

COMET DETECTION OF DNA DAMAGE FROM BUCCAL MUCOSA COMET ASSAY IMAGES UTILIZING 2D OTSU METHOD

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Received October 2025; revised February 2026

ABSTRACT. *The comet assay is used to assess DNA damage levels, producing images that resemble a comet shape. This study implements comet detection in buccal mucosa cells using digital image processing with a detection algorithm based on three features: the number of objects within a comet, the comet's intensity, and its area. The number of objects within a comet was obtained through the reject overlapping object process, the comet's intensity was derived from the reject artifact process, and the comet's area was determined using the reject unwanted region process. The preprocessing step involves preprocessing sequencing including segmentation using 1D and 2D Otsu method and some other preprocessing methods. The test results show that the detection system performs best with the processing sequence: rejects overlapping, rejects artifacts, rejects unwanted regions, rejects artifacts again, and adds padding. The accuracy achieved was 84.12% with 1D Otsu thresholding and 84.16% with 2D Otsu thresholding.*

Keywords: Comet assay, Buccal mucosa image, Object detection, Otsu method, Accuracy

1. Introduction. The comet assay, also known as single-cell gel electrophoresis (SCGE), is a powerful technique for detecting DNA damage at the single-cell level. It is particularly valuable in biomedical research, including in studies on genotoxicity and DNA repair mechanisms [1]. This assay can identify low levels of DNA damage such as single-strand breaks (SSBs) and double-strand breaks (DSBs), which are critical for assessing cellular responses to various genotoxic agents [2-4].

The versatility of the comet assay extends beyond its ability to detect DNA damage, as it can also be used to evaluate DNA repair kinetics and cellular responses to various

environmental stressors [5,6]. This technique involves embedding cells in agarose, lysing them to expose their DNA, and then subjecting the DNA to electrophoresis. The resulting image resembles a comet, with the head containing intact DNA and the tail composed of damaged DNA fragments [6,7]. Multiple studies have demonstrated that the length and intensity of the comet tail correlate with the extent of DNA damage, enabling quantitative analysis [8-10].

In the context of image segmentation, the traditional Otsu or 1D Otsu thresholding method has been widely employed to separate objects from the background by maximizing between-class variance [11,12]. 1D Otsu methods have been experimentally validated to provide improved segmentation results in various biomedical contexts, including DNA damage analysis in comet assays. For instance, Sreelatha et al. [13] proposed an automatic detection technique for comets in silver-stained comet assay images, which demonstrated better performance in terms of true positive detection, false negatives, and false positives compared to existing methods. However, this method can be compromised in noisy environments, leading to suboptimal segmentation results [14,25,26]. For example, high-intensity artifacts or uneven illumination in comet assay images can obscure boundaries between DNA fragments, reducing segmentation accuracy.

To address these limitations, this study proposes comet detection approach based on the two-dimensional (2D) Otsu thresholding method. Unlike conventional one-dimensional (1D) Otsu thresholding, which relies solely on pixel intensity distributions, the 2D Otsu method incorporates both intensity and spatial correlation information through a two-dimensional histogram. This approach enables more robust segmentation in noisy biomedical microscopy images, where uneven illumination, overlapping structures, and low-intensity comet tails often hinder accurate detection. Building upon prior studies, the proposed approach is specifically optimized for buccal mucosa comet assay images, addressing challenges that are not sufficiently handled by general-purpose comet detection systems or conventional Otsu-based methods.

The major contributions of this work are fourfold: 1) proposing a comet detection approach tailored for buccal mucosa comet assay images; 2) systematically evaluating and comparing the performance of 1D and 2D Otsu thresholding methods in this domain; 3) introducing an optimized multi-stage preprocessing and detection sequence involving artifact rejection, overlapping rejection, unwanted region elimination, and adaptive padding; and 4) demonstrating substantial performance improvements over prior methods, including OpenComet and previous Otsu-based comet detection approaches.

The reminder of this paper is organized as follows. Section 2 reviews Otsu-based segmentation methods and related comet detection studies. Section 3 presents the proposed methodology, including preprocessing and detection mechanism. Section 4 reports the experimental results and performance comparison. Section 5 discusses the finding, practical implications, and limitations of the proposed approach. Finally, Section 6 concludes the paper and outlines future research directions.

2. Otsu Method and Related Work. Several studies have highlighted that the conventional Otsu method (1D Otsu) can result in incorrect classification and unsuccessful segmentation when objects are considerably smaller than the background. This problem stems from 1D Otsu method's assumption of a bimodal distribution of pixel intensities and its aim to maximize between-class variance. This method fails when objects are much smaller than the background, causing the histogram to become unimodal or nearly unimodal [15]. In such situations, the threshold tends to favor the predominant background class, leading to misclassified pixels and inadequate segmentation of small objects.

Researchers have developed various enhancements to Otsu method to tackle this issue. For example, Wang and Dong [15] developed a new weighting approach that ensures the threshold value is positioned at the valley between two peaks or at the bottom edge of a single peak, thereby enhancing segmentation for both bimodal and unimodal distributions. Zheng et al. [16] introduced an adaptive windowed range-constrained Otsu method that utilizes local information, resulting in better detail preservation and more satisfactory area overlap measures compared to traditional methods.

Li et al. [17] proposed an improved Otsu method based on uniformity measurement, which adjusts threshold selection to more effectively segment small floating objects on water surfaces. In summary, although the traditional Otsu (1D Otsu) method struggles with images containing small objects against large backgrounds, numerous improvements have been made to overcome this limitation. These enhanced techniques incorporate local information, adaptive windowing, or modified weighting schemes to achieve more precise segmentation results for images with unbalanced object-to-background ratios.

Zhang and Hu [18] conducted a study to enhance 2D Otsu method using a dataset of kidney biopsy images. Due to the objects being significantly smaller than the background, the traditional Otsu method (1D Otsu) often resulted in misclassified pixels and segmentation failures. To address this, they proposed a histogram analysis approach using wavelet transformation to refine Otsu thresholding. Similarly, Zhu et al. [19] worked on improving 2D Otsu method by projecting a 2D histogram onto the diagonal to create a 1D histogram with distinct peaks and valleys. They then applied 2D Otsu thresholding along a line perpendicular to the diagonal to determine the optimal threshold, thereby reducing the search space from 2D to 1D. Furthermore, they employed a lookup table to increase computational speed by avoiding redundant calculations inherent in 2D Otsu method. This improvement significantly enhanced the segmentation speed: while the original 2D Otsu method required 45.067 s, their proposed method required only 0.025 s to perform segmentation on blood cell images.

Sansone et al. [20] conducted research on an algorithm designed for comet assay analysis, developing an automated segmentation system for comet assays. This system aims to reduce user interaction and provide reproducible measurements. The algorithm operates in two main steps: 1) comet identification using Gaussian filtering and morphological operations and 2) comet segmentation through fuzzy clustering. The algorithm was evaluated using comet images of human leukocytes treated with commonly used DNA-damaging agents. Compared with existing commercial systems, the proposed fuzzy clustering segmentation approach demonstrated improved sensitivity. The method achieved a positive predictive value (PPV) of 67%, whereas existing commercial software yielded a PPV of only 53%.

Sreelatha et al. [13] studied an effective automated method for detecting comets in noisy comet assay images. Their study used images from fluorescent-stained comet assays. The silver staining method, commonly applied in clinical settings in developing countries such as India, was utilized because of its affordability, long-term slide preservation capability, and lower health risks. The segmentation process involved Gaussian filtering for smoothing and Otsu thresholding method. Based on the results and clinical expert evaluations, the proposed method outperformed the existing methods, achieving %TP of 89.33%, %FN of 10.67%, %FP of 5.18%, PPV of 94.5%, and sensitivity of 89.3%.

In contrast, recent studies have continued improving Otsu-based segmentation frameworks through optimization and computational refinement. For example, Ma et al. [27] investigated hybrid fractional-order butterfly optimization into the conventional 2D Otsu thresholding process. Their method improved global search capability and threshold optimization efficiency through fractional-order differentiation and sine-cosine search str-

ategies. Similarly, Guo et al. [28] proposed an adaptive double-mutation differential evolution approach to optimize multi-threshold Otsu segmentation, significantly improving segmentation precision while reducing computational complexity compared with conventional thresholding strategies.

Although various comet detection and segmentation methods have been proposed, many existing approaches were developed using different biological samples, staining protocols, or imaging modalities, such as leukocyte or silver-stained comet assay images. These methodological and dataset differences make direct quantitative comparison challenging. Therefore, this study primarily focuses on evaluating the effectiveness of a specialized Otsu-based detection framework for buccal mucosa comet assay images, where image characteristics present distinct analytical challenges. OpenComet was selected as a practical comparative baseline due to its widespread use as a general-purpose comet assay analysis tool.

3. Methodology.

3.1. Dataset acquisition. The dataset was obtained by swabbing patients' inner mouth, performing comet assay with a specialized kit, and capturing the resulting slides with fluorescence microscopy. The analysis used 43 microscope images with a total of 66 objects.

3.2. Preprocessing. First, buccal mucosa comet assay images undergo a sequential preprocessing workflow, including image resizing, grayscale conversion, shading correction, contrast stretching I, Gaussian filtering, contrast stretching II, thresholding, boundary object elimination, morphological opening and closing. The detailed workflow for the preprocessing steps is illustrated in Figure 1.

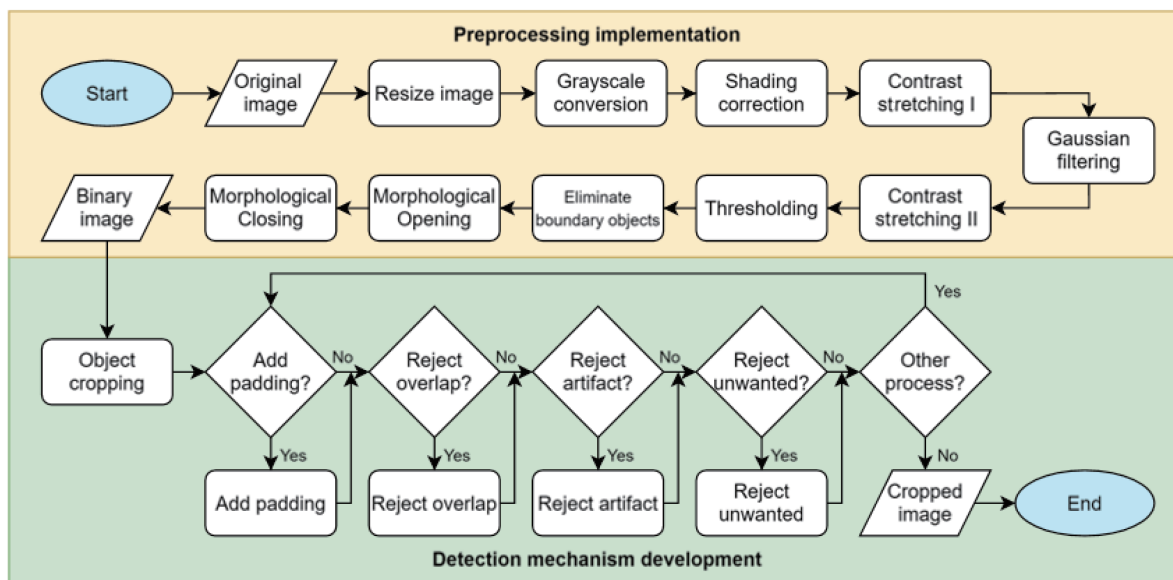


FIGURE 1. A schematic representation of the preprocessing and detection mechanism workflow applied to buccal mucosa comet assay images

3.2.1. Resize image. Image resizing was performed to reduce computational complexity and make the dataset uniform in size, making it easier to compute performance. This process involves resizing the images to 640×853 or 853×640 pixels. To facilitate performance calculation, the images are resized back to their original dimensions in the final steps.

3.2.2. Grayscale conversion. A grayscale image, similar to monochromatic black-and-white or gray, consists of shades of gray, ranging from black with the weakest intensity to white with the strongest intensity. To convert the RGB (Red, Green, Blue) color space into the grayscale Y' color space, Equation (1) can be used.

$$Y' = 0.2989R + 0.5870G + 0.1140B \quad (1)$$

3.2.3. Shading correction. Shading correction aims to remove shadows from the comet by applying a morphological opening to the grayscale image. The absolute difference between the morphologically opened image and the grayscale image was then calculated using Equation (2).

$$T_w(f) = f - (f \circ b) \quad (2)$$

where $T_w(f)$ is white top-hat image, f represents grayscale image, b represents kernel morphology, and $\circ$ represents opening.

3.2.4. Contrast stretching. Contrast stretching is performed to enhance the contrast between the object and background by expanding the range of intensity values to reach the desired range. Consequently, the object becomes easier to detect. Contrast stretching can be performed either before or after Gaussian filtering.

$$P_{out} = (P_{in} - P_{min}) \times \left(\frac{b - a}{P_{max} - P_{min}} \right) + a \quad (3)$$

Contrast stretching can be calculated using Equation (3), where P_{out} represents the pixel intensity value after the contrast stretching process, and P_{in} denotes the input pixel intensity value. The parameters P_{min} and P_{max} correspond to the minimum and maximum pixel intensity values, respectively. In this study, the parameter a is set to 0, while b is set to 255.

3.2.5. Gaussian filtering. Gaussian filtering aims to smooth the image and remove the “noise” [21]. This technique is beneficial for converting a comet’s head and tail segment into one during the thresholding process in the future. The Gaussian function is represented by Equation (4):

$$G(x, y) = \frac{1}{2\pi\sigma^2} e^{-\frac{x^2+y^2}{2\sigma^2}} \quad (4)$$

where x is the distance from zero along the horizontal axis, y is the distance from zero along the vertical axis, and σ represents the standard deviation of the Gaussian distribution.

3.2.6. Thresholding with Otsu method. The segmentation method was used to separate objects from the background in this study, where two thresholding methods were tested: 1D and 2D Otsu method.

1D Otsu Method. At each step, pixel intensities are used as threshold values to minimize or maximize within-class variance. The formula for calculating within-class variance (σ_W^2) is provided in Equation (5), while Equation (6) contains the formula for calculating between-class variance (σ_B^2).

$$\sigma_W^2 = W_b\sigma_b^2 + W_f\sigma_f^2 \quad (5)$$

$$\sigma_B^2 = W_bW_f(\mu_b - \mu_f)^2 \quad (6)$$

where W_b represents the background weight, W_f represents weight foreground, σ_b^2 represents background variance, σ_f^2 represents foreground variance, μ_b represents background mean, and μ_f represents foreground mean.

2D Otsu Method. In the grayscale image, neighboring pixel values were identified using Equation (7).

$$g(x, y) = \frac{1}{(2k+1)^2} \sum_{i=-k}^k \sum_{j=-k}^k f(x+i, y+j) \quad (7)$$

where x represents distance from the origin along the horizontal axis, y represents distance from the origin along the vertical axis, k represents distance to neighboring pixels, $f(x, y)$ represents gray level value of the original pixel, and $g(x, y)$ represents gray level value of the averaged pixel.

Gray level values range from 0 to $L-1$, where L represents the distinct gray levels in the original and average pixels [19]. The occurrence probability (P_{ij}) of pixel pairs (i, j) in the histogram can be determined by dividing the frequency (f_{ij}) of occurrences by the total number of pixel pairs (N) in the image, as shown in Equation (8).

$$P_{ij} = \frac{f_{ij}}{N}, \quad \sum_{i=0}^{L-1} \sum_{j=0}^{L-1} P_{ij} = 1 \quad (8)$$

Using a threshold (s, t) , the histogram is divided into four regions (Figure 2), where regions A and C represent the background and object, while regions B and D represent edges and noise [19]. From this division, two classes, C_0 and C_1 , are formed, and their probabilities are calculated using Equation (9).

$$\omega_0 = \sum_{i=0}^{s-1} \sum_{j=0}^{t-1} P_{ij}, \quad \omega_1 = \sum_{i=s}^{L-1} \sum_{j=t}^{L-1} P_{ij} \quad (9)$$

The optimal threshold (s, t) is determined by identifying the pixel pairs that maximize the discrete matrix $t_r(S_b)$ value, which can be calculated using Equation (10).

$$t_r(S_b) = \frac{(\mu_{T_i}\omega_0 - \mu_i)^2 - (\mu_{T_j}\omega_0 - \mu_j)^2}{\omega_0(1 - \omega_0)^2} \quad (10)$$

where $\mu_{T_i} = \sum_{i=0}^{L-1} \sum_{j=0}^{L-1} iP_{ij}$, $\mu_{T_j} = \sum_{i=0}^{L-1} \sum_{j=0}^{L-1} jP_{ij}$, $\mu_i = \sum_{i=0}^{s-1} \sum_{j=0}^{t-1} iP_{ij}$, $\mu_j = \sum_{i=0}^{s-1} \sum_{j=0}^{t-1} jP_{ij}$.

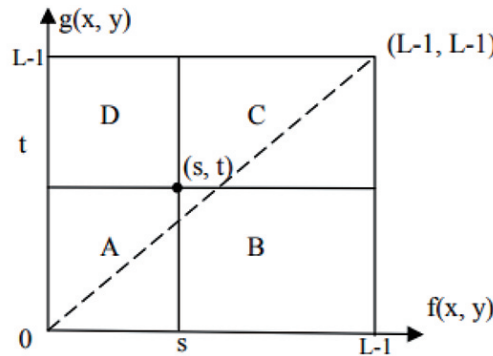


FIGURE 2. 2-dimension histogram

3.2.7. Eliminate boundary object. The dataset used had circular edges, making it necessary to remove objects located along the image borders. To detect a circle, a grayscale image is first processed with morphological opening, followed by thresholding on the resulting opened image with a threshold value of $T = 1$, producing a binary image containing a single circular object.

3.2.8. Opening and closing morphology. In addition to the shading correction process, morphological opening was also performed after eliminating the boundary object. The difference lies in the size of the kernel used for shading correction, which is 65×65 . After eliminating the boundary object, the size of the kernel used for morphological opening and closing was 11×11 . Opening and closing are morphological operations applied to binary images using structuring elements. Opening involves performing erosion followed by dilation to remove residual noise after thresholding, whereas closing applies dilation followed by erosion to filling gaps within objects in the image [22]. The mathematical formulations for opening and closing are presented in Equations (11) and (12), respectively.

$$A \circ B = (A \ominus B) \oplus B \quad (11)$$

$$A \bullet B = (A \oplus B) \ominus B \quad (12)$$

where A represents the input image, while B denotes the kernel or structuring element. The symbols used include $\oplus$ for dilation, $\ominus$ for erosion, $\circ$ for opening, and $\bullet$ for closing operations.

3.3. Detection mechanism. After preprocessing, experiments were conducted using various combinations and sequences of processing techniques, such as adding padding, rejecting overlapping, rejecting artifacts, and rejecting unwanted regions. These combinations may vary in sequence, incorporate repeated steps, or apply only a subset of techniques. An overview of the detection mechanism, along with its relation to the preprocessing steps, is presented in Figure 1.

3.3.1. Add padding. To enhance object detection accuracy, padding was added around each bounding box of the cropped objects. This padding increases the Intersection over Union (IoU) score, helping to capture the object more completely within the bounding box. The amount of padding was determined by multiplying the larger dimensions (either width or height) by a constant factor of 0.35.

Specifically, if the width is greater than the height, the padding is calculated based on 35% of the width; if the height is greater than the width, the padding is calculated based on 35% of the height. Once padding is determined, the bounding box is expanded equally on all sides to make it square, ensuring uniform padding around the object. This process standardizes the bounding box dimensions, leading to a more consistent and reliable object detection across images.

3.3.2. Reject overlapping object. When two comets are close together, they can be detected as a single object. To address this, objects were thresholded at 80% of their maximum intensity, Equation (13), and objects containing multiple comets were removed [13].

$$t = 0.80 \times \maxIntensity \quad (13)$$

First, the RGB image is converted to grayscale. Next, the threshold value t was calculated using Equation (13). This threshold was applied by setting the pixels below t to 0 and those above t to 255. The area of each object was then measured by counting the pixels valued at 255. Objects with an area greater than 10% of the maximum area ($0.1 \times \maxArea$) were identified. If more than one object satisfies this condition, it is considered overlapping and subsequently rejected.

3.3.3. Reject artifact. To remove non-comet objects, their intensity can be analyzed, because non-comet objects tend to have lower intensities. The intensity of an object can be determined by analyzing its comet profile, P_n . The profile of the n th comet can be calculated using Equation (14).

$$P_n(x) = \frac{1}{m} \sum_{y=y_1}^{y_m} l_n(x, y) \quad (14)$$

where m represents the number of pixels in the n th comet, y_1 represents the smallest y -coordinate of the n th object at position x , y_m represents the largest y -coordinate of the n th object at position x , and l_n represents the intensity level at coordinates (x, y) of the n th object.

$$d_n = \max[P_n(x)] - \text{average}[P_n(x)] \quad (15)$$

The n th object with a low d_n value is considered a non-comet object. To calculate d_n in Equation (15), an object is not detected as a comet if it has a value of $d_n < T_d \frac{1}{N} \sum_{j=1}^N d_j$ where T_d is 0.7 [13] and N is the number of objects in the image.

3.3.4. Reject unwanted region. Objects with an area ($Area_n$) smaller than the average area of all comets ($avgArea$) will be rejected [13]. In this study, objects with areas larger than the average comet area ($regionArea_n > avgArea$) were also rejected. To reject unwanted region, first calculate the comet area ($Area_n$) from the pre-processed cropped binary image by counting all pixels with a value of 255 (white). Then, reject cropped images with an area $regionArea_n > avgArea$.

3.4. Performance evaluation metrics. In this experiment, accuracy serves as the primary metric to evaluate detection performance for each thresholding method, 1D and 2D Otsu. Accuracy is calculated as the ratio of correctly detected objects (true positives and true negatives) to the total number of objects and background areas analyzed.

4. Results and Analysis. This study assessed object detection accuracy using 1D and 2D Otsu thresholding methods across seven different variations in detection technique sequences and parameter configurations. Each experiment applied a combination of object rejection and padding techniques, with varying thresholds and region rejection criteria. These variations aimed to identify optimal configurations that would enhance object detection accuracy.

In terms of accuracy trends, the conventional Otsu method (1D Otsu) displayed a steady increase in accuracy, starting from 56.76% in Experiment 1 and reaching 84.12% by Experiment 7. Similarly, the 2D Otsu method, introduced in Experiment 2, demonstrated an upward trend, beginning at 57.07% and peaking at 84.16% in Experiment 7. Both methods achieved their highest accuracy levels in Experiments 6 and 7, with accuracies exceeding 83%, underscoring the effectiveness of these particular experimental setups. Detailed results of accuracy trends and parameter configurations can be found in Table 1.

When comparing the performance between the two methods, the 1D Otsu method showed slightly better results in Experiments 2 to 5, whereas the 2D Otsu method surpassed it in Experiments 6 and 7. The most notable difference in accuracy was observed in Experiment 6, where the 2D Otsu method outperformed the 1D Otsu method by 0.73%. Experiment 7 yielded the highest accuracy for both methods, indicating that this sequence of techniques was optimal. Specifically, positioning the Add Padding technique at the beginning or end of the sequence positively influenced the final accuracy.

Several key observations emerged from this analysis. First, higher artifact rejection thresholds (0.9) contributed to improved accuracy in both methods, and experiments that used the $regionArea > avgArea$ criterion for rejecting unwanted regions showed better performance. Second, the sequence of detection techniques significantly impacted accuracy, with Experiments 6 and 7 – which incorporated strategic padding adjustments and optimal rejection criteria – achieving the highest accuracy. These results suggest that

TABLE 1. Comparison of detection accuracy using 1D and 2D Otsu methods on comet assay buccal mucosa images

Order of detection		Otsu acc. (%)	
		1D	2D
1	Reject OL. $\rightarrow$ Reject A. ^a $\rightarrow$ Reject UR. ^c	56.76	—
2	Add P. $\rightarrow$ Reject OL. $\rightarrow$ Reject A. ^a $\rightarrow$ Reject UR. ^c	57.79	57.07
3	Add P. $\rightarrow$ Reject OL. $\rightarrow$ Reject A. ^a $\rightarrow$ Reject UR. ^d	68.28	67.66
4	Add P. $\rightarrow$ Reject A. ^a $\rightarrow$ Reject UR. ^d	60.64	60.18
5	Add P. $\rightarrow$ Reject A. ^b $\rightarrow$ Reject UR. ^d	70.27	69.88
6	Add P. $\rightarrow$ Reject OL. $\rightarrow$ Reject A. ^b $\rightarrow$ Reject UR. ^d $\rightarrow$ Reject A. ^b	83.02	83.75
7	Reject OL. $\rightarrow$ Reject A. ^b $\rightarrow$ Reject UR. ^d $\rightarrow$ Reject A. ^b $\rightarrow$ Add P.	84.12	84.16

P. = padding; OL. = overlapping; A. = artifact; UR. = unwanted region.

Note: ^aReject artifact (A.) threshold: 0.7

^bReject artifact (A.) threshold: 0.9

^cReject unwanted region (UR.) threshold: $regionArea < 1/2 \text{ avgArea}$

^dReject unwanted region (UR.) threshold: $regionArea > avgArea$

including the Add Padding step at key points in the sequence leads to more precise object detection.

5. Discussion. In this study, we examined the effects of various threshold values, detection sequence configurations, and padding applications on object detection accuracy using 1D and 2D Otsu methods. Our results demonstrate that careful tuning of parameters, such as artifact rejection thresholds, and the strategic placement of detection steps significantly enhance detection accuracy. Each adjustment contributes uniquely to refining the detection process and optimizing the overall segmentation quality. In the following subsections, we discuss the specific impacts of threshold adjustments, the use of padding, area-based threshold conditions, and the addition of a second artifact rejection step.

5.1. Impact of increasing T-Reject Artifact value. Increasing the T-Reject Artifact threshold from 0.7 to 0.9 resulted in a notable improvement in detection accuracy. This change created a more stringent boundary for filtering artifacts, allowing the system to be more selective in retaining valid objects. By setting the threshold at 0.9, the detection process was better able to eliminate artifacts with low to medium intensity, effectively reducing noise and enhancing the detection of true objects. Additionally, this higher threshold reduced the likelihood of noise or artifacts being misclassified as valid objects, particularly in areas with moderate intensity, which often contain artifacts. This led to fewer false positives and a more reliable detection process. Ultimately, the 0.9 threshold ensured that only objects with characteristics closely matching the target were retained, leading to higher consistency and precision in segmentation outcomes.

5.2. Comparison of area threshold conditions. Applying the $regionArea > avgArea$ condition was more effective than $regionArea < 1/2 \text{ avgArea}$ for improving detection accuracy. This condition aligns better with the natural characteristics of valid objects, which tend to have larger areas than the average. In contrast, artifacts and noise are generally smaller, making the $regionArea > avgArea$ condition a more suitable filter. This filtering approach effectively eliminated small noise artifacts, reducing the chances of minor artifacts being classified as valid objects and maintaining the integrity of primary objects. Additionally, the $regionArea > avgArea$ condition provided more consistent results across

different images, reducing variability in segmentation and increasing the system's overall reliability. This discussion underscores that the observed gains in accuracy were not merely the result of additional processing steps, but also of strategic parameter optimization and a carefully planned process sequence. Each modification contributed specifically to the improvement of segmentation quality, refining the overall detection process.

5.3. Effectiveness of adding a second Reject Artifact step. The inclusion of a second Reject Artifact step in Experiments 6 and 7 significantly boosted accuracy by enabling a more layered approach to artifact filtering. The first stage effectively eliminated clear artifacts, while the second step captured residual artifacts missed in the initial pass, resulting in a more comprehensive validation system. This dual-layered approach allowed the first step to focus on filtering out major artifacts, while the second step refined the results by targeting minor details and smaller artifacts, thereby enhancing segmentation accuracy. Additionally, this approach facilitated parameter optimization, as different parameters could be applied at each stage; the first step used more lenient settings, while the second stage applied stricter criteria. This sequential filtering approach not only improved the precision of segmentation but also optimized detection performance as a whole.

5.4. Factors contribute to the system's failure to detect comets. Several factors contribute to the system's failure to detect comets. First, the absence of padding addition in Experiment 1, which replicated the methods used by Sreelatha et al. [13], caused detection issues. Without padding, the bounding boxes for detected comets were too small, leading to an Intersection over Union (IoU) value of less than 0.5, which in turn resulted in false positives (FP), as demonstrated in Figure 3(a).

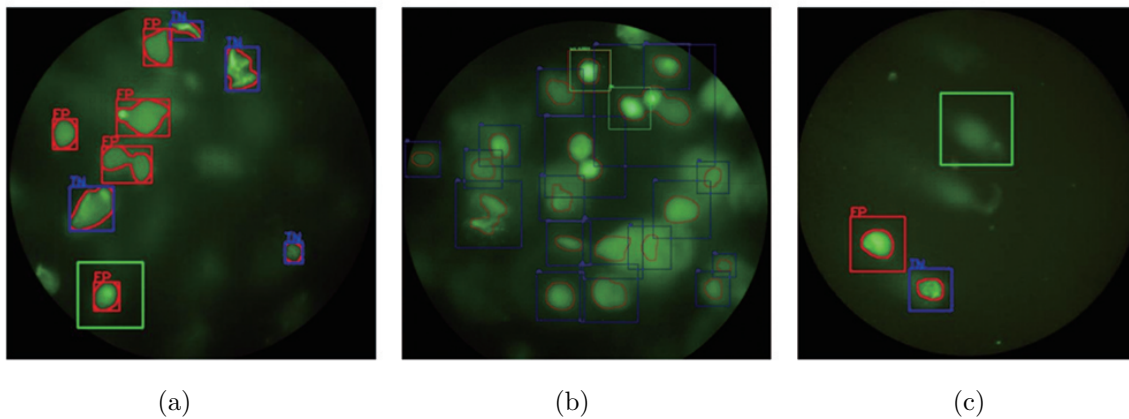


FIGURE 3. (color online) Factors contributing to comet detection failures: (a) Absence of padding addition; (b) overlapping objects; (c) low comet intensity

Another issue was segmentation failure due to overlapping. In some cases, comets were located too close to other objects, and during thresholding, these comets merged with adjacent objects, forming a single segment. This caused the system to reject them in the overlapping object rejection step, as illustrated in Figure 3(b). Finally, comet detection was hindered by low intensity levels. Many comets, particularly those with tails, exhibited low intensity, making them challenging to segment effectively during thresholding. Even if segmentation succeeded, comets with low intensity were often rejected during the artifact rejection process, as shown in Figure 3(c).

5.5. Performance far exceeding OpenComet. The research results demonstrate remarkable improvements in comet object detection for buccal mucosa comet assay images. Even the lowest performance achieved in this research, using traditional Otsu (1D Otsu) with an accuracy of 56.76%, substantially outperforms OpenComet's 11.5% accuracy. More impressively, the implementation of the 2D Otsu method achieved a peak accuracy of 84.16%, demonstrating a dramatic enhancement in comet object detection capability.

This substantial performance gap (from OpenComet's 11.5% to 2D Otsu's 84.16%) indicates that OpenComet, despite being a widely used tool [23,24], has significant limitations in analyzing comet assay images from buccal mucosa cells. This marked improvement highlights the importance of developing specialized biomedical image analysis methods that are specifically tailored to the unique structural and imaging characteristics of particular sample types. In the context of buccal mucosa comet assay images, domain-specific methodological adaptation enables substantially greater detection accuracy than generalized tools, demonstrating that customized computational frameworks are essential for reliable performance in specialized biomedical applications.

This superior performance can be attributed to several key characteristics of the developed method. First, the 2D Otsu approach demonstrates the ability to simultaneously consider spatial and intensity information, providing a more comprehensive image analysis [18,19]. Second, the proposed method also exhibits enhanced robustness against intensity variations and image noise, which are common challenges in microscopic image analysis. Third, the developed algorithm has been specifically designed to accommodate the unique characteristics of buccal mucosa images, enabling more accurate detection.

The research findings demonstrate a significant progression in comet assay image analysis accuracy. Starting from OpenComet's modest 11.5% accuracy, the implementation of traditional Otsu (1D Otsu) achieved a substantial baseline performance of 56.76%, culminating in the optimal performance of the 2D Otsu method at 84.16%. This progressive improvement in performance validates the effectiveness of the approach developed in this research. This progression underscores the significant advancements made in the analysis of buccal mucosa comet assay images. While OpenComet has been a standard tool in comet assay analysis, these results demonstrate substantial room for improvement when dealing with specific cell types such as buccal mucosa.

5.6. Practical applications and limitations. The proposed comet detection approach has significant practical potential in biomedical and clinical application, particularly support genotoxicity screening, early disease risk assessment, and clinical biomonitoring. Beyond these applications, the primary practical significance lies in its suitability for resource-limited settings. By relying on efficient digital image processing techniques, it offers a cost-effective interpretable, and reliable solution for laboratories healthcare facilities. This makes the system valuable in developing regions or under-resourced clinical settings, where accessible and affordable biomedical image analysis tools are essential.

Despite these advantages, several limitations remain. First, the dataset used in this study was relatively limited in size, which may restrict the broader generalizability of the findings across diverse populations, imaging devices, or clinical settings. Second, detection performance can still be affected by severe object overlap, extremely low comet intensity, and substantial imaging artifacts, which remain challenging in certain cases. Third, while the proposed method demonstrated substantial improvements over prior approaches such as OpenComet, further comparative validation with a wider range of contemporary comet detection methods would strengthen its broader applicability.

6. Conclusions. This study on comet detection in buccal mucosa comet assay images, based on digital image processing and a modified detection method from Sreelatha et al. [13], leads to several conclusions. First, comet detection in buccal mucosa comet assay images using this digital image processing method has been successfully implemented. Second, the order of detection algorithms can impact comet detection performance. Third, certain characteristics – such as the number of objects in a comet, comet intensity, and comet area – are not entirely compatible for detecting comets in buccal mucosa comet assay images. Lastly, system testing results show that the best detection performance, with an accuracy of 84.16%, was achieved using 2D Otsu thresholding, a T-Reject Artifact value of 0.9, and an algorithm sequence as follows: first, reject overlapping objects; second, reject artifacts; third, reject unwanted regions; fourth, reject artifacts again; and finally, add padding.

Future research should focus on validating the proposed approach on larger and more diverse datasets, including broader patient populations, varied imaging conditions, and additional comet assay sample types to improve generalizability. Further refinement may also enhance robustness against severe overlap, low-intensity comet structures, and complex imaging artifacts. Expanding and adapting this approach for wider biomedical and clinical applications could further strengthen its practical value as an accessible automated tool for DNA damage assessment. Future studies should also incorporate broader comparative evaluations with additional comet detection methods across standardized datasets to further establish the proposed framework's relative performance.

Acknowledgment. This research was funded by The Directorate General of Higher Education, Research, and Technology of the Ministry of Education, Culture, Research, and Technology, grant number 0459/E5/PG.02.00/2024; 2650/UN1/DITLIT/PT.01.03/2024.

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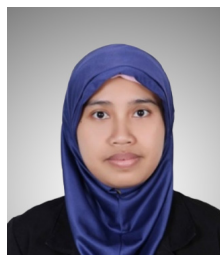
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