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Original Research Article

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Title a Novel Approach to Cell Viability Measurement in Paclitaxel Cytotoxicity Experiments

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ABSTRACT

Cell viability and drug cytotoxicity measurements conducted for triple-negative breast cancer cells are the core measurements in chemotherapy treatment protocols. Spectrophotometric methods that depend on the use of harmful biochemicals are used to measure cell viability. This study proposes the use of digital image analysis techniques to measure cell viability. The digital image analysis results were compared to the results of conventional biochemical assays. MATLAB's Image Analysis Toolbox was used to perform the viable cell count. The results of the cell count were compared to the trypan blue assay using hemocytometer slides and MTT assays using microtiter plates. There was strong to moderate statistical significance seen between trypan blue assay, MTT assay, and digital image analysis assay. The study confirmed that traditional biochemical methods contribute to cell death and incorrect viable cell count. Image analysis assays were less laborious and took minimal time to complete the analysis once the image was obtained. The research results recommend the use of digital image analysis techniques to perform more precise and accurate drug kinetic studies in the popular field of personalized therapy.

1. Introduction

Cell viability experiments are used to measure the number of viable cells in a sample. It is used in testing the cytotoxic effect of chemical compounds. Colorimetric, dye exclusion, fluorometric, and luminometric assays are used to determine cell viability. All of the assays require an incubation period where the cells are incubated with the reagents. The viable cells convert the substrate to a colored substance or generate a fluorescence that can be detected or measured using a plate reader. For the fluorescent assays, the strength of the signal is proportional to the number of viable cells [9]. Conventional biochemical assays are not the ideal method to determine cell viability. It utilizes chemical reagents that contribute to cell death. The results are dependent on the concentration of reagents, and the length of the incubation. The pH of the solution plays a vital role In the experiments too. Therefore, each experiment is exposed to multiple variables that may lead to an increased chance of false-positive or false-negative results [10].

Although the Tetrazolium reduction assay (MTT) method is considered a reliable method to measure the number of viable cells, the cytotoxic effect of formazan and other chemicals that are present in the cell culture impact the accuracy of the procedure. Formazan products precipitates accumulate inside the cell and cause false spectrophotometric reading.

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Formazan crystals may harm the cells by puncturing the cell membrane, which contributes to cell death and falsely decreased viable cell count [8]. The insoluble precipitates must be solubilized before measuring the absorbance. Dimethylformamide, Acidified isopropanol, and other detergents are used to solubilize the precipitated salts. The color of the phenol red that is present in the cell culture interferes with the absorbance reading. Acidifying the medium turns the phenol red to yellow, which has less impact on the reading [1,6]. A falsely elevated number of dead cells during cell viability experiments can lead to the wrong decision made regarding the efficacy of the medication under investigation. A new approach is needed to replace conventional spectrophotometric and manual methods.

1.1. Preliminaries

Biological samples produce images that are noisy, full of artifacts that can impact the decision made by the performing personnel. Imperfections of the slide or the stain can prevent the human eye from distinguishing or extracting cellular features. Image analysis extracts quantitative information from pictorial data. Automated image analysis instruments can apply techniques to correct any imperfections before extracting features. It can capture the nuclear orientation, structure, shape, and texture of cells and nuclei from hematoxylin and eosin slides [14]. It is capable of performing features analysis or use deep learning techniques to extract and classify features or objects. Deep learning is the process of training the computer to read slides using a set of pre-programmed features. During this process, the computer continues to learn new features based on user inputs.

1.2. Image Analysis Techniques

Image analysis starts with acquiring the image; this step is performed using a digital camera. The images are often obtained from a microscope. One of the most common issues with microscope images is uneven illumination and noise at low levels of light. The image processing step improves the picture quality by removing the noise and adjusting the image contrast. Features extraction is the step where computer techniques and algorithms are used to extract patterns and morphological features from the image. This step is called image segmentation. Data collection is the final step in the process; quantitative data is collected during this step using computational tools that are applied to determine the properties of a specific region or quantitate object based on its features.

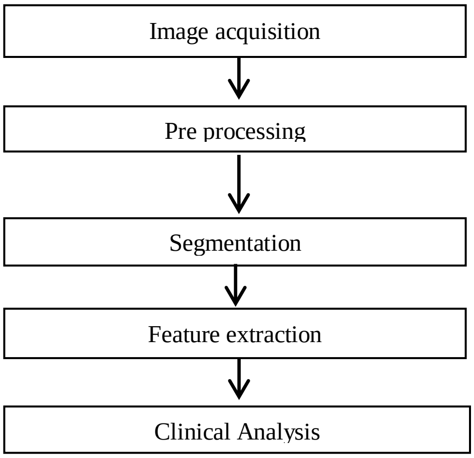
2. Methods

Paclitaxel (Taxol) is a cytotoxic agent used to treat multiple types of cancers, including breast, ovarian, lung, cervical, and pancreatic cancers [2,13]. It kills the cells by causing chromosome missegregation on multipolar spindles. The effect of paclitaxel is directly proportional to its concentration in the cells [13]. A Variety of conventional biochemical assays are used to measure cell viability to evaluate the effectiveness and efficacy of drugs. The process includes the incubation of cells with a reagent or a chemical dye. The cells convert the substrate to a colored or a fluorescent product. The wavelength or the degree of fluorescence can be detected using a spectrophotometer or a plate reader. The intensity of the color or the amount of fluorescence is directly proportional to the number of viable cells [12].

This proposal discusses the use of image analysis techniques to measure the viability of cells instead of using conventional biochemical assays. The image analysis assay is performed using high-resolution cameras without adulterating the cells with reagents or dyes. The experiment included comparing the results of standard biochemical assays to image analysis when measuring cell viability.

The images obtained were processed and analyzed using Matlab. The number of cells was counted then the results of manual cell counter were compared to the results of the digital image analysis technique. Figure 1 indicates the flow of image processing and analysis.

Figure 1: Flow of image processing and analysis



Human breast cancer cells (MDA-MB-231) were cultured in DMEM medium supplemented with 10% FBS, 1% penicillin-streptomycin. Cells were suspended in media at a concentration of approximately 76,000 cells/mL. The cells were cultured in 50ml culture vials and were stored in a 5% CO_2 incubator at 37^oC. At the end of 24 hours, the cells were harvested by trypsinization and loaded in 96 well plates. These cells are treated with 10µM paclitaxel concentrations ranging from 0.6-6,000 ng/ml (logarithmic dilution- 1:10) and stored in 5% CO_2 incubator at 37^oC. Some cells remained untreated and were used as a control. After 72 hours of incubation, the treated and untreated cells were quantified using MTT dye and trypan blue assays.

2.1. Biochemical Assays

At the end of the treatment phase of 72 hours, the following staining assays were carried out for analysis.

2.1.1. MMT Dye Assay

10 µl of MTT reagent was added to each well and incubated for 2 hours. The media was aspirated carefully without disturbing the cells at the surface of the wells. 100 µl of 10% acidified isopropanol was added to all wells. The plate was incubated for 10 mins at 37ºC and was prepared to be read at 570nm in a microtiter plate reader.

2.1.2. Trypan blue assay using a hemocytometer

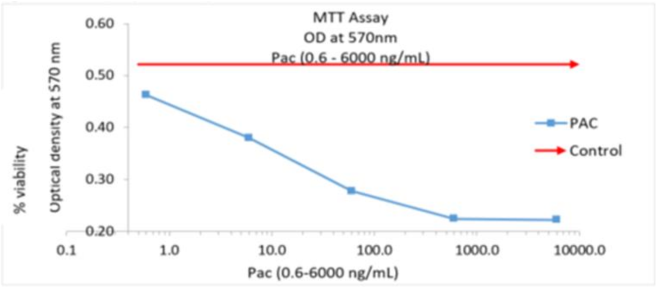
The adherent cells were harvested with trypsin EDTA. The cells were stained with 0.4% trypan blue reagent. A cell culture suspension of 100 µl was studied on a hemocytometer counter to conduct a cell viability count using trypan blue diluted in PBS (1:3). The spatial distance that is the distance between the nuclei of cells was eyeballed.

3. Results

3.1. MMT Dye assay using ELISA

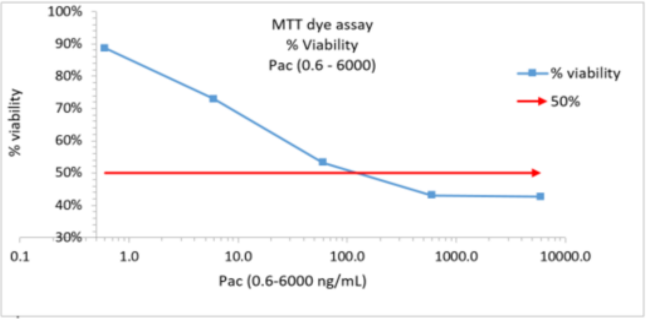
The cells quantified using MTT reagent was read at 570 nm on the ELISA reader (microplate spectrophotometer). Dose-response of paclitaxel effect on breast cancer cells MDA-MB-231 is shown in figure 2.

Figure 2: MTT assay- optical density measurements.



The cell titration curve observed and percent cell viability measured showed an exponential decrease in cell viability at and beyond 600ng/mL of paclitaxel onwards, as shown in figure 3.

Figure 3: MTT assay percent cell viability.



3.2. Trypan blue assay using a hemocytomete

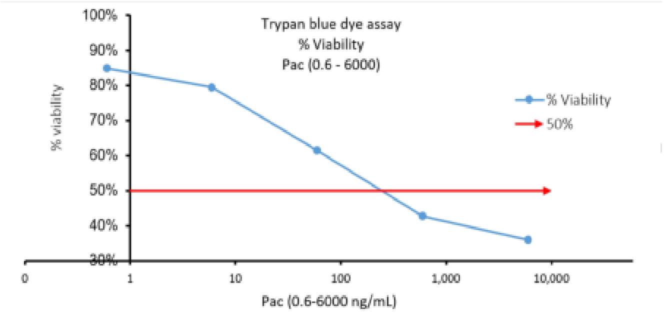
The laborious and time-consuming method to quantify viable and non-viable cells using a hemocytometer and an inverted microscope is shown in table 1 below.

Table 1: Percent cell viability measured using Trypan blue assay and hemocytometer.

Paclitaxel (*ng/mL*)	**Count**		**Cell Viability** $\left\{\frac{Live}{Live+Dead}\right\}\%$
	Viable	**Nonviable**	
0 *(Control)*	567	24	95.96 %
0.6 *ng/mL*	478	85	84.90 %
6 *ng/mL*	452	117	79.44 %
60 *ng/mL*	342	215	61.40 %
600 *ng/mL*	102	137	42.68 %
6,000*ng/mL*	37	66	35.92 %

Untreated cells (control) show 96% viable cells because the cells continue to go through the life cycle. Figure 4 shows the percentage cell viability curve for the Trypan blue assay.

Figure 4: Trypan Blue assay using hemocytometer – percent cell viability.



3.3. Image analysis for quantification of cells

Digital images are prone to a variety of noise. Noise is a result of errors in the image acquisition process that result in pixel values of the image not to reflect the true intensity value of the real scene. There are numerous filters available for noise removal. The median filter is used to replace the intensity of each pixel in the image with the median of intensities in the predefined neighbourhood. It eliminates intensity spikes by forcing areas with very distinct intensities to match the surrounding area; therefore, smoothing the image [4]. The Median filter was used to remove the salt and pepper noise without reducing the sharpness of the image. Figure 5 indicates the original image (a) and the image after applying the median filter (b).

Figure 5: a) Original noisy grey image b) noise removed using a median filter

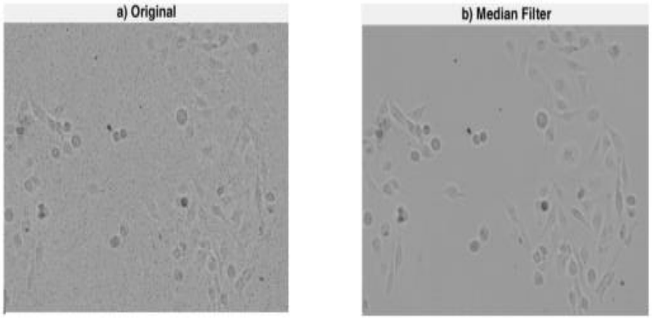


Image enhancement improves the interpretation and perception of information. The main objective of image enhancement is to modify the attributes of the image. Mathematically, image enhancement is transforming an *image ‘I’* into *image ‘J’* using transformation *‘T’* The intensity values of each pixel in input and output (*resultant*) images are a and b, respectively [3]. Hence, the relationship between a and b is given: $a = T(b)$

Contrast enhancement is done using the histogram equalization command. The command improves the quality of the image by stretching the contrast. The process enhances the process of detail visualization. Figure 6 depicts the histogram equalization Process. The intensity of the image was equally distributed throughout every pixel in the image, which increased the contrast producing a highly enhanced image. Figure 7 is a side by side comparison between the original gray image and the adjusted image.

Figure 6: a) Gray image b) Gray image histogram c) Adjusted image d) Adjusted image histogram

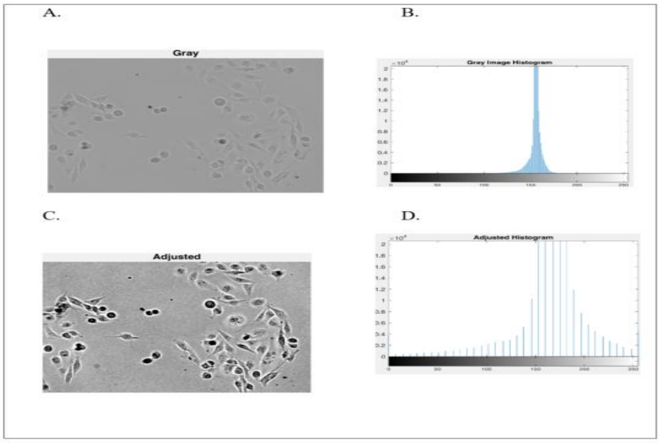
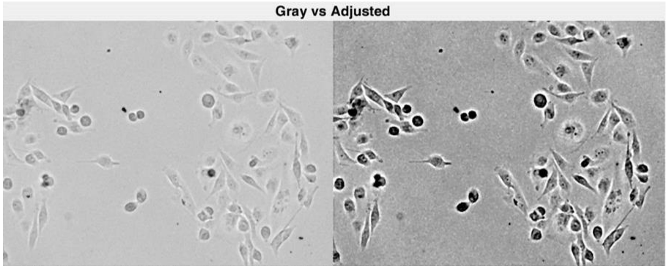
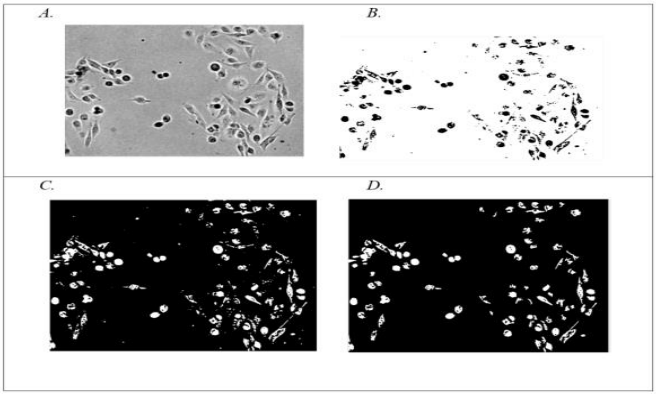


Figure 7: Original gray image and adjusted images



The image must be segmented to start the cell count step. The first step in the segmentation process is to isolate the image background from the objects using a process called thresholding [5,11]. In this case, the image has cells that have a different intensity than the background. Identification and segmentation using threshold segmentation allowed the proper distinction of viable cells from non-viable cells and other cell debris in the image. As a result of image enhancing and processing steps, the clear visible intensity values of viable cells and non-viable cells make segmentation and identification the first milestone achieved using digital image processing. The resultant binary images were further processed to quantify cells based on morphological characteristics such as size and shape. This added step filtered out the structures that were either smaller or larger than the average size of a viable cell. This step was performed for both intensities of viable and non-viable cells that make this quantification technique a robust model, as indicated in figure 8.

Figure 8: A) High contrast grayscale image B) Thresholded image C) Morphological preprocessing of the image D) Live-cell segmentation.



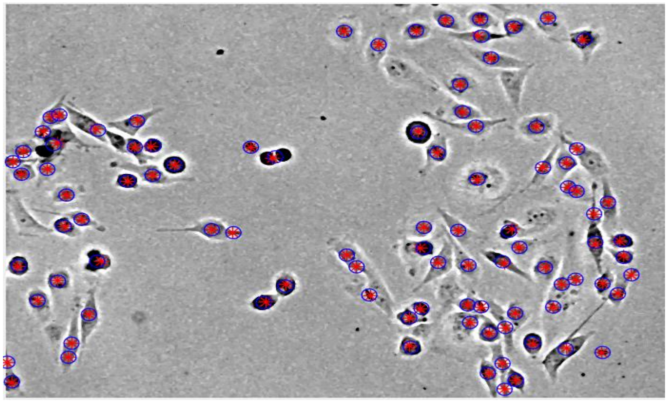
The spatial distance was measured from the nuclei of viable cells identified, and the consequential Euclidean distance was analyzed.

Euclidean distance is calculated by

$$d^2_{euclidean} = (x_1 - x_t)(x_1 - x_t)'$$

Where x is the centroid location matrix, x_1 is an individual nucleus, and x_t are all the other nuclei [4,5,7]. The average scattered euclidean distance was directly proportional to drug toxicity or indirectly proportional to the number of viable cells. The analysis of the data collected from the above techniques aided in identifying viable, non-viable cells and cell debris. The void spatial Euclidean distance further confirmed the percent cell viability. Figure 9 depicts nuclei identification using centroids.

Figure 9: Spatial distance (Euclidean distance) - nuclei identified.

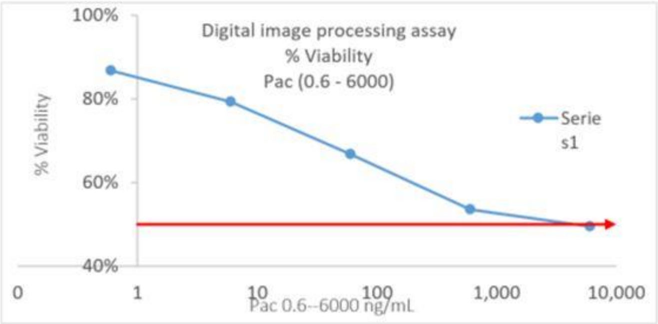


An average count of four random images for each well in the titration was collected and analyzed. The table below shows the average (with variance) spatial distance measured, count of viable cells, and count of non-viable cells in untreated and serial diluted treated cells. As shown in Table 2 and figure 10.

Table 2: Percent cell viability measured using digital image processing and analysis.

	Average spatial distance	**Average number of viable cells**	**Average number of non-viable cells**	**% Viability**
Control (0 ng/mL)	624.53 (± 52.00)	63.5 (±18.70)	4.5 (± 2.30)	100.00%
0.6 ng/mL	627.56 (±55.38)	53.8 (±30.00)	5 (± 4.60)	91.12%
6 ng/mL	630.79 (±10.63)	40.0 (±42.00)	8 (±7.14)	83.20%
60 ng/mL	648.59 (± 97.30)	35.25 (±13.32)	12 (± 5.43)	70.00%
600 ng/mL	663.73 (± 88.61)	31.40 (±23.55)	14 (± 3.40)	56.24%
6000 ng/mL	673.93 (± 35.62)	24.4 (±23.51)	25 (±16.60)	51.90%

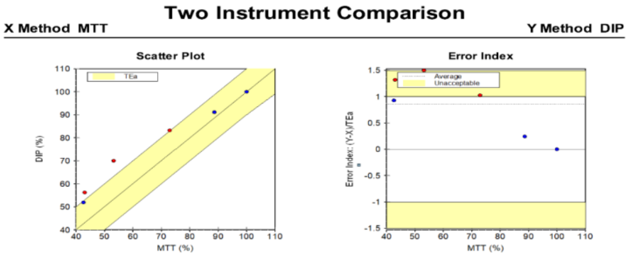
Figure 10: Digital image processing assay - percent viability.



3.4. Comparison study

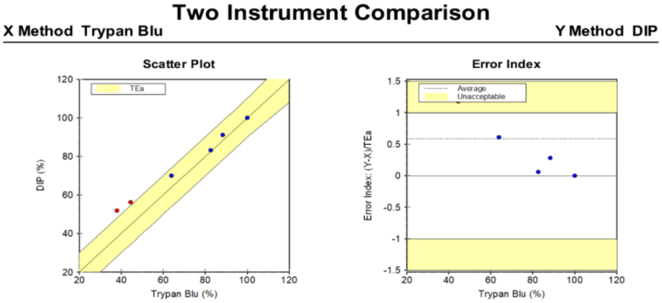
A Cell Count was analyzed by methods MTT and DIP to determine whether the methods are equivalent within the allowable total error (TEA) of 10 %. Six specimens were compared over a range of 42.62 to 100.00 %, as indicated in figure 11.

Figure 11: MTT and DIP two method comparison.



The difference between the two methods was within allowable error for 3 of 6 specimens (50.0%). The average error index (Y-X)/TEA was 0.87, with a range of 0.00 to 1.68. The largest error-index occurred at 53.21%. Cell count was analyzed by methods trypan blue and DIPS to determine whether the methods are equivalent within the allowable total error of 10 %. Six specimens were compared over a range of 37.90 to 100.00%. See figure 12.

Figure 12: MTT Two method comparison.



The difference between the two methods was within allowable error for 4 of 6 specimens (66.7%). The average error index (Y-X)/tea was 0.59, with a range of 0.00 to 1.40. The largest error-index occurred at a concentration of 37.90 %.

Table 3 and figure 13 briefly compare the percent viability of the biochemical assays to the digital image processing method. The known differences in the techniques studied are the additional reagents and dyes used that are known to have positive or negative effects on viable cells, whereas digital image processing is a combination of the sample and the drug studied. The table and the graph below clearly show an added toxicity.

Table 3: Biochemical assays and digital image processing percent viability comparison table.

	MTT	**Trypan Blue and hemocytometer**	**Digital image processing**
Control (0 ng/mL)	100.00%	100.00%	100.00%
0.6 ng/mL	88.69 %	88.30%	91.12%
6 ng/mL	72.94 %	82.60%	83.20%
60 ng/mL	53.21 %	63.90%	70.00%
600 ng/mL	43.05 %	44.44%	56.24%
6000 ng/mL	42.62 %	37.90%	51.90%

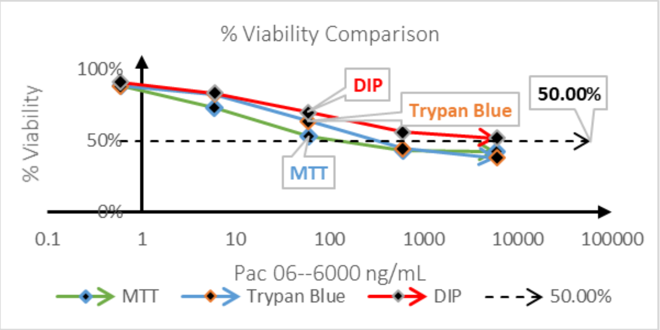


Figure 13: Percent viability comparison chart (biochemical assays and digital image processing).

4. Conclusion

For the cell viability experiments, statistical significance studies of percent viability among the methods conclude that the methods are significant to each other. The significance studies between MTT and DIP conclude that the methods are highly significant to each other where p = 0.0209. Similarly, the significance studies between trypan blue assay (using hemocytometer) and DIP conclude that the methods are faintly significant to each other where p = 0.0578.

In addition, the correlation coefficient of Euclidean distance (in pixels) and percent cell viability was measured to be -0.98357. This concludes that spatial distance (Euclidean distance) is inversely correlated to percent cell viability.

Although the techniques to measure cell viability indicate that they are significant to each other, literature expresses strong evidence of error constants to be considered while using both MTT and trypan blue assays. Errors in the range of 8-10% are expected in a hemocytometer count, or trypan blue assay method due to pipetting errors, chamber volume errors, errors from the volume of sample introduced into the chamber, cytotoxicity errors as trypan blue adds to the toxicity values in toxicity studies. Also, trypan blue stains cannot be used to distinguish between viable, healthy cells and the cells that are viable but losing cell functions. Similarly, about 7-10% of the errors are considered during cytotoxicity studies as the amount of signal generated depends on several factors like incubation periods after the addition of MTT dye and solubilizing chemicals like acidified isopropanol, cell confluence and pH of the solution after adding the dye. Digital image processing and analysis is a method where either minimal or no added stress is shown on the sample cells in cell viability and cytotoxicity studies.

Future studies should focus on using digital image processing and analysis in drug efficacy studies by quantifying Euclidean distance and viable cell count at specified intervals of time to calculate and study the drug kinetics on the sample cell.

The information gathered as a result of image processing makes analysis a simple task in comparison to the laborious biochemical techniques. The analysis of measuring cell viability was compared to the concept of spatial distance in the image that inversely relates to percent cell viability or the number of viable cells present. As the drug concentration in the titration increased, the Euclidean distance increased, and the count of viable cells decreased. The direct relationship between the distance between cells and count of non-viable cells or cell death cannot be measured using standard biochemical assays, whereas when it is measured using digital image processing and analysis, it strengthens the model. In conclusion, the study shows promising results and a promising future for digital image processing in cytotoxicity experiments.

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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