

Developing the Vertical Comet Assay and Image Handling Software for the Radiological Research Accelerator Facility Microbeam II

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Abstract

The vertical comet assay is a relatively new technique for quantitatively measuring radiation induced DNA damage in cells. The vertical comet assay provides potential for measuring DNA strand breaks in a dense monolayer of plated cells. This paper explores the successes and challenges of developing a vertical comet assay system for use with the current Microbeam II alpha particle source at RARAF. Additionally, the program BioComet, which was developed for confocal comet imaging, is explored in depth.

Comet Assay

The traditional 2D DNA comet assay is a widely used technique for quantitatively measuring DNA strand breaks. In the traditional comet assay, nuclei of damaged cells are placed in an electrophoresis buffer and then lysed to leave free DNA molecules in the gel.¹ The gel is placed in an electrophoresis buffer, and a current is applied across the system. The DNA has a negatively charged phosphate group that is attracted towards the cathode, causing the molecules to migrate.² Smaller fragments from double-strand breaks move through the gel more quickly than undamaged sections. The DNA is then fluorescently stained and imaged using a nuclear stain. The stain is excited with a laser and imaged with an optical microscope.

The resulting formation of DNA has the appearance of a comet with a tail composed of DNA fragments. In the standard 2D comet assay, a single image for each comet is collected and analyzed for parameters such as comet tail length, tail/head ratio, comet moment and comet radius. A longer tail implies greater damage, as the DNA is more highly fragmented. The comet is aligned along the electric field such

that the tail is pointing towards the cathode, and the head is nearer the anode.

Vertical Comet Assay

The vertical comet assay is a new technique that provides increased flexibility for DNA damage characterization. The only previous work on the vertical comet assay used a loose collection of cells in a gel³. After irradiation and gel electrophoresis, the comets were imaged at several slices in the z-plane using a confocal microscope. The resulting image stack was analyzed for the usual comet parameters.

The vertical comet assay is appealing because it allows for the study of bystander effects in dense cell monolayers without having to separate the cells from the monolayer for damage quantification (Figure 1). Additionally, because the images are taken confocally, there is more information for each comet, including the possibility of 3D reconstruction of the comets. This method of obtaining 3D data from a series of 2D slices closely resembles the image analysis found in MRI (Magnetic Resonance Imaging) and CT (Computed Tomography) scans.

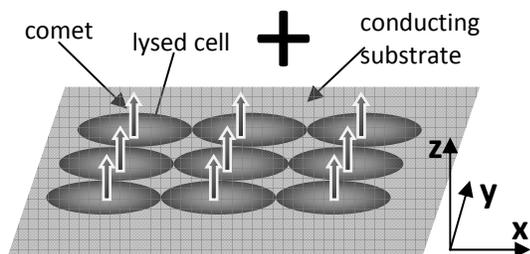


FIG. 1: Vertical comet assay for studying dense cell monolayers. Confocal images are taken in the z-plane

Developing the Assay Hardware

One objective of our vertical comet assay was to plate the cells, irradiate them with alpha particles, and perform the gel electrophoresis all in a single Microbeam dish, thus allowing for studies of dense monolayers. The cells are plated in 25 ml Corning polystyrene cell culture dishes with a 1 cm diameter hole drilled in the bottom to form a well when a substrate is attached (Figure 2).

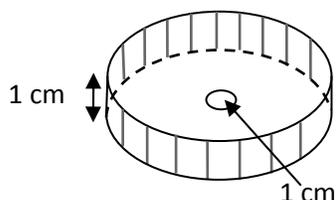


FIG. 2: Microbeam II 25 ml cell culture dish with 1 cm hole drilled in bottom.

The main challenge in developing a vertical comet assay system for the RARAF Microbeam was the development of a thin, biocompatible surface onto which to plate the cells. Initial tests were performed on 9.8 μm Mylar aluminized with 15 nm of aluminum. This thin yet durable material was easily attached to the microdishes using Master Bond epoxy. The aluminized Mylar was able to form a flat, tight substrate for the cells to plate onto.

Small Airway Epithelial Cells (SAEC) were removed from the cell culture dish with 0.05% Trypsin, and centrifuged to produce a 10^6 living cells/ml cell suspension in Small Airway

Cell Basal Medium (SABM). 2 μl of this suspension were added to the microdishes to produce a yield of around 2000 plated cells per well. The dishes were allowed to incubate and plate for 40 minutes before the addition of 2 ml of SABM to the well to support growth.

In addition to plating cells onto aluminized Mylar, dishes were prepared with a thin layer of Cell-Tak, a coating designed for plating cells. This allowed us to compare the aluminum only cell-plating with the Cell-Tak plating. However, the conductance of Cell-Tak was insufficient for our purposes.

To test the cell plating onto the aluminum and Cell-Tak, cells were stained with Hoechst 33342 UV nuclear cell stain two days after preparation. Light from a UV laser was passed through a filter cube in order to produce the 350 nm wavelength needed to excite the Hoechst stain. Figure 3 shows successful plating of the cells onto the aluminum, comparable to cells plated onto Cell-Tak.

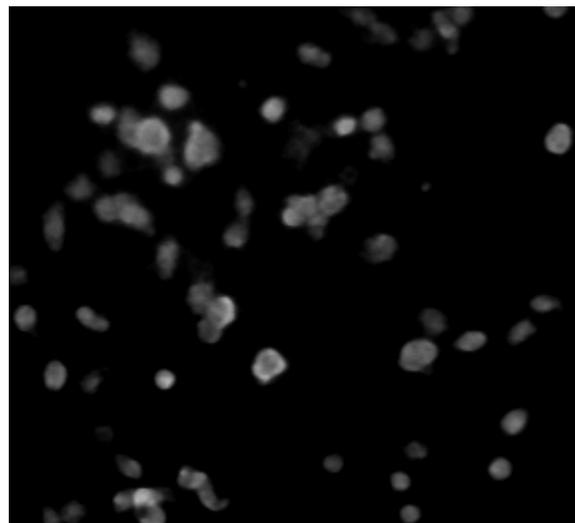


FIG. 3: Small Airway Epithelial Cells (SAEC) on aluminum labeled with Hoechst 33342 nuclear stain after 24 hours.

Initial electrophoresis tests on the aluminum substrate were unsuccessful, as the electrophoresis buffer reacted with the aluminum. Instead, a more inert conductor, such as gold, is required. Like aluminum, mammalian cells have been shown to successfully plate onto gold⁴. However, obtaining a thin film of gold was

difficult, especially without the availability of an evaporator or working electroplating solution. As a proof-of-concept design, gold leaf was obtained from L.A. Gold Leaf. The gold leaf has a thickness of roughly 100 nm, and is attached to 30 μm of tissue paper. The gold leaf was glued to the bottom of the microdish, along with an aluminum electrode for better electrical contact (Figure 4).

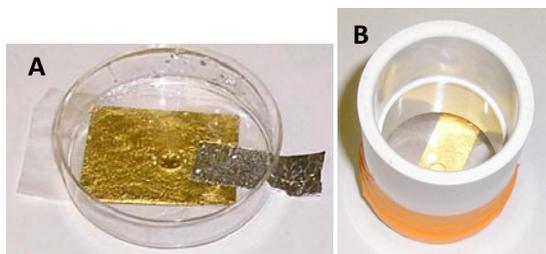


FIG. 4: a) Microbeam dish with gold leaf and aluminum electrode. b) Gel electrophoresis device without top electrode.

Gel Electrophoresis

The gel electrophoresis was performed in the microdish (see Appendix). 100 μl of LMP agar was placed onto the well and allowed to gel. The dish and 10 cm PVC pipe buffer attachment (Figure 4) were filled with 75 ml 1x TBE electrophoresis buffer. A current of 150 mA at 70 volts was applied across the two electrodes. The high current produces excess heat, and is plotted in Figure 6.

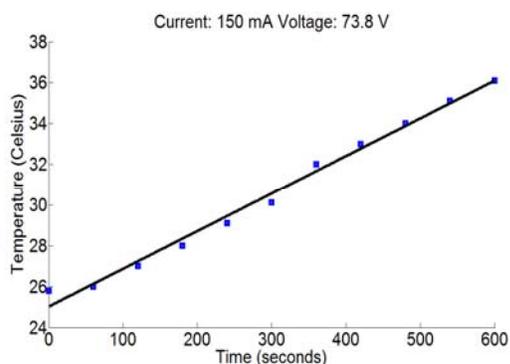


FIG. 6: Gel electrophoresis induced temperature change in sample.

The temperature plot indicates an average power lost to heating of 6.3 Watts. This small amount would be easily handled by a cooling device such as a Peltier cooler, which could be easily acquired for final development of the assay system.

Unfortunately, the gold leaf substrate did not provide enough structural support, and its loose attachment to the tissue was not sufficient under the stresses of the electrophoresis.

Radiation Simulations

While DNA damage can be applied using a variety of techniques. This particular comet assay is designed for use with alpha particle irradiation. The RARAF Microbeam II facility is capable of producing 6 MeV alpha particles at a beam-spot diameter of 3.5 μm ⁵. To test the energy loss associated with alpha particle irradiation of the microdish and substrate, SRIM⁴ (Stopping and Ranges of Ions in Matter) Monte Carlo simulations were run for the Mylar substrate. The results show minimal energy loss across 9.8 μm layer of Mylar used in the initial tests.

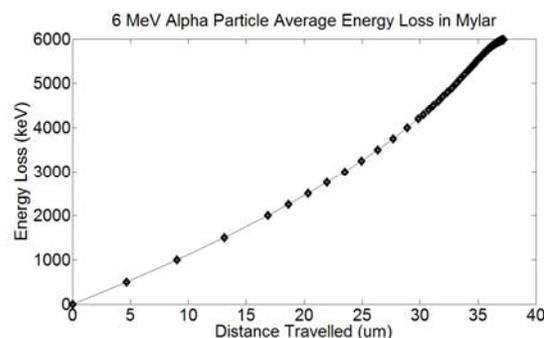


FIG. 7: SRIM calculations for 6 MeV alpha particle energy loss across Mylar and gold.

Additionally, it was necessary to simulate the scattering of ions as they pass through the substrate. The Transport of Ions in Matter (TRIM) Monte Carlo simulation (Figure 8) demonstrates minimal scattering and near full transmission of alpha particles through our barrier⁶.

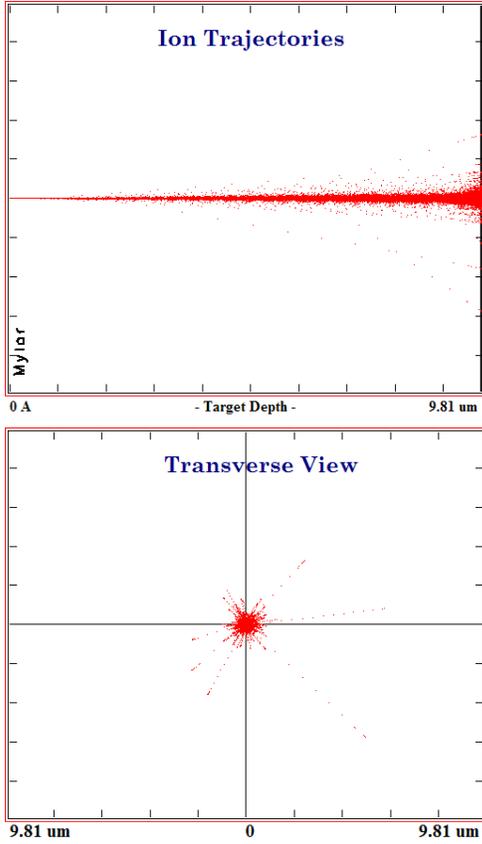


FIG. 8: a) 5000 Alpha particle trajectories through 9.8 μm Mylar barrier
 b) Looking down the beam line direction, we see that scattering is small relative to the beam spot and cell nucleus

Figures 7 and 8 give us a general idea of the thickness limits that must be adhered to when producing a substrate for the cells. However, to determine the exact amount of energy loss in our gold layer, we go to the Bethe-Bloch equation, which describes the rate of energy lost due to ionization of a particle traveling through a medium:

$$\frac{dE}{dx} = \frac{4\pi N_A Z \rho}{m_e c^2 A} \frac{z^2}{\beta^2} \left(\frac{e^2}{4\pi\epsilon_0} \right)^2 \left[\ln \left(\frac{2m_e c^2 \beta^2}{(10\text{eV})Z(1-\beta^2)} \right) - \beta^2 \right] \quad (1)$$

Where m_e is the mass of an electron, N_A is Avogadro's number, Z is the target atomic number, A is the target atomic weight, ρ is the target density, z is the ratio of the particle charge to the electron charge, and e is the charge of an

electron. At a He^{2+} particle energy of 5 MeV ($\beta = 0.05174$), the energy loss rate in a gold substrate is 440.47 KeV/ μm .

A thin layer of gold (15 nm) evaporated onto Mylar would provide the best alpha particle transmission. An alpha particle with an initial energy of 6 MeV would lose around 1 MeV in the Mylar and only tens of KeV in the gold. This would allow the cells to receive a majority of the alpha particle energy.

Image Analysis

Motivation

In order to obtain quantitative information about DNA strand breaks, image analysis software must be applied to the fluorescently labeled comets. Currently, there are numerous commercial software packages available for the analysis of traditional 2D comets⁷. However, this software is unsuitable for use with 3D comets, which use multiple images per comet rather than a single image. Software which runs algorithms similar to those in the 2D software, but on a 3D image stack, needed to be developed.

To analyze the images, the "BioComet" software package was developed to obtain statistics from confocal images of fluorescently labeled DNA. BioComet was written in Java because of its cross-platform capabilities, and because unlike Matlab or LabVIEW, Java is free to use and only requires the Java Virtual Machine. Furthermore, Java contains an extensive library of Application Programming Interfaces (API) for graphics handling, file input and output, and image analysis. Specifically, the Java Advanced Imaging (JAI) API was used most extensively because of its wide range of image handling abilities. The JAI package has shown great success with a wide range of biomedical imaging applications, including gel electrophoresis⁸ and multidimensional images from MRI and CT scans⁹. Additionally, JAI is able to handle many different file formats, including the TIFF format, which is commonly used as a file type for images obtained from microscopes.

BioComet was designed to be user friendly, providing both an appealing Graphical User Interface (GUI) and allowing the user to customize the image analysis performed by the program. The program contains many familiar GUI components, such as file choosers and text areas to allow the user to become familiar with BioComet with little training. Furthermore, the source code was commented in HTML to provide users with a full Javadoc^{††} for the BioComet class library, providing for further customization of the program, as well as future development.

Choosing the Image Stack and Setting Output Options

BioComet comes with a file chooser and filter, displaying only supported image files. The user chooses a directory containing the desired image stack, and may click on any file to scale and display the icon in the display panel. Figure 9 shows a sample image being loaded from a directory containing TIFF files of SYBL Green labeled cell nuclei under confocal fluorescence imaging.

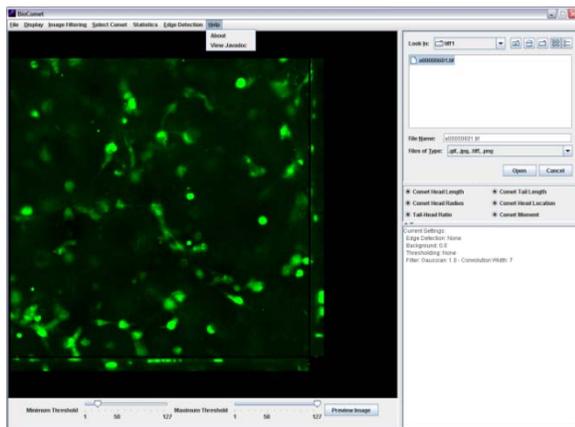


FIG. 9: BioComet GUI displaying scaled TIFF file of fluorescently labeled cell nuclei

BioComet allows the user to write the final analyzed images to an image file (PNG format). The user may choose the directory to write to, and the files are grouped by comet, into folders “PicFolderX” where X is the comet number. Additionally, the comet statistics can be

written to a DAT file for processing in a program such as Matlab. The files are stored in a folder labeled “DataFolder.”

BioComet automatically sorts image files based on the number with which they end. This allows for immediate processing of images from the confocal microscope, which typically end in a number defining their position in the stack. Only images ending in a number are analyzed.

The image stack is run through a series of filters and algorithms to extract the most accurate data possible. At every step of the image handling, the user is able to customize the algorithms to his or her purposes, including leaving out or adding certain filters or algorithms. The primary algorithm sequence run on the image stack is the Canny Edge Detection method¹⁰. However, at various points in the algorithm sequence, data was collected on the images to give information on certain comet statistics.

Grayscale

The first step is to convert the image to grayscale, a common technique for analyzing fluorescently labeled comet images¹¹. Image files contain a standard weighted ratio of colors. Equation 1 provides the necessary weighting to convert the image to grayscale¹².

$$P_{xy}(r, g, b) = 0.2125r + 0.7154g + .0721b \quad (2)$$

Where $P_{xy}(r, g, b)$ is the grayscale pixel at location (x, y) , r is the red value of the original pixel, g the green value, and b the blue value.

Background Filter

BioComet allows the user flexibility in filtering the background noise out of the image before further processing is performed. The user selects the background of the image using a selection tool (Select Comet -> Background), and can then preview and set the background (Image Filtering -> Set Background). The background filter works by summing the pixel values of the selected area to find a mean

intensity per pixel (Equation 3). The entire image is then looped through, and pixels with a value less than the background value are set to 0.0 (black). Pixels above this threshold remain unchanged.

$$\bar{I}_z = \frac{\sum P_{xy}}{x \cdot y} \quad P = \begin{cases} 0 & \text{if } P < \bar{I}_z \\ P & \text{if } P > \bar{I}_z \end{cases} \quad (3)$$

Gaussian Filter

To reduce noise, the images are then filtered with a normalized Gaussian function with standard deviation σ (Equation 4). The filter is applied by means of discrete convolution filtering (Equation 5). A graphical representation is shown in Figure 10.

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

$$P_{xy} = p_{xy} \otimes k_{xy} = \sum_{m=0}^{M-1} \sum_{n=0}^{N-1} K_{mn} P_{x-m, y} \quad (5)$$

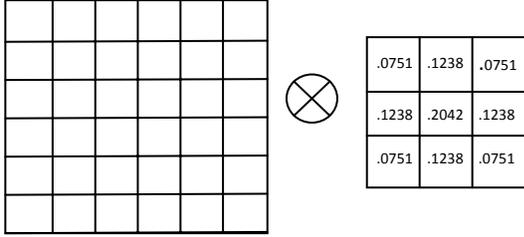


FIG. 10: Graphical representation of convolution filtering of image (left) with $\sigma = 1.0$ Gaussian kernel (right).

To evaluate the discrete components of the kernel, the Gaussian function is evaluated at each pixel of the kernel (0,0), (0,1), (1,1), etc, and then normalized so that the sum of the kernel values is unity. Notice that if the input image is x by y pixels, the output image will only be $x-1$ by $y-1$ pixels for a 3 by 3 kernel (a 5 by five kernel yields $x-2$ by $y-2$, etc).

BioComet defaults to $\sigma = 1.0$, which seems to give sufficient Gaussian smoothing for most purposes. A higher magnitude of smoothing is available, but is generally unnecessary for most reasonably well defined images.

Intensity Measurements

Before the image is further operated on, it is necessary to obtain statistics on the intensity of the fluorescence of each image in the stack. Equation 6 sums all the pixel values in a certain slice of the image stack. This operation is performed for each image to obtain a set of I_z values for the image stack. The first I_z above the background is defined to be the beginning of the comet. The largest I_z value then corresponds to the location of the center of the comet head. Finally, the location of the comet tail is defined when I_z converges again to the background intensity.

$$I_z = \sum P_{xy} \quad (6)$$

The length of the comet head is defined to be twice the distance from the beginning of the comet to the center of the comet head. Similarly, the length of the comet tail is defined to be the distance from the comet head center to the end of the tail.

Gradient Operation

The next convolution performed on the image is the gradient magnitude operation, which uses two Sobel kernel¹³ operators, one in the x direction and one in the y direction (Figure 11) to approximate the first derivate of the image. Using Equation 7 after applying the two convolutions, we obtain the new P_{xy} pixel values:

$$P_{xy} = \sqrt{G_x^2 + G_y^2} \quad (7)$$

where G_x is the result of the x -direction convolution, and G_y the y -direction.

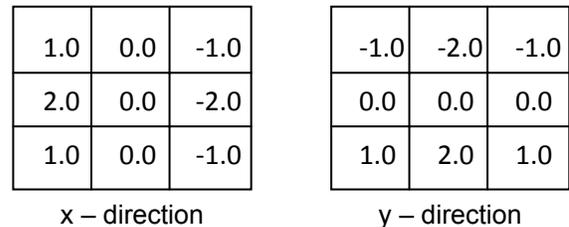


FIG. 11: Sobel kernel operators for edge detection

Edge Thinning and Hysteresis Thresholding

The final set of algorithms performed on the image is edge thinning and hysteresis thresholding. The algorithm takes the Gaussian filtered image and applies the Sobel convolution and stores the results in a matrix of gradient values for each pixel. Using these matrices (G_x and G_y), a new matrix storing the angle of the gradient is computed using Equation 8. The angles are then rounded to the nearest value of 0, 45, 90, or 135, corresponding to the location of a neighboring pixel.

$$\theta = \text{Tan}\left(\frac{G_y}{G_x}\right) \quad (8)$$

Next, the technique of “non-maximum suppression” is applied to the original image using the angles defined by the gradient matrix.⁹ Pixels from the original image on either side of the gradient are tested. If either of the neighboring pixels is greater than the source pixel, then the source is set to zero (Figure 12).

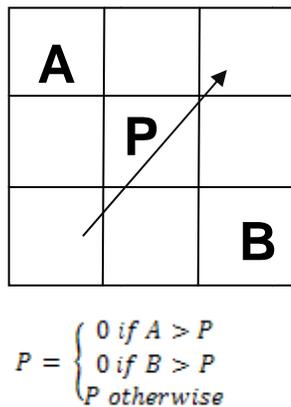


FIG. 12: Example operation on pixel P with gradient indicated by arrow.

Finally, the resulting image is converted to binary by the process of “hysteresis thresholding.” Pixels above a set “maximum threshold” are turned on (intensity of 255), and pixels below the minimum threshold are turned off (intensity of 0). Pixels with intensities between the two thresholds are reconsidered after the initial thresholding: if these pixels are neighbored by any “on” pixels, they are set to on”. Otherwise, they are set to “off.” This method

of thresholding supports continuous shapes, but rejects noise.

The advantage of using the Canny edge detection method are in the significant noise reduction. Figure 13 shows a Gaussian smoothed, background filtered image that contains two cells, as well as background noise not associated with any cells. This improved noise reduction is valuable for determining the length of the comet head and tail.

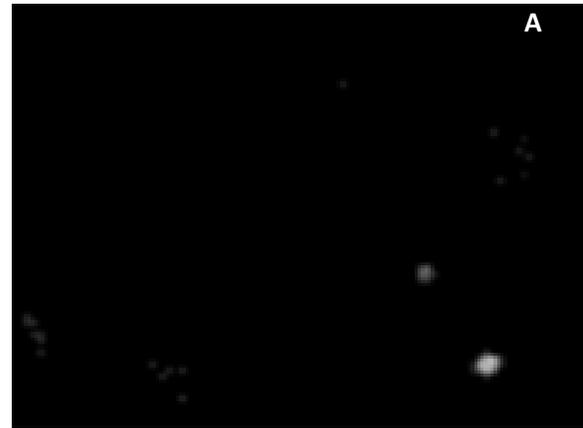


FIG. 13: a) Original image of two cells and background noise. b) Two functions representing I_x , (pixels summed vertically to create vector) one without edge detection (picture shown), and one with the Canny edge detection algorithm applied. The Canny edge detection retains information for the more well-defined cells, and filters out the smaller, less well defined noise on the left hand side of the image.

Comet Head Radius

Once the location of the center of the comet head is determined, BioComet can calculate the comet head's radius. The algorithm works by first converting the two-dimensional pixel matrix into a one-dimensional vector. The result is shown in Figure 14. The left edge of the comet head is set when the pixels increase over the background level. The right edge is set in the same way, and the difference is the comet radius. This is done twice, once for the I_x vector and once for I_y . The radius is taken to be the average of the two calculations.

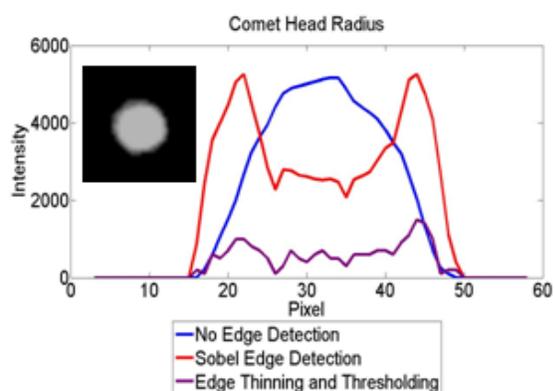


FIG. 14: Comet head radius analysis at various points in the Canny edge detection algorithm.

Despite the improved noise reduction for detecting the location of the comet, it is not advantageous to apply the full Canny algorithm for determining the comet radius. Figure 14 shows the I_x vector for the given image at three different points in the Canny edge detection sequence. The best representation of the image for calculating comet head radius is the function with the greatest slope at the edges and most clearly defined contrast from the background. Therefore, BioComet performs the comet head radius algorithm after the Sobel edge detection, but before edge thinning.

Conclusions

Development of the vertical comet assay for Microbeam II still requires some additional work. The proposed setup is sufficiently radiation transparent and would allow

the cells to receive a high radiation dose. A gel electrophoresis device was designed for the vertical comet assay, assembled, and tested. The concerns of heating in the electrophoresis process have been quantitatively analyzed, and are easily remedied.

Additionally, aluminized Mylar was successfully placed onto the dishes and provided the necessary tension required to keep the surface flat. And although the buffer reacted with the aluminum, cells were able to plate very well, further suggesting that gold evaporated onto Mylar would be a successful substrate.

To finalize the project, a cooling system (such as Peltier) and a supply of evaporated gold or platinum onto Mylar or some stretchable plastic is required. Additionally, a green or red DNA stain (such as SYBL Green) is needed, along with a confocal microscope with overhead laser illumination.

The program BioComet was developed with confocal fluorescence imaging of vertical comets in mind. However, the program's convenient graphical interface, vast image processing capabilities, extensive user flexibility, included Javadoc and ease of editing all provide possibilities for other types of applications involving image stack analysis. The operations tree design (see Appendix) allows for quick and easy additions to the image processing algorithms without the need to rewrite the GUI and many standard image processing techniques.

After final development, future work could include measuring the bystander effect by analyzing the difference in comets between irradiated cells and nearby cells. Additionally, more automation in BioComet could provide full comet analysis without requiring the user to specify where comets are located. Rather, the edge detection algorithms currently in place could be developed to detect comet locations from the image stack. ■

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Appendix

Cell Handling Procedures*

Placing aluminized mylar on microdishes

Materials: Microbeam dishes, large plastic dish, electronic balance, ethanol, Compounds A and B, microscope slide, sandpaper, roller, aluminized mylar

Procedure:

1. Lightly sand the bottoms of the dishes with sandpaper. Set aside
2. Mix 4 parts A to 1 part B, by weight, by measuring in electronic balance. Measure to nearest milligram.
3. Stir for 5 minutes with microscope slide. Then let sit for five minutes. Turn on oven to 100 C.
4. Roll compound onto bottom of dishes. Place aluminized mylar onto bottom of dishes.
5. Turn off oven, and when at 90 C, place dishes in oven and let sit overnight.
6. Mix 70% ethanol, 30% water, and place dishes in mixture for 45 minutes to sterilize.
7. Allow to dry standing on side in sterile hood
8. Wash roller with ethanol.

Applying Cell-Tak

Materials: Cell-Tak, 0.2 M NaCo₃, Microbeam dishes, small capsule large plastic dish

Procedure:

1. Mix 4 μ l Cell-Tak, 36 μ l, and 40 μ l NaCo₃ in small capsule
2. Mix using autoclave, place 16 μ l of solution in each dish
Make sure solution covers entire base
3. Place in large plastic dishes and place in low-temp oven for 40 min
4. Wash 3 times each with 5 mL sterile water. Aspirate off using sterile aspirator
5. Replace in plastic dishes, allow to dry for a few hours.
6. Label large plastic dishes and microbeam dishes.

Plating Cells

Materials: SABM, 0.05% Trypsin, microdishes, large plastic dishes, hemocytometer, glass coverslips, centrifuge capsule (and counterbalance).

Procedure:

1. Sterilize aspirator and aspirate off medium.
2. Use 5 ml Hank's Solution to remove serum
3. Add .5 ml 0.05% Trypsin (2.5% diluted in Hank's Solution)
4. Shake lightly, place in incubator for a few minutes. Check that cells are removed.
5. Add 4 mL SABM buffer to inactivate trypsin.
6. Place small amount (2 μ l or so) of cell mixture into hemocytometer
7. Count cells (only those reflecting light). One big square signals 10^4 cells per ml.
8. Place cells centrifuge capsule. Place capsule in centrifuge with counterweight.
9. Centrifuge at 1500 rpm for 5 min (Program 3)
10. Aspirate off excess media, leaving cell pellet.
11. Add enough SABM to make 10^6 cells per mL
12. Place 2 μ l on each microdish. Immediately cover with glass coverslip.
13. Place in incubator (20-25 min for fibroblasts, 45-50 min for EC).
14. Remove glass coverslips and add 2 mL SABM per microdish.

15. Replace in incubator
16. Wash hemocytometer.

Staining the Cells

Procedure:

1. Dilute Stain 33342 1 in 10 (we want between 50 nM - 100 nM concentration on the cells)
Initial stain concentration = 1 μ M
To create the 50-100 nM concentration, remove liquid currently in dish, add stain to get desired concentration, place in dish.
2. After placing stain on cells, allow to sit for 30 min in incubator.

*Procedures compiled by Jeremiah Wala from existing RARAF protocols

Operation Sequence

```
/** Sends images through operations sequence. Calls methods for ripping pixels, converting to grayscale,
 * filtering image, and writing files.
 * Contains a {@link PrintWriter} that writes both image and data files to local disk. Calls {@link getHist} to
 * obtain {@link Histogram} object containing statistics of grayscale image.
 * @see {@link ImageCalculator}
 */
public void sumForCometHead() {
```

```
    currentImage = grayScale(false, currentImage);
    area = height*width;
    DataBuffer buffDB = currentImage.getRaster().getDataBuffer();

    try {

        //filters image
        if (!standardDeviation.startsWith("No"))
            currentImage = gaussianFilter(standardDeviation, currentImage);

        //gets intensity total
        total = getTotalIntensity(currentImage);

        //edge detection
        if (DETECTION != 0)
            currentImage = edgeDetection(currentImage);

        post_thin_total = getTotalIntensity(currentImage);

        //computes radius and total
        if (!gotRadius)
            finalRadius = getCometHeadRadius(currentImage);
        gotRadius = false;

        //Thresholding
        if (THRESHOLD != 0)
            currentImage = thresholding(currentImage);

        if (writeImageFile || FrontWindow.PREVIEW)
            for (int i = 0; i < width*height; i++)
                buffDB.setElem(i, (byte)buffDB.getElemDouble(i));
```


Gel Electrophoresis Setup

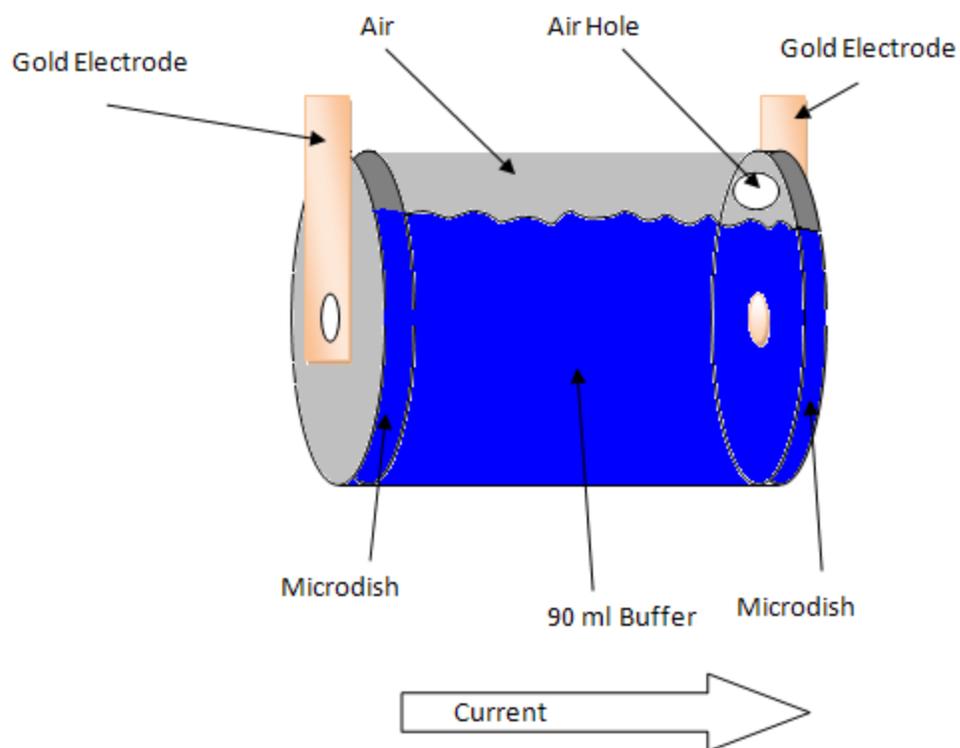


FIG. 16: Schematic of PVC pipe gel electrophoresis setup. The pipe is water tight and provides the necessary hardware for a proof-of-concept vertical comet assay device. The air hole is required to keep the pressure constant, but could also be used to filter through electrophoresis buffer or as a place to pour the buffer in.