attractive choice for measuring DNA damage, including the need for a relatively low number of cells per sample and the availability of data at the individual cell level [11]. Measurements at the single-cell level allow robust statistical analysis and provide a way

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to assess variations in response to DNA damaging agents between cells of the same exposed population. The comet assay is used to study processes involving DNA damage in many fields such as environmental toxicology [12], bio-monitoring [13], radiation biology [14], nutritional studies and cancer studies [15,16]. While the comet assay has widespread use, a common issue in all its applications is the process of analyzing the microscope images. The time needed for scoring these images is one major drawback of the comet assay. Further, the non-availability of free automated tools for this task still poses considerable challenges.

Comets can be identified and scored by visual inspection or by using image analysis software packages [11]. Visual scoring gives a simple qualitative indication of DNA damage. However it is very subjective [17]. In contrast, the use of comet analysis software provides quantitative and reproducible measurements. The currently available comet analysis tools can be broadly classified as manual or automated. Manual analysis typically requires an expert to set threshold brightness values separating the background, to select the nucleus, and to mark the comet head [17–19]. Automated tools employ image analysis techniques to recognize and measure comets, and are generally much faster than manual scoring. Due to the efficiency gained through automation, one can typically afford to measure larger sample sizes, which is critical for statistically significant results [20]. Algorithms for automated analysis [21–25] have been proposed in recent years, however, these are coupled with in-house microscope setups and the source code is not accessible to the public. Commercial automated tools such as IMSTAR Pathfinder (www.imstarsa.com) are also available, and have been used in comet assay studies [20] (for a list of commercial comet assay analysis software, see http://www.cometassay.com) but, they are expensive and provide no possibilities for examining and modifying the underlying image processing algorithm.

Here we present OpenComet, a tool specifically developed to address the difficulties in the automatic analysis of comet assay images. In OpenComet, we have implemented novel image processing algorithms for finding comets and segmenting the comet head. The overall analysis time is reduced significantly compared to tools requiring manual selection of comets. Another important advantage of our tool is that it is open source, thus enabling users to modify the program for customization and improvement. OpenComet has been validated on both alkaline and neutral comet assays across different levels of damage. Our validation results demonstrate that the automated image processing methods used by the tool lead to fast and robust comet analysis. A comparison with manual measurements shows that OpenComet achieves high accuracy and significantly shortens the analysis time. We have also compared the features and capabilities of OpenComet with two of the widely used free tools for comet assay image analysis (CaspLab [17] and CometScore [18]) in Table 1. As shown in the table, other than automating the analysis process, OpenComet also overcomes some technical restrictions of these tools.

In the following, we first introduce the interface of OpenComet, and then describe the image processing steps involved in analyzing comets. Next we describe the comet assay experiments used to validate OpenComet. The experiments involved measuring the DNA damage and DNA repair kinetics upon medium sub toxic hydrogen peroxide (H_2O_2) induced stress. We then show the performance of OpenComet on the images obtained from these experiments.

Materials and methods

OpenComet user interface

OpenComet is deployed as a plug-in for the popular image processing platform, ImageJ. The ImageJ software (www.rsbweb.nih.gov/ij/) can display, process and analyze images, and is mainly intended for use with microscopy images. It is an ideal framework for OpenComet to run in due to its cross-platform nature, its ability to interpret a variety of image formats and due to the fact that it implements a large number of elementary image processing operations. OpenComet, once installed, can be launched from the Plugins menu of ImageJ.

OpenComet has a user-friendly interface (Fig. 1) to select a set of images and run the analysis. The user first selects a set of input images to be analyzed. Any number of images inside a single folder can be selected for analysis, and the images can be of any of the widely used image formats (typically BMP or TIFF). Next, one selects an output folder, where the result spread sheet as well as copies of the input images with the analysis overlay will be saved.

The measurement results are saved in a spreadsheet which includes measurements obtained for each individual comet, as well as statistics for the population of comets extracted from all input images. Further, for each input image, a result image is generated which shows the comet and head outlines, the associated profiles as well as identification numbers to easily link the comets to the measurements. The output images and the spreadsheet are saved automatically in the chosen output folder. After the automated analysis is complete, the user has the option to review the images and click on any comet to remove it from the output if needed.

We have developed a live analysis function in OpenComet, which allows users to analyze images as soon as they are acquired through a microscope. This functionality can be used when launching OpenComet as a plug-in in Micro-Manager [26], an open-source microscope control software, which provides an interface between ImageJ and a large number of microscope models. To start analysis, the user captures an image of the current

Table 1
Comparison of features and capabilities of OpenComet with two of the leading free comet assay analysis software, CometScore and CaspLab.

Features	OpenComet	CometScore	CaspLab
Scoring	Automated	Manual	Manual
Manual input	None/optional manual review	Comet boundary and head position selection	Comet boundary selection
Output format	Tab delimited file with XLS extension	Tab delimited text file	Tab delimited text file
Saving scored images	Automatic saving	Manual	Manual
Background correction	Yes, automated	Yes, based on manually set background cut-off	Yes, manual selection
Multiple objective calibration	Yes, 5 × –20 × objectives	Yes for 5 × and 10 × objectives. For 20 × objective, measurement is difficult	Ideal for 20 × objective images. For 5 × and 10 × images, comet head not found correctly.
Input image format	All common image formats	Only BMP	Only TIFF
Operating system	Windows, Mac OS X, Linux	Windows	Windows, Mac OS X, Linux