



# CometAnalyser: A user-friendly, open-source deep-learning microscopy tool for quantitative comet assay analysis



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## ABSTRACT

Comet assay provides an easy solution to estimate DNA damage in single cells through microscopy assessment. It is widely used in the analysis of genotoxic damages induced by radiotherapy or chemotherapeutic agents. DNA damage is quantified at the single-cell level by computing the displacement between the genetic material within the nucleus, typically called "comet head", and the genetic material in the surrounding part of the cell, considered as the "comet tail". Today, the number of works based on Comet Assay analyses is really impressive. In this work, besides revising the solutions available to obtain reproducible and reliable quantitative data, we developed an easy-to-use tool named *CometAnalyser*. It is designed for the analysis of both fluorescent and silver-stained wide-field microscopy images and allows to automatically segment and classify the comets, besides extracting Tail Moment and several other intensity/morphological features for performing statistical analysis. *CometAnalyser* is an open-source deep-learning tool. It works with Windows, Macintosh, and UNIX-based systems. Source code, standalone versions, user manual, sample images, video tutorial and further documentation are freely available at: <https://sourceforge.net/p/cometanalyzer>.

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## 1. Introduction

Comet Assay is a sensitive *in vitro* method to assess DNA damages in individual cells based on the technique of microgel electrophoresis [1]. This assay was originally described by Ostling and Johanson in 1984 [2]. Its first significant variation, which is the most common today, was proposed 4 years later by Singh *et al.* [3]. The term "Comet" was introduced by Olive *et al.* in 1990 [4] to describe the shape of the DNA visualised upon observing agarose gels. Besides they introduced the concept of "Tail Moment", computed as the product of two other features, "Tail Length" and "Tail Fluorescence Percentage", which are some of the most important features considered upon evaluating this assay.

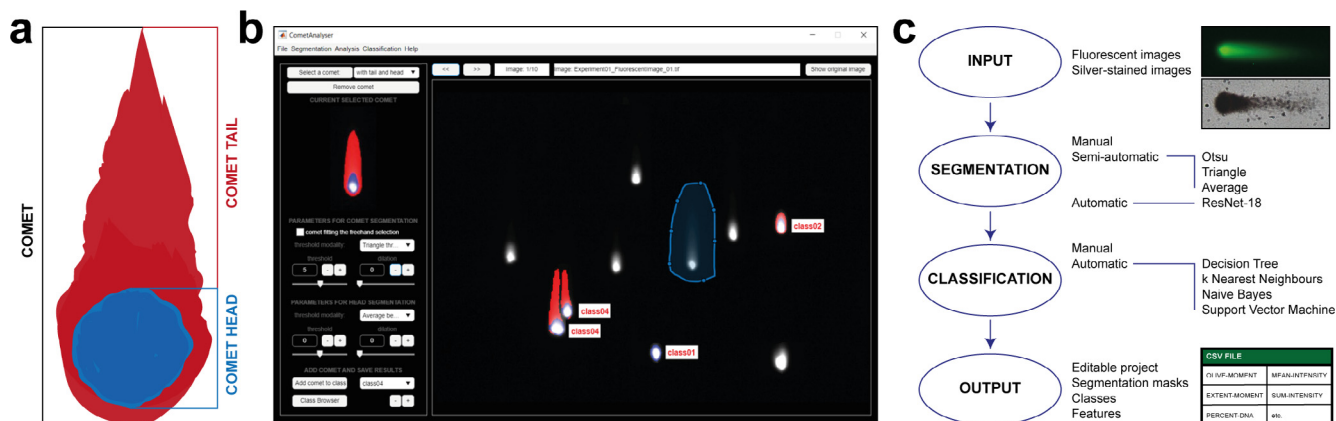
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Today, an impressive number of works are based on Comet Assay analyses, especially in the field of cancer research where it is largely used to evaluate different DNA damages induced by ionising radiation or anticancer agents [5]. This significant interest has led to the foundation of an international interest group that offers information, protocols and a forum for discussions on Comet Assay (<https://cometassay.com>, [6]). In a Comet Assay, cells embedded in agarose are: (a) lysed, (b) subjected to an electric field, (c) fluorescent/silver stained, and (d) observed using a fluorescence/bright-field microscope. As broken DNA migrates farther in the electric field than the non-damaged genetic material, the cells harbouring DNA damage resemble a "comet" (Fig. 1a) with a near-spherical head and a tail region, the latter increasing as DNA damage increases [7].

Originally, comet assay was performed as a qualitative analysis, but nowadays commercial and freely available tools are available to obtain reproducible quantitative data. However, none of the freely available tools works on both fluorescent- and silver-



**Fig. 1.** *CometAnalyser*. (a) In a Comet Assay, cells resemble comets, with the length of the tails increasing as DNA damage increases. (b) *CometAnalyser*'s GUI. (c) Flow chart summarising the main possibilities available and steps performed when using *CometAnalyser* to obtain quantitative results.

stained images, which would allow the user to easily segment the comets, and by utilising machine learning methods, automatically identify the most appropriate class for the unannotated comets before extracting several intensity/morphological features, and save the segmentations for future analysis.

In this work, besides revising the existing solutions for performing Comet Assay analysis, we present *CometAnalyser*, an open-source deep-learning tool designed for easy segmentation and classification of comets in fluorescent- and silver-stained images. Source code, standalone versions, user manual, sample images, video tutorial and further documentation are freely available at: <https://sourceforge.net/p/cometanalyzer>.

The next sections are organized as follows: **Sect. 2** presents a short overview of the tools designed for performing quantitative analyses of Comet Assay microscopy images. **Sect. 3** provides a detailed description of *CometAnalyser*, **Sect. 4** describes the material used and the results obtained in the experiments performed to validate the method proposed. Finally, **Sect. 5** summarises the main findings of the work.

## 2. Available tools

In this Section we briefly report a description for *CASP* [8], *CellProfiler* [9–10], *CometScore* [11], *HiComet* [12], *OpenComet* [13], the tools today freely available for performing quantitative Comet Assay analyses. Fig. 2 reports a representative print-screen of the Graphical User Interface (GUI) for each of them, whilst their main features are summarised in Table 1. In addition, Table 2 reports a reference publication for *CoMat* [14], *CometQ* [15], *DeepComet* [16], *LACAAS* [17], and *SCGE-ProSoftware* [18], tools mentioned in the literature but today not downloadable/available, and Table 3 the links of the commercial tools today accessible for quantitative Comet Assay analysis, but not freely available for the community.

*CASP* [8]: *CASP* is a user-friendly C/C++ open-source tool developed by Kořica et al. in 2003. It works with either silver-stained or fluorescence-stained comets saved in “.tif” format. An unlimited number of images can be marked, and the program will load them successively into an “image view” window. To recognize the comet head and tail it assumes 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. Accordingly, only comets oriented from left to right can be analysed correctly, but images can be pre-rotated. The tool is a semi-automatic one. Practically, to proceed with the analysis, the user must define a rectangle around the comet of interest. Then, he/she can adjust various

thresholds of sensitivity and save the adjustments for future use. An intensity profile of the currently selected comet shows up on a “profile” window together with selected feature values (e.g. tail moment and several other features). When measurements are terminated, the feature values are exported into a text file and the full project can be saved and loaded back for future analysis.

*CellProfiler* [9–10]: *CellProfiler* is a popular MATLAB/Python software suite worldwide used to set several microscopy image-based analyses by aligning in a pipeline customised modules for standard image processing tasks. Among the different analyses, the comet assay is a typical one, directly reported also in the list of the common applications on the *CellProfiler*'s website. Over the years, several *CellProfiler*'s pipelines have been designed by different groups to optimise the comet assay analysis for different scenarios, including both the silver-stained and fluorescence images. However, the pipelines can be modified with a limited programming experience, and the segmentation masks and the features computed can be then exported in different formats.

*CometScore* [11]: *CometScore* is a freely available software tool developed by Rex Hoover in 2005, then supplied in an extended PRO version by the *TriTek Corporation* (Sumerduck, VA). *CometScore* is developed for Windows systems only and requires as the input “.bmp” images where all comets are oriented with head on the left and tail on the right. It provides fully-automatic, semi-automatic and manual methods to segment comets based on intensity thresholds defined by the user. Head and tail profiles are visualised directly on the images. *CometScore* also provides an intuitive wizard for comet classification. Feature values can be exported into a text file.

*HiComet* [12]: *HiComet* is an automated tool for high-throughput comet-assay analysis. It was developed in MATLAB and PHP by Lee et al. with the idea to freely provide an online implementation to analyse fluorescence comet assay images. It is optimised for rapidly recognising and characterising a large number of comets using little user intervention. It is based on a histogram-thresholding technique for automatically segmenting the comets and no manual correction opportunity is provided to modify the obtained layouts. The feature values appear in a pop-up window shown directly on the image and the comets are then automatically classified on the basis of the features extracted. However, unfortunately, there is no user guide and info about how to run the code available on the *GitHub* repository, no standalone version available, and the Corresponding Author of the reference paper said that today *HiComet* is not maintained anymore. Accordingly, although *HiComet* seems a very promising tool, it is very hard to run it.

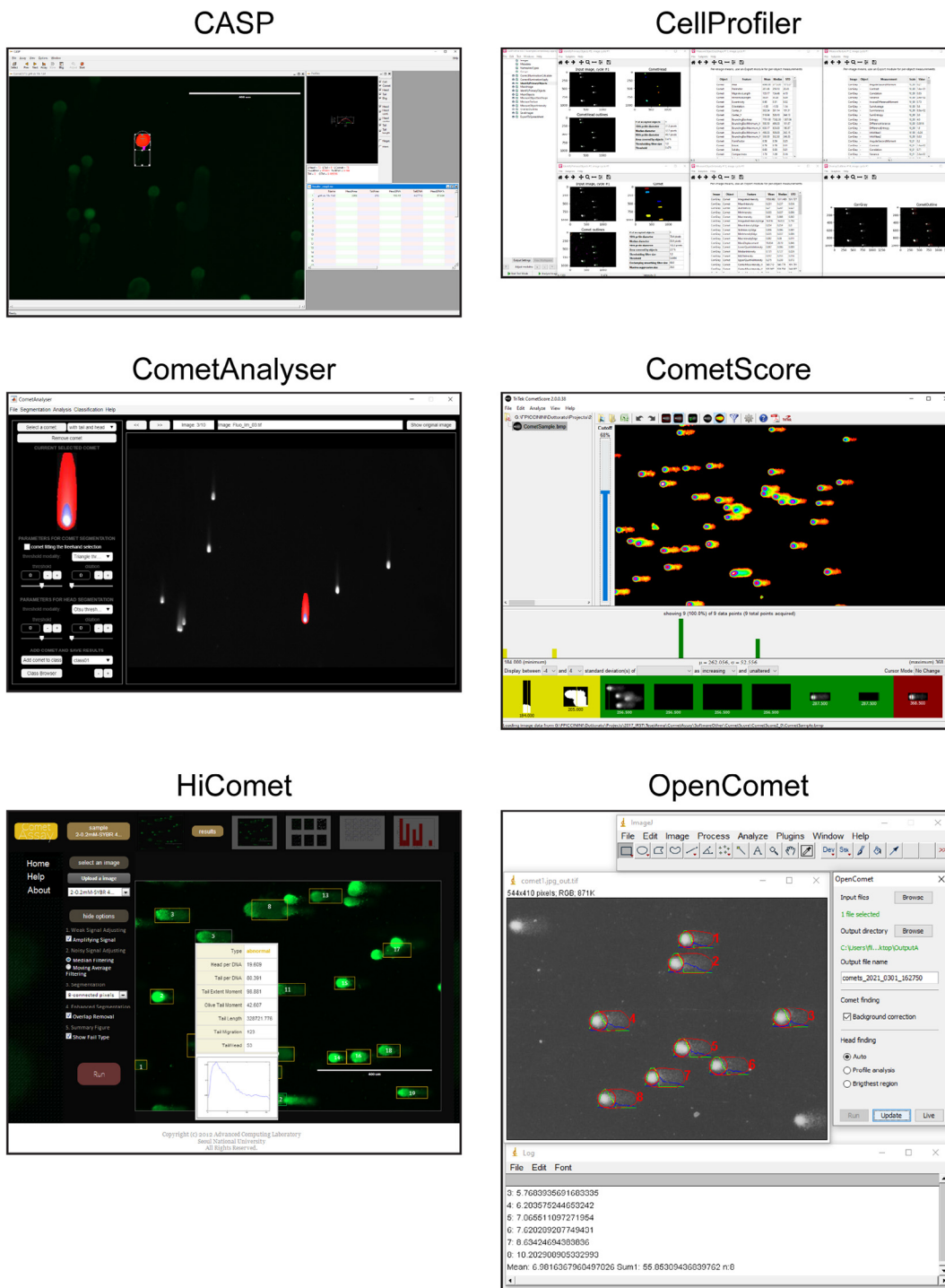


Fig. 2. Tools freely available - main window screenshots.

**OpenComet [13]:** *OpenComet* is a popular *ImageJ/Fiji* plugin implemented by Gyori et al. in 2014. It is extremely easy-to-use but it works with fluorescence images only where all comets are oriented with head on the left and tail on the right. It requires just the definition of the input and output folders and a few pre-processing decisions. It uses a robust method for finding comets based on geometric shape attributes and segmenting the comet heads through image intensity profile analysis. Head and tail profiles are then visualised directly on the images, while feature values are exported into a spreadsheet file. No manual correction opportunity is provided to modify the comet layout, but 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. Finally, images with overlaid profiles and the spreadsheet files are saved automatically in the chosen output folder.

### 3. – CometAnalyser

*CometAnalyser* is written in *MATLAB*. It provides an easy solution for accurate comet segmentations and quantitative analysis. It exploits advanced processing methods with minimal user interaction to quantitatively evaluate the cell's viability by assessing DNA

**Table 1**

Tools freely available - characteristics (X = available/yes; O = not available/no, last access: 30/07/2022).

	<b>CASP</b>	<b>CellProfiler</b>	<b>CometAnalyser</b>	<b>CometScore</b>	<b>HiComet</b>	<b>OpenComet</b>
<b>VERSION</b>						
<i>Year first release</i>	2003	2012	2021	2005	2018	2014
<i>Current version</i>	1.2.3 beta2	0	1.0	2.0	0	1.3.1
<i>Link</i>	<a href="https://sourceforge.net/projects/casp/">https://sourceforge.net/projects/casp/</a>	<a href="https://cellprofiler.org/examples">https://cellprofiler.org/examples</a> ; <a href="https://ars.els-cdn.com/content/image/1-s2.0-S1383571812002288-mmc1.pdf">https://ars.els-cdn.com/content/image/1-s2.0-S1383571812002288-mmc1.pdf</a>	<a href="https://sourceforge.net/p/cometanalyzer">https://sourceforge.net/p/cometanalyzer</a>	<a href="https://rexhoover.com/index.php?id = cometscore">https://rexhoover.com/index.php?id = cometscore</a>	<a href="https://github.com/taehoonlee/hicomet">https://github.com/taehoonlee/hicomet</a>	<a href="https://cometbio.org">https://cometbio.org</a>
<b>DOCUMENTATION</b>						
<i>User guide</i>	X	X	X	X	O	X
<i>Website</i>	X	X	X	X	O	X
<i>Video tutorial</i>	O	O	X	X	O	X
<i>Sample dataset</i>	O	X	X	X	X	O
<i>Open source</i>	X	X	X	O	X	X
<i>Implementation language</i>	C++	Python	Matlab	O	Matlab & PHP	Java
<b>USABILITY</b>						
<i>Input image format</i>	TIF only	All common	All common	BMP only	All common	All common
<i>No programming experience required</i>	X	O	X	X	O	X
<i>User-friendly GUI</i>	X	X	X	X	O	X
<i>Intuitive visualisation settings</i>	O	X	X	X	O	X
<i>No commercial licences required</i>	X	X	X	X	X	X
<i>Portability on Win/Linux/Mac</i>	X	X	X	Win only	X	X
<b>FUNCTIONALITY</b>						
<i>Silver-stained images</i>	X	X	X	O	O	O
<i>Fluorescence images</i>	X	X	X	X	X	X
<i>Automatic comet segmentation</i>	O	X	X	X	X	X
<i>Semi-automatic comet segmentation</i>	X	O	X	X	O	O
<i>Manual comet segmentation/correction</i>	O	O	X	X	O	O
<i>Feature selection</i>	X	O	X	X	O	O
<i>Automatic comet classification</i>	O	O	X	X	X	O
<b>OUTPUT</b>						
<i>Segmentation masks</i>	O	X	X	O	X	O
<i>Feature values</i>	X	X	X	X	X	X
<i>Classification statistics</i>	O	O	X	X	X	O
<i>Editable project</i>	X	O	X	O	O	O



**Table 2**  
Tools mentioned in the literature but today not downloadable/available - references.

<b>CoMat</b>	Mani, U., Manickam, P. "CoMat: An Integrated Tool for Comet Assay Image Analysis." <i>Journal of Pharmaceutical Sciences and Research</i> 9.6 (2017): 919.
<b>CometQ</b>	Ganapathy, S., et al. "CometQ: An automated tool for the detection and quantification of DNA damage using comet assay image analysis." <i>Computer Methods and Programs in Biomedicine</i> 133 (2016): 143–154.
<b>DeepComet</b>	Hong, Y., et al. "Deep learning method for comet segmentation and comet assay image analysis." <i>Scientific Reports</i> 10 (2020): 18,915
<b>LACAAS</b>	LAI's Automated Comet Assay Analysis System (LACAAS, (Loates Associates, Inc, Westminster, MD)
<b>SCGE-Keohavong,</b>	<b>ProSoftware</b> Phouthone, and Stephen G. Grant, eds. <i>Molecular toxicology protocols</i> . Vol. 291. Humana Press, 2005.

damage. This is quantified by computing several intensity/morphological features for every single comet to evaluate the displacement between the genetic material within the nucleus, *i.e.* the "comet head", and the genetic material in the surrounding part, considered as the "comet tail". An early command-line version of our tool has been used by Pignatta *et al.* for studying DNA damage of cancer cells treated according to different radiotherapy protocols [19]. Since then *CometAnalyser* has been extended by (I) implementing machine learning methods to automatically segment and classify the comets; (II) developing modules to analyse/modify the segmentations; and (III) designing a user-friendly GUI (Fig. 1b) subdivided into 4 main modules: (a) image/project processing, (b) comet segmentation, (c) comet classification, (d) feature extraction and data sharing (Fig. 1c).

### 3.1. Comet segmentation: Fully-automatic modality

Comets are segmented by exploiting a deep convolutional neural network [20]. Pre-trained segmentation networks for both fluorescent- and silver-stained images are provided, and an easy-to-use wizard guides the user in training more specific segmentation models. To create the models we used a built-in *MATLAB* implementation of a convolutional neural network 18 layers deep known in the literature by the name "ResNet-18". Additional details on the network's architecture are reported at: <https://www.mathworks.com/help/deeplearning/ref/resnet18.html>.

To train the models we used two different datasets of images (Fig. 3) with these characteristics: (a) *Fluorescent-stained dataset*: The dataset is composed of 33 1200 × 1600 pixels ".tif" images, containing 20–100 comets for each image. The comets were manually annotated by an expert microscopy user. The slides were prepared according to a standard neutral comet assay manufacturer's protocol (Comet assay, Trevigen, Gaithersburg, MD). Briefly, 5000

**Table 3**  
Commercial tools today available - links (last access 30/07/2022).

<b>Andor - Komet 7.0</b>	<a href="https://andor.oxinst.com/products/komet-software/komet-7">https://andor.oxinst.com/products/komet-software/komet-7</a>
<b>Helix3</b>	<a href="https://www.helix3inc.com">https://www.helix3inc.com</a>
<b>Imstar- SCREENTOX</b>	<a href="https://www.imstara.com/products-applications/">https://www.imstara.com/products-applications/</a>
<b>Instem - Comet Assay IV</b>	<a href="https://www.scorecomets.com">https://www.scorecomets.com</a>
<b>MetaSystems - Metafer</b>	<a href="https://metasystems-international.com/en/products/solutions/comet-foci/">https://metasystems-international.com/en/products/solutions/comet-foci/</a>
<b>Trevigen - Comet Analysis Software</b>	<a href="https://trevigen.com/docs/protocol/manual_Trevigen%20Comet%20Analysis%20Software.pdf">https://trevigen.com/docs/protocol/manual_Trevigen%20Comet%20Analysis%20Software.pdf</a>

cancer cells (A549, Non Small Cell Lung Cancer cell line, American Type Culture Collection, ATCC, Rockville, MD, USA) were suspended in LMAgarose at 37 °C and immediately transferred onto the comet slide. The slides were immersed for 1 h at 4 °C in a lysis solution, washed in the dark for 1 h at room temperature in a neutral solution, and electrophoresed for 30 min at 21 V. Slides were then dipped in 70 % ethanol and stained with the Syber Green (Bio-Rad Laboratories, Hercules, CA, USA). Images were captured using an AMG EVOS FL microscope (Thermo Fisher Scientific, Waltham, MA, USA), equipped with a Sony ICX285AL CCD camera (Tokyo, Japan) at 10 × magnification. (b) *Silver-stained dataset*: The dataset is composed of 54 1280 × 1024 ".tif" images, containing 2–20 comets for each image. The comets were manually annotated by an expert microscopy user. The slides were prepared according to a standard alkaline comet assay manufacturer's protocol (Comet assay, Trevigen, Gaithersburg, MD). Briefly, 5 × 10<sup>5</sup> fibroblast cells (normal fibroblast lung cell line MRC-5, American Type Culture Collection, ATCC, Rockville, MD, USA) were suspended in LMAgarose (at 37 °C) and immediately transferred onto the comet slide. The slides were immersed for 1 h at 4 °C in a lysis solution, washed in the dark for 1 h at room temperature in an alkaline solution, and electrophoresed for 30 min at 21 V. Slides were then dipped in 70 % ethanol and stained with the Silver Staining Kit (Trevigen) according to the manufacturer's protocol. Images were captured using an Olympus IX51 microscope (Tokyo, Japan), equipped with a Nikon DS-Vi1 camera (Tokyo, Japan) at 10 × magnification.

The deep learning pre-trained segmentation models created, the images used in the training sets and segmentation masks obtained are freely available at: <https://sourceforge.net/p/cometanalyser>. It is worth noting that the provided pre-trained deep-learning segmentation models logically reliably perform just on images with characteristics similar to those of the images used for training the models. However, the user can easily train a new segmentation model following these steps: (1) Annotate an appropriate number of comets. Please note that all the comets in the images included in the training set must be segmented (*i.e.* images in the training set cannot be just partially segmented). (2) Export the segmentation masks using the "Segmentation - Export Annotation" button of *CometAnalyser*. (3) Train a new deep-learning model using the "Segmentation - Train New Model" button of *CometAnalyser*. Network parameters can be modified using the "Segmentation - Training Options" button.

### 3.2. Comet segmentation: Semi-automatic modality

Currently, 3 different threshold-based semi-automatic modalities have been implemented in *CometAnalyser*. All of them are based on the analysis of the histogram of the grey-level intensities of the local ROI surrounding the comet to be segmented. The first is the classical *Otsu thresholding* segmentation [21]. The second is the *triangle segmentation* defined by Zack *et al.* [22]. Finally, we implemented an additional segmentation strategy called "*Average between Otsu and Triangle*", with the final threshold defined as the average value of the ones defined according to the two methods just cited. To segment a comet using one of the semi-automatic modalities available, first the user should simply surround the single comets by drawing a circle. Then, he/she has to select one of the available threshold modalities for segmenting the comet from the background and the head from the tail. Finally, using additional commands for scaling the thresholds and dilating the segmentation masks, he/she can adjust the contours proposed for the head and the comet in general. Once defined the parameters, the settings can be reused for the segmentation of similar comets. *Semi-automatic modality*: the user simply marks single comets by drawing circles.

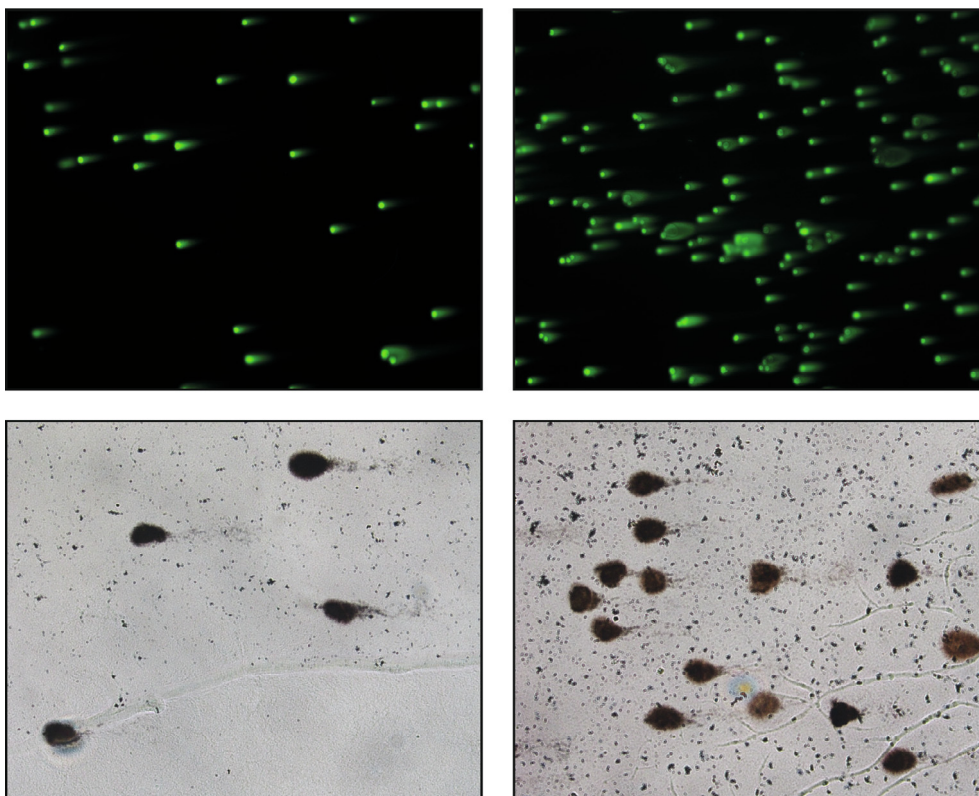


Fig. 3. Representative images of the two datasets used for training the deep-learning segmentation models.

Table 4  
Intensity/morphological features automatically extracted by CometAnalyser.

Features extracted for the entire comet	Features extracted for the comet head	Features extracted for the comet tail
Area	Area	Area
Max Length	Max Length	Max Length
Mean Intensity	Mean Intensity	Mean Intensity
Std Intensity	Std Intensity	Std Intensity
Sum Intensities	Sum Intensities	Sum Intensities
Sphericity	Sphericity	PercentDNA
	PercentDNA	Extent Moment
		Olive Moment

### 3.3. Comet segmentation: Manual modality

By switching on the “comet fitting the freehand selection” checkbox present on the left side of the CometAnalyser’s GUI, the user enables the manual segmentation modality and can precisely define the border of the comet by directly drawing the corresponding contour.

### 3.4. Comet classification

In the literature, comets are typically classified into 5 different categories, with the first class representing undamaged cells (i.e. comets with no or barely detectable tails), while class 5 includes just comet tails without visible nuclei [23]. To increase flexibility by providing the opportunity to define sub-classes or perform regression analysis [24], CometAnalyser allows the user to define an infinite number of classes. Accordingly, the user can manually define the class for each segmented comet, or may use built-in machine learning algorithms to train a classifier based on previously classified comets, and then use it to automatically define the class of newly segmented ones. It is worth noting that through

CometAnalyser, the user can easily train a new classifier (i.e. classification network) following these steps: (1) Define the classes. (2) Manually classify an appropriate number of comets for each class. (3) Export the features (i.e. “Analysis – Export Measurements” button of CometAnalyser) of the classified comets. (4) Define the machine learning algorithm to be used for creating the network. Currently, CometAnalyser provides four different machine learning algorithms to define the classification network: “Decision Tree”, “k Nearest Neighbours”, “Naive Bayes”, “Support Vector Machine”. The machine learning algorithms implemented in CometAnalyser are based on MATLAB built-in functions. A more detailed description of them is available at: [https://it.mathworks.com/help/stats/classification.html?s\\_tid = CRUX\\_lftnav](https://it.mathworks.com/help/stats/classification.html?s_tid = CRUX_lftnav).

### 3.5. Feature extraction and data sharing

Typically, several intensity/morphological features are evaluated in a Comet Assay analysis [13]. Table 4 reports the list for the 21 features extracted automatically by CometAnalyser sepa-

Table 5  
Jaccard Index values (the better value for each row reported in Italics).

Jaccard Index	CometAnalyser	CellProfiler
Image 01	0.7235	0.6611
Image 02	0.6598	0.6730
Image 03	0.6039	0.5464
Image 03	0.8121	0.5690
Image 04	0.7280	0.3952
Image 05	0.7446	0.6129
Image 06	0.5773	0.6124
Image 07	0.7146	0.7982
Image 08	0.5577	0.7798
Image 09	0.6723	0.6721
Average Jaccard Index [mean ± std]	0.6794 ± 0.1110	0.6320 ± 0.1528

rately considering the comet’s head, the comet’s tail, or for the whole comet. Briefly:

- *Area* [pixels]: number of pixels belonging to the object.
- *Max Length* [pixels]: length in pixels of the maximum diameters passing through the centroid of the object.
- *Mean Intensity* [grey levels]: average of the intensity values of the pixels of the object.
- *Std Intensity* [grey levels]: standard deviation of the intensity values of the pixels of the object.
- *Sum Intensities* [grey levels]: sum of the intensity values of the pixels of the object.

- *Sphericity* [range 0–1]: sphericity value computed according to the following equation:  

$$\text{Sphericity} = \pi * \sqrt{a * \text{Area} / \pi} / \text{Perimeter}.$$
- *PercentDNA* [%]: percentage between *Sum Intensities* of the object (i.e. head or tail) and *Sum Intensities* of the entire comet:  

$$\text{PercentDNA} = 100 * (\text{Object\_Sum\_Intensities} / \text{Comet\_Sum\_Intensities}).$$
- *Extent Moment*: ratio between *Sum Intensities* of the comet tail and *Sum Intensities* of the entire comet, multiplied by the *Max Length* of the comet tail:  

$$\text{Extent\_Moment} = \text{Tail\_Max\_Length} * (\text{Tail\_Sum\_Intensities} / \text{Comet\_Sum\_Intensities}).$$

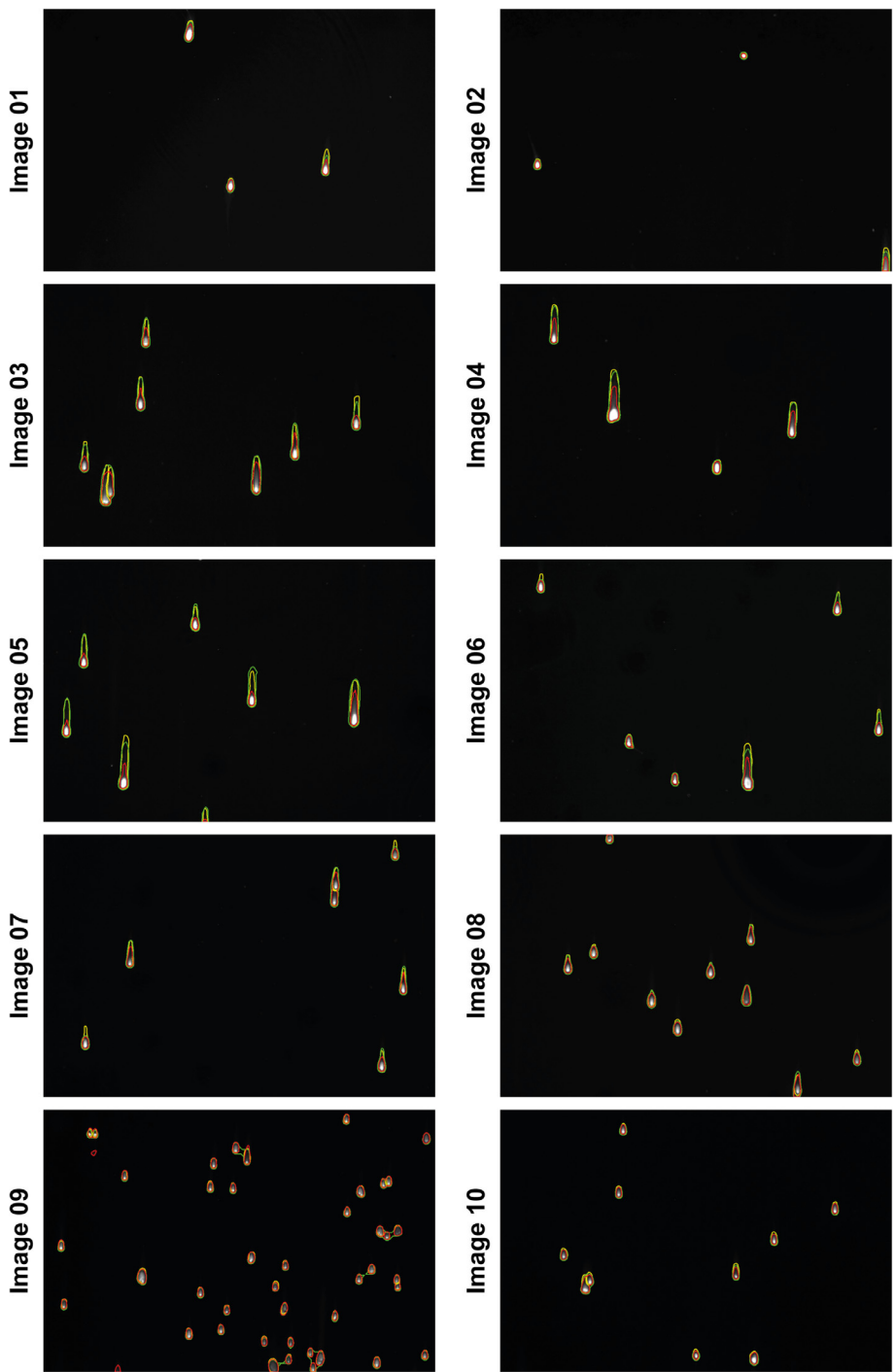


Fig. 4. Segmentations. Ground truth (yellow outline), CometAnalyser’s masks (green outline), CellProfiler’s masks (red outline). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)



- **Olive Moment:** ratio between *Sum Intensities* of the comet tail and *Sum Intensities* of the entire comet, multiplied per the absolute value of the module of the distance between the centroid of the head and the centroid of the tail:  
Olive Moment = | Head\_centroid\_position - Tail\_centroid\_position | \* (Tail\_Sum\_Intensities / Comet\_Sum\_Intensities).

For an extensive description of the features please check also the “Appendix: Equations” in the tutorial of the *CometScore* tool, available at: <https://dokument.pub/ql/cometscore-tutorial-automatic-comet-assay-flipbook-pdf>. Moreover, *CometAnalyser* provides an easy solution for the users to define new features. The extracted features are saved in a spreadsheet file, and the segmentation masks are saved as “.png” files. All the segmented comets are shown as thumbnails in a pop-up wizard called “*Comet Browser*”, and the user can navigate the original images with just a click to find and eventually modify the segmented comets which are highlighted in red (DNA) and blue (nucleus). The project can be saved and shared, allowing future modifications and analysis.

#### 4. Experimental RESULTS

*CometAnalyser* was compared with the competitor tools. First, we discarded from the comparison the tools reported in Table 1 but not providing output masks of the segmented comets (i.e. *CASP*, *CometScore* and *OpenComet*). In addition, we also discarded *HiComet* because today it is not maintained and currently there are no available instructions on how to run the tool. Concluding, we defined a testbed dataset, and using the ground truth (GT), we quantitatively compared the masks automatically obtained by *CometAnalyser* and *CellProfiler*.

In particular, we analysed the masks of the comets automatically segmented using the fluorescence pre-trained deep-learning segmentation network provided at: <https://sourceforge.net/p/comet-analyser>. First, we defined a dataset of 10 new fluorescent images with characteristics similar to those used for training the segmentation network. Then, we asked an expert microscopist to manually segment the comets for defining the GT masks. To evaluate the segmentation quality of the comets’ masks, we computed the Jaccard Index (JI) [25]. JI, also known as Intersection over Union (IoU) or the Jaccard Similarity Coefficient. As described in [26], JI is a well-known metric used for evaluating the similarity of two sample sets (e.g. *A* and *B*).  $JI(A,B)$  is mathematically defined as the size of the intersection (i.e.  $|A \cap B|$ ), the number of overlapping voxels) divided by the size of the union (i.e.  $|A \cup B|$ ) of the sample sets:

$$JI(A,B) = |A \cap B| / |A \cup B| = |A \cap B| / (|A| + |B| - |A \cap B|).$$

An open source MATLAB code for computing the JI for intensity-coded masks for multiple objects (i.e. masks with a unique ID for each object) is provided at: <https://www.3d-cell-annotator.org/download.html> (Supplementary Material 1 file - “SM1”). In the provided implementation, the JI calculation method pairs the objects in the GT (i.e. the mask of objects created by the expert microscopist manually segmenting the single objects) with the object of the predicted masks having maximum overlap, assigning a zero value to objects with less than 50 % of overlap with any of the predicted masks. Then, for each GT object, it computes a single JI value. Finally, the output JI is calculated by averaging the JI values obtained for the single objects.

Testbed dataset, GT masks, *CometAnalyser*’s masks, *CellProfiler*’s pipeline and masks and the customised version of MATLAB code to compute the JI are provided in a “.zip” folder named: “*CometAnalyser vs CellProfiler*”, available at: <https://sourceforge.net/p/cometanalyser>.

The JI values obtained for each image are reported in Table 5. It is worth noting that originally we used the *CellProfiler*’s pipelines

available at the links reported in Table 1 without any parameter setup, but we obtained poor results. Then, we edited the *CellProfiler*’s pipelines directly using the images of the testbed dataset to be analysed in the validation tests. Practically speaking, for *CellProfiler* we reported in Table 5 the best values we were able to obtain. At the end, both tools obtained very good segmentation masks (Fig. 4), however, *CometAnalyser* achieved a slightly better JI (i.e. 0.68 versus 0.63). Concluding, we think that *CometAnalyser* should be preferred to *CellProfiler* for several reasons: (a) *CometAnalyser* works with fluorescent and silver-stained datasets without requiring any manual modifications, while *CellProfiler* needs severe parameter settings for obtaining good results; (b) Today there is no an unique official *CellProfiler*’s pipeline for Comet Assay analysis and the currently available do not nicely work with different datasets; (c) The users should have at least some limited computational skills to be able of interacting with *CellProfiler* at least to decide which output features and masks exporting; (d) *CellProfiler* does not provide any opportunity for manual correction in case of segmentation errors, or the need of improvements for some contours; (e) The currently available *CellProfiler*’s pipelines do not provide any classification stage. Consequently, all the sub-groups analyses should be performed as a post-processing step. (f) Finally, the quantitative results achieved with *CometAnalyser* were slightly better.

#### 5. Conclusions

*CometAnalyser* is an open-source tool to quantitatively assess DNA damage, for instance to analyse genotoxic effects induced by radio- and chemotherapy. *CometAnalyser* is developed in MATLAB and it works for both fluorescence and silver-stained comet assay microscopy images. It provides fully-automatic, semi-automatic and manual methods to segment comets in several scenarios. The main aim of *CometAnalyser* is to perform accurate comet segmentations and quantitative analysis using advanced image processing methods with minimal user interaction. It is well documented with a user manual, a video tutorial, sample images and results a very easy-to-use tool. A deep-learning model for automatic comet segmentation is provided. Furthermore, an intuitive GUI helps the user in defining interesting classes and annotating representative comets. Finally, the projects can be saved and loaded back for future analysis. Concluding, *CometAnalyser* is currently the most complete freely available solution for 3 main reasons: (a) it works with both fluorescent- and silver-stained images; (b) it provides easy-to-use machine learning modules for the segmentation and classification of the comets; and (c) it offers several opportunities to edit the segmentations and export the data.

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### CRedit authorship contribution statement

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### Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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