**Automated Quantification of Cellular Apoptosis and DNA Damage**

**Ross Jones, Singh Lab, December 12, 2013**

**Report First Draft (Introduction)**

**Abstract <will update closer to end>**

Apoptosis is an important constituent of the cell life cycle, and is relevant when considering tissue remodeling, cancer elimination, aging, drug genotoxicity, and cell sample health status. Current techniques in apoptosis evaluation lack sensitivity, can be inaccurate, and require relatively expensive procedures, making them not ideal for low-resource research where high accuracy is needed. Two techniques for apoptosis and DNA damage evaluation solve many of these issues by individually analyzing cells embedded in an agarose gel matrix: the DNA diffusion and fast halo assays. These assays suffer from an inability to analyze adherent cells and the labor intensive process of individually scanning thousands of cells. This project aims to address these shortfalls in the DNA diffusion and fast halo assays. We will first conduct research on methods of adherent cell detachment in order to find a procedure that best preserves frail apoptotic cells. We will then compare the DNA diffusion and fast halo assays to find an optimized procedure for assaying both apoptosis and DNA damage. Automatic image analysis algorithms will be designed to rapidly scan and find the level of apoptosis, necrosis, and DNA damage in a given sample. These algorithms will be written using MATLAB’s image analysis toolkit. A program to implement algorithms, collect and present data, and provide tools to analyze and compare results will then be written in Python. A GUI for the program will be devised in the Python-variant language Kivy. Software will be assessed in terms of processing speed, accuracy, and ease of use. Manual image scoring methods will be used to evaluate algorithm sensitivity and false positive generation rate. In summary, we aim to reduce the cost and time required for clinical studies and research involving apoptosis. This will be achieved with a simple, robust assay protocol and software for automated image analysis.

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# 1 Introduction

## 1.1 Definition of Project

Current techniques in apoptosis evaluation suffer from a variety of functional issues, such as poor sensitivity, requiring large-cell-count samples, artifact labeling, loss of detection of some cell types, false positive results, and relatively expensive procedures. This makes these assays lacking in the key factors of speed, cell concentration efficiency, and sensitivity. Many of these issues can be resolved by analyzing apoptosis at the single cell level. Based on cellular morphology observed, each cell can be individually classified as apoptotic, necrotic, or live. Of particular interest are the DNA diffusion assay (DDA) [e] and fast halo assay (FHA) [f], two single-cell techniques for quantifying apoptosis and DNA damage, respectively. While these assays are fast, sensitive, inexpensive, and can analyze low cell count samples, there is a long and labor intensive analysis required to individually assess each cell.

Our overarching goal is to assess apoptosis and DNA damage at low cost, high speed, and reliable sensitivity. Here, we deliver a platform for the systematic analysis of apoptosis at the single-cell level. In order to address the issues stated above with the DDA and FHA, we started a collaboration with Dr. Liyuan Ma’s group at Worchester Polytechnic Institute. The Ma Laboratory has designed microfabricated slides for regular patterning of cells while performing the FHA [ac], which they have referred to as “HaloChips”. In correspondence, I have developed software to quantify apoptosis and DNA damage in cells patterned on these HaloChips. Included in this software are automated image analysis algorithms designed to rapidly survey fluorescent images of cells taken post-assay, providing the levels of apoptosis, necrosis, live cells, and overall DNA damage of a sample. In addition, the software can implement algorithms, store experimental data, compute statistics, and present data.

In summary, we aimed to reduce the cost and time required for research and clinical evaluations of DNA damage and apoptosis with a regularized, high-throughput assay coupled with analytical software for automated image algorithm implementation.

## 1.2 Significance

Apoptosis, a generally non-inflammatory and naturally occurring form of programmed cell death, is known to be a major factor in tissue remodeling, cancer elimination, aging, and age-related diseases [a], [b], [c]. Apoptosis is initiated 50-70 million times in the average adult human every day [ad], and as such, issues with apoptosis pathways are important in the progression of many diseases. For example, excessive apoptosis can lead to atrophy and insufficient apoptosis can lead to tumor formation. Determining the apoptotic index – the ratio of apoptotic to live cells – along with the level of DNA damage in a cell samples is an important diagnostic for genotoxicity and health status. Additionally, in many clinical evaluations and research studies, apoptosis is contrasted with necrosis, an irregular and premature method of cell death that is initiated by many pathogens, diseases, and traumatic events, such as temperature shock.

The combination of HaloChips’ regular cell patterning and my software for apoptosis and DNA damage evaluation will allow researchers and clinicians to rapidly measure apoptosis, necrosis, live cells, and DNA damage with a high degree of sensitivity on a cell by cell basis. This will aid in genotoxic, developmental, cancer, drug design, and aging related research in particular. This software could be used to monitor the efficacy of chemotherapy in various cell types or in sperm for evaluations of fertility and gamete health. Importantly, our choice of assay will allow for inexpensive and high throughput quantification even with low cell counts, such that research can be performed when sample sizes are limited.

The collaboration between the Ma Laboratory and my own will allow for the rapid analysis of DNA damage and apoptosis in cell samples without requiring the use of expensive equipment and highly trained technicians. This project’s deliverables will be beneficial in both clinical and research settings where cost minimization is being sought. Additionally, our approach will be highly beneficial for research and clinical evaluations in low-resource settings, such as in developing countries without access to large, well-equipped medical facilities and largely rural populations.

## 1.3 Issues Considered

### 1.3.1 Social and Ethical Issues

Since the program and algorithms designed will be used to analyze apoptosis, one must consider the applications of apoptosis research. One such application is embryonic stem cell research, which is deemed by some to be highly contentious. By benefitting stem cell research, this platform could also fall under scrutiny. In addition, the assay and algorithms could be used in DNA damage and cancer drug studies. If these studies are performed on large populations on people, it will be important for them to have high accuracy. Poor sensitivity could lead to misplaced conclusions and any ill consequences involved.

### 1.3.2 Economic Issues

In general, most facets of this project will be easy, fast, and inexpensive for the user. The software will likely be released as open-source freeware, allowing any person to use and improve its abilities on their own. The limiting factor as far as economics are concerned is the cost and availability of a high-resolution image capturing fluorescent microscope, as well as the filters associated with different dyes for cell imaging. These microscopes and accessories can range price from tens to hundreds of thousands of dollars for a high quality device. In low-income areas, this may not be feasible to purchase or available in close proximity to the area of need for the user.

### 1.3.3 Legal and Regulatory Issues

While this project is relatively benign in terms of regulatory issues, it must comply with the FDA’s CDRH Laboratory of Image Analysis’ guidelines for computer-aided diagnostics if it hopes to be applicable in clinical evaluations. When published, this project’s source code will need to be licensed; a likely candidate will be GNU General Public Licence, the most widely used open source / free software license. Of note, though the MATLAB IDE software and image processing toolbox are not free, they are commonly used and compiled standalone applications written can be freely distributed by the author.

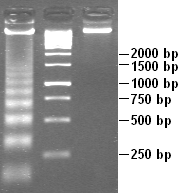
## 1.4 Engineering Standards

Our complete assay and image analytic platform conforms to the Good Laboratory Practices (GLP) standards set forth to ensure reproducibility, uniformity, consistency, reliability, and quality, of laboratory work.

## 1.5 Technical Background

### 1.5.1 Theory: Apoptosis & DNA Damage Analysis

Apoptosis is characterized by reduced cellular volume, chromatin condensation, plasma membrane blebbing, and regularly fragmented DNA. DNA is commonly found tightly wrapped around histones at regular intervals of about 180 kilobases, a structure referred to as a nucleosome. During apoptosis, the endonuclease Capase-Activated DNase (CAD) splices DNA at the internuclosomal linker sites between the nucleosomes, causing DNA to break at regular intervals. In contrast, DNA in cells undergoing necrosis is broken at random sizes, causing the two modes of cell death to be easily distinguishable by inspecting DNA breakage patterns. This property of apoptosis was first described by AH Wyllie in 1980, where it was shown that DNA electrophoresed from a sample of apoptotic cells formed into regularly repeating bands, as opposed to the random distribution seen in samples of necrotic cells [d]. This so-called DNA laddering effect can be seen in Figure 1.



**Figure 1:** Apoptotic DNA Laddering (Wiki-media Commons). A cell sample is lysed and DNA is electrophoresed in a gel column. The left channel is DNA from a sample of apoptotic cells, the center channel is known sized DNA, and the right channel is DNA from control cells. This effect is caused by excision of nucleosome chains during apoptosis, which are wrapped in DNA with length in multiples of about 180 kilobases. The bands in the apoptotic channel are thus roughly separated by this margin.

In DNA halo style assays such as the DDA and FHA, cells are lysed in a gel to allow their DNA to radially diffuse from the nuclei. A greater distance and amount of DNA spread is correlated with a greater amount of DNA damage. There are several methods of calculating the level of DNA damage, such as the nuclear diffusion factor (NDF) [ac], adjusted NDF, and halo moment. NDF is calculated by comparing the surface area of the halo to that of the nucleus (R = radius of halo; r = radius of nucleus):

Adjusted NDF is calculated by multiplying NDF by the ratio of the halo intensity to the nuclear intensity, effectively adjusting the measurement to take variable contrasts into account (I = intensity, taken as the average of the subscripted region):

Halo moment takes a different approach by calculating the intensity of each point in the halo multiplied by its distance from the center of the nucleus, then summing over the entire cell (I(x, y) and r(x, y) refer to the point intensity and radius from the center of a cell at a given location, defined by the coordinates x and y):

As with many methods of identifying apoptosis, we looked for the characteristic regular DNA fragmentation. DNA fragmentation can be identified by examining the frequency domain of an image, where apoptotic cells have a unique frequency spike associated with this fragmentation in their halo region (note: this is hypothetical at this point in the project). To convert images to frequency domain, the Fourier transform in two dimensions is calculated by the following formula ( = angular frequency):

### 1.5.2 Theory: Image Analysis

Images are acquired by staining cell features and capturing images using a microscope. Once imported into an image processing program, images are typically converted to 8 bit gray scale in order to minimize computational time. For the purposes of this project, the specific tasks involved include filtering, thresholding, and segmenting images before criteria can be assessed for DNA damage and apoptosis quantification.

There are a vast number of filters used in image analysis, finding use in many tasks such as noise reduction, edge detection, and image sharpening. Two common filters for smoothing and noise reduction are the median and Gaussian filters. Median filters simply work by taking the mean of nearby pixels, which is beneficial in that it preserves edges. Gaussians are defined by the following function in two dimensions ( = the Gaussian’s standard deviation):

Prewitt and Sobel operators are two common filters used for edge detection in images. These filters are applied by convolving images with 3x3 kernels to determine the x- and y-direction derivatives. Using the distance and atan2 functions on these derivatives gives the image gradient’s magnitude and direction, respectively.

Laplacian filters are also used for edge detection and enhancement. Laplacian kernels approximate the following function (I = intensity values):

Thresholding is employed to normalize and maintain desired features of an imaged sample. M Sezgin and B Sankur have thoroughly assessed many thresholding techniques [p], which was a useful resource for this project. Thresholding techniques come in many forms, and can make threshold decisions based on criteria such as greyscale histogram patterns and clustering. One example of a grayscale thresholding described is used in the MATLAB function, multithresh. Following N Otsu’s technique, this function defines threshold levels by utilizing zeroth- and first-order cumulative moments to maximize seperability [ae].

A clustering analysis technique commonly used for thresholding is the k-means algorithm. K-means works by minimizing the within-cluster sum of squares over several iterations, such that n observations (x) are partitioned into k sets (S, mean = ):

Separating overlapping cells is achieved by segmentation algorithms. An example of contour segmentation works by creating a negative of the image and “filling in” the darker “craters” where cells are located [q]. When fills from two cavities meet, the boundary between two cells is established; this example can be seen in Figure 2. An example of centroid segmentation is the regularized centroid transform (RCT), which collapses image regions to their centers [r], which has the benefit of identifying completely overlapping objects.

### 1.5.3 Review of DNA Damage Literature

In 1984, the single cell gel electrophoresis (SCGE) assay, better known as the comet assay, was introduces as a method of quantifying DNA damage at the single cell level [al]. The comet assay works by encapsulating cells in a gel matrix, lysing the cells, and then placing the cells in an electric field to drive DNA migration. This migration forms a comet-like appearance, with the head being the nucleus and the tail being the damaged DNA. The original assay was performed under neutral conditions, and was sufficient for visualizing DNA single strand breaks. In 1988, my advisor, Dr. Narendra Singh, modified the comet assay to be performed in alkaline conditions [v], allowing researchers to visualize DNA double strand breaks as well as single strand breaks. The alkaline version of the comet assay has become the standard method, thought there are many modified forms for visualizing various specific occurrences, such as a method for integrating fluorescence *in vitro* hybridization (FISH) [aj]. A table describing comet assay variants can be found in a recent 2011 review paper [ah].

Since the comet assay has come into common practice, it has been the study of intensive amounts of research. Olive *et al* found that the number of DNA double strand breaks identified by the comet assay is cell cycle dependent for most cells [af]. Collins [ag] and Klaude [aj] discuss many details of comet assay implementation, including its limitations. Due to the high sensitivity of the assay to any form of DNA damage, researchers must be extremely cautious to maintain laboratory conditions that will not lead to extra damage, i.e. keeping lights dim. If conditions are not carefully controlled, there is a propensity for the assay to deliver highly variable results.

In 1999, the alkaline halo assay (AHA) was developed to measure single strand DNA breaks in DNA “halos” around cells [an]. The principle of this assay is similar to the alkaline comet assay, with the exception that electrophoresis is not performed. Instead, DNA is allowed to radially diffuse out of the nucleus into the surrounding gel matrix. It has been shown that results from this assay closely match those by comet assay [ao]. This assay has since been modified, with the fast halo assay (FHA) being an important improvement which drastically speeds up the assay and improves image quality [o]. Recently, a mechanism was proposed for the formation of DNA halos under alkaline and saline conditions [f].

In 2012, Dr. Liyuan Ma’s laboratory released a paper describing a technique in which slide microfabrication was used with a modified AHA protocol to capture, encapsulate, and visualized DNA halos around cells in a regularly patterned grid [ac]; the slides are referred to as “HaloChips”. In the normal AHA, FHA, and comet assay techniques, cells are at variable heights in the gel matrix, causing some to be out of focus during image acquisition and limiting the ability of automated analysis. This technique reduces variability by locking cells at the same height on the slide. The procedure also has the ability to be adapted to a 96-well format, and thus could be very high-throughput.

### 1.5.4 Review of Apoptosis Literature

The concept of DNA laddering originated with the canonical study mentioned earlier by AH Wyllie [d]. This technique works by lysing and staining cells with ethidium bromide (EB), then applying an electrophoretic field on the sample to induce DNA migration. Apoptotic cells show a regularly repeating band at multiples of 180 kb, allowing researchers to identify the level of apoptosis in the sample based on the presence and intensity of these bands. Necrosis causes DNA to be randomly cleaved, and thus in DNA ladder assays, necrosis is seen as lanes with continuous DNA distributions. Of particular interest is a non-enzymatic version of the DNA ladder assay described by Suman *et al*. which addresses many of the short-comings of the DNA ladder assay, such as the loss of DNA fragments, having many procedural steps, and using expensive reagents [g]. While these improvements have significantly benefitted the DNA ladder assay, it still requires high-cell-count samples and a timely electrophoresis stage to be performed. In addition, apoptotic index evaluation is less quantifiable than other techniques, as gel DNA fragmentation bands are insensitive to small changes in apoptosis.

The terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay is a widely used method for detecting DNA fragmentation caused by apoptosis. The TUNEL assay works by labeling “nicks” in the terminal ends of DNA with the enzyme terminal deoxynucleotidyl transferase [h]. The accuracy of this method has been called into question with regard to its inability to differentiate between apoptotic, necrotic, and live cells [i], as well as its propensity to issue false positive results [j]. Methods to improve the TUNEL assay sensitivity have been put forward [k], but it remains contentious as to its false positive generation and loss of frail apoptotic cells during processing.

The Annexin V assay is a highly common method of measuring apoptosis. Annexin V is a calcium-dependent protein that strongly stains phosphatidylserine, a protein which is externalized to the cell surface during the early stages of death [l]. While relatively rapid and sensitive, this technique suffers from false positives when live cells incur membrane damage, as phosphatidylserine is an abundant intracellular protein [m]. Additionally, this technique utilizes flow cytometry, which is not widely available in low-resource settings.

A more recently studied method of identifying apoptosis is by using probes for caspases. Caspases, or cysteine-aspartic proteases, are directly involved in apoptosis signaling [aq]. Many probes have thus been designed to target caspase activity as a method of imaging apoptosis, specifically in tissues. Caspase probe imaging suffers from high background noise and thus the propensity for false positive generation; newer smart probes are being developed to combat this issue [ap]. A recent review goes into depth discussing the effectiveness of different DNA probes in identifying apoptosis [x].

It was recently shown that ligation-mediated real-time polymerase chain reaction (LM-RT-PCR) could be used to detect DNA fragmentation in human sperm [y]. They compared their method to the TUNEL assay and sperm chromatin dispersion to determine that DNA fragmentation patterns were similar among all three methods, an indication that LM-RT-PCR can effectively detect apoptosis either alone or in combination with the other methods.

In 2000, Dr. Singh modified the AHA in order to visualize apoptotic cells at the single-cell level [e]. This method is referred to as the DNA diffusion assay (DDA); as with the AHA, it is a simple, relatively rapid and highly sensitive technique. Apoptotic cells are identified as having small, potentially fragmented nuclei surrounded by a large, diffuse halo of DNA. Necrotic cells are identified as not containing a centralized nucleus and having a large, random DNA spread pattern. An updated version of this protocol was used to study the induction of apoptosis by the compound artemisinin [ak]. This assay has found criticism for its use of YOYO-1 dye rather than EB, as well as potential issues in defining necrotic cells [n]. Simultaneous use of the DDA with the comet assay has been shown to be a strong method for total evaluation of cell status with respect to apoptosis and DNA damage levels [w].

### 1.5.5 Relevant Image Analysis Programs

Cell profiler is an open-source project with the aim of providing image analysis modules for biologists [s]. These modules are built with the intention of automatically assessing phenotypes from microscope images. Modules can be combined to build a pipeline which will perform several tasks, such as first identifying target objects, then further dissecting and analyzing them depending on what modules the user lines up. NumPy and SciPy, two major Python libraries, are implemented by modules to perform their operations. Currently, there are no modules that can perform operations pertinent to identifying DNA damage and apoptosis in nuclear halo style assays.

Comet Assay IV is a commercial software for the assessment of comet assay images [am]. This software interfaces with microscopes, allowing the user to dynamically interact with cell samples and visualize comets while obtaining images. Users simply click on cells to have the program identify and return results about the selected cell. Comet Assay IV also uses a spreadsheet generator to deliver the results such that users can further analyze data. Analytics include all major metrics for DNA damage in comet assay images.

## 1.6 Prior Relevant Work

Several years ago, our laboratory collaborated with Dr. Sayan Pathak to develop algorithms for automated quantification of apoptosis in DDA images. Two major algorithms were developed: an adaptive version of the Markov random fields [t] algorithm (A-MRF) and a hierarchical thresholding (H-Thresh) technique. The A-MRF algorithm worked by localizing cell regions using a k-means algorithm [u] modified to adapt to region intensity variations. The H-Thresh algorithm worked by analyzing the image intensity histogram and iteratively defining two image thresholds to divide the image into three segments: background, nucleus, and diffused DNA. Both algorithms implemented a regularized centroid transform [r] to identify overlapping cells. H-Thresh was found to be approximately ten times faster than A-MRF. Both algorithms were found to be effective in quantifying apoptosis relative to manual scoring techniques with a 95% level of certainty. This unpublished work demonstrated the potential of automated image algorithms as a means of quantifying apoptotic cells at the single cell level, which we aim to expand to live and necrotic cell types. Unfortunately, we have been unable to contact Dr. Pathak regarding guidance for this project, and the algorithms are lost.

## 1.7 Outstanding Technical Issues at Outset

<None, this project was virtually brand new, so this section is irrelevant.>

# 2 Design of Tools, Devices, and Experiments

## 2.1 Overview of Design Plan

The original design goals of this project were to develop MATLAB-based automatic image algorithms and Python-based program. These designs were to progress through iterative changes in content, quality evaluations, optimization of code and output, and piecewise-developed builds and architectures. The following is an overview of the original plan.

### 2.1.1 Phase I: Assay Research

The purpose of the first project phase was to research procedures relevant to single cell apoptosis assays. The two major aims were to: 1) study methods of cell detachment to preserve apoptotic cells and 2) directly compare and assess the DDA and FHA. Current methods in cell detachment, along with different combinations of such, were to be analyzed with the goal of identifying procedures that best preserve frail apoptotic and necrotic cells. The primary interests were combinations of trypsin, EDTA, and citric saline. The DDA and FHA were to be compared based on image clarity, artifact propensity, and cell morphology. Combinations of each assay were to be tested to best optimize an alkaline gel diffusion assay.

### 2.1.2 Phase II: Software Development

The purpose of the second project phase was to design automatic image analysis tools for apoptosis and DNA damage evaluation. There were two major constituents to this phase: 1) the creation of an array of image analysis algorithms for the quantification of apoptosis and DNA damage in fluorescently imaged cells from the previously designed assay and 2) design of a program which would implement the algorithms and collect results for analysis. The image algorithms were to be written using MATLAB and would implement thresholding and segmentation techniques to discern between cell morphologies and quantify DNA spread from cell nuclei. The program was to be written with Python and the app-building language Kivy in order to effectively build and manage a GUI for user interaction, as well as communicate with the MATLAB algorithms. The output of this phase was to be complete software for the rapid assessment of images obtained from the optimized assay.

### 2.1.3 Phase III: Additional Features

The purpose of the third project phase was to provide optional features which would improve the program and user interaction. This phase would involve constant tweaking of the software to optimize the look and usability of the GUI, as well as establishing methods of data visualization. In addition, there may have been time to develop a Python-based apoptosis module for Cell Profiler by translating ATLAB algorithms. The output of this phase would be program features that improve users’ workflow and analyses.

## 2.2 Overview of Revised Design Process

With the collaboration initiated between our group and the Ma Laboratory, the first phase of the project became irrelevant, as we had decided on using HaloChips as our assay method. Thus, the following design process reflects the pivoting of the project direction.

<will add later next quarter when it is more developed.>

## 2.3 Materials and Methods

All materials were purchased from Sigma Aldrich Corporation (St Louis, MO, USA) unless mentioned otherwise.

### 2.3.1 Cell Culture

MOLT-4 cells were purchased from American Type Culture Collection (ATCC, Manassas, VA, USA). They were cultured in RPMI-1640 media (Life Technologies, Grand Island, NY, USA) with 10% fetal bovine serum (ATCC) at 37 ºC with 5% CO2 and 100% humidity to reach a density of 6x105 cells/mL before diluting to a density of 6x104 cells/mL approximately 24 hours prior to a treatment. Twenty-four hours of pre-incubation allowed the cells and media to be conditioned prior to exposure to apoptosis and necrosis inducing conditions and experiments to begin at a density of approximately 1x105 cells/mL. At this time, cells were in the log phase. Cell counts were performed using a hemocytometer and trypan blue.

### 2.3.2 Fast Halo Assay

The FHA was performed as described by Galaz-Leiva *et al*. [f]. Briefly, MOLT-4 cells were spun down and resuspended in 150 uL of RPMI-1640 media. Cells were then mixed with 150 uL molten 2% SFR agarose (Amresco, Solon, OH, USA). 100 uL of the cell-gel mix was spread on clear-window frosted MGE slides (Erie Scientific Co, Portsmouth, NH, USA) under a cover glass (24 x 50 mm2, Corning Glass Works, Corning, NY, USA) and allowed to solidify at 4 ºC. The cover glass was removed and the slides were immersed in a glass Couplin jar containing lysing solution (0.3 M NaOH in PBS) for 15 minutes at room temperature. Slides were then transferred to a jar containing neutralizing solution (0.4 M Tris-HCl pH 7.6) for 2 minutes at room temperature. Slides were washed in deionized water twice for 5 minutes at room temperature, then allowed to air dry. Prior to imaging, slides were stained with either 10 uM EB or 0.25 uM YOYO-1.

### 2.3.3 DNA Diffusion Assay

The DDA was performed as described by Singh & Lai [ak]. Briefly, MOLT-4 cells were spun down and resuspended in 300 uL of molten 0.7% agarose 3:1 (Amresco). 100 uL of cell-gel mix was spread on MGE frosted microscope slides under a cover glass and allowed to solidify at 4 ºC. The cover glass was removed and 100 uL of molten 2% SFR agarose was spread on the solidified gel under a cover glass and allowed to solidify again at 4 ºC. The cover glass was again removed and the slides were immersed in a Couplin jar containing cell lysing solution (1.25 M NaCl, 1mM tetrasodium EDTA, 5 mM Tris, 0.01% sodium lauroyl sarcosine, 0.2% DMSO, and 0.3 M NaOH) for 10 minutes at room temperature. Slides were removed and placed in jars containing CTAB solution (1 mg/mL cetrimonium bromide, 40 mM Tris-base pH 10) twice for 10 minutes at room temperature, then removed again and placed in jars containing neutralizing solution (2 mM Tris in 75% EtOH) three times for 10 minutes at room temperature. After, slides were allowed to air dry. Prior to imaging, slides were stained with either 10 uM EB or 0.25 uM YOYO-1.

### 2.3.4 Comet Assay

The comet assay was performed as described by Krieg Jr. *et al*. [ar]. Briefly, preparations of slides with MOLT-4 cells was completed exactly the same as previously described for the DDA. Prepared slides were immersed in pre-warmed lysing solution (1.25 M NaCl, 50 mM tetrasodium EDTA, 10 mM Tris-base pH 10, 0.5 mg/mL proteinase K (Amresco), 1 mg/mL reduced glutathione) and incubated at 37 ºC for 1 hour. The slides were then placed in an MGE 2222 electrophoretic unit (Ellard Instrumentation, Monroe, WA, USA) with 1 L alkaline solution (300 mM NaOH, 1 mM EDTA, 0.2% DMSO) and allowed to equilibrate for 20 minutes at room temperature in low light conditions. Electrophoresis was commenced for 20 minutes at 18 V (0.6 V/cm), 450 mA. During electrophoresis, the solution in the unit was circulated at about 100 mL/min using a peristaltic pump. Slides were then immersed in CTAB solution twice and neutralizing solution thrice for 10 minutes each (same solutions as DDA). Slides were allowed to air dry before staining with 0.25 YOYO-1 dye for imaging.

## 2.4 Costs (1pg)

The following table set the budget for materials required for my laboratory to undertake this project. Materials needed are to perform DDA, FHA, and comet assays on cell samples to capture and compare images. The total cost of all materials is approximately $1200. The cost of materials in the Ma Laboratory is not calculated.

**Table 1.** Project Budget

|  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- |
| **Item** | **Manufacturer** | **Item** | **Pieces** | **Units** | **Price** | **Quantity** | **Total** |
| Frosted slides | Mac & Sons (MGE) | Box | 72 | slides | 45 | 3 | 135 |
| Cover glasses | Corning | Pack | 100 | glasses | 25 | 2 | 50 |
| 5 mL pipettes | VWR | Case | 200 | pipettes | 23.14 | 1 | 23.14 |
| 15 mL centrifuge tubes | VWR | Case | 500 | tubes | 85 | 1 | 85 |
| Medium nitrile gloves | VWR | Case | 200 | boxes | 140.35 | 1 | 140.35 |
| T-25 50 mL culture flasks | VWR | Case | 200 | flasks | 88.78 | 1 | 88.78 |
| 1 mL pipette tips | VWR | Case | 1000 | tips | 22.31 | 1 | 22.31 |
| 200 uL pipette tips | VWR | Case | 1000 | tips | 122.16 | 1 | 122.16 |
| L-15 complete media | Invitrogen | Case | 10 | 500 mL | 262 | 1 | 262 |
| RPMI-1640 complete media | Invitrogen | Case | 10 | 500 mL | 204 | 1 | 204 |
| Trypsin | Amresco | Bottle | 1 | grams | 53.96 | 1 | 53.96 |

## 2.5 Details of Design Process

### 2.5.1 Discussion of Realistic Constraints Considered

Criteria for the software were set to ensure that the processing of images is robust, rapid, and not prone to many mistakes As such, it was agreed that individual images must be analyzed within 15 seconds with a 98% sensitivity and only 1-2% false positive generation when considering apoptosis. Longer times than 15 seconds would mean excessive waiting by users if there were 100+ images to score. As is self-evident, it was decided that DNA damage algorithms must deliver measures of DNA damage for each cell which are insignificantly different (p < 0.05) than those found using manual techniques. Additionally, we determined that the assay and algorithms must capture scales of DNA damage that can be described as a linear transformation of results of the same samples using the comet assay and VisCOMET analytical software. As most laboratory personnel use Microsoft Windows for their data acquisition and machine-interfacing computers, it was determined that the software must be compatible with all Windows operating systems since XP, and at the very least handle the common PNG, JPEG, GIF, and TIFF image formats for analysis.

### 2.5.2 Table of Design Specifications

The following table details the design specifications discussed in the previous section.

**Table 2.** Design Specifications

|  |  |  |
| --- | --- | --- |
| **Design Specification** | **Target values and tolerances** | **Validation Tool** |
| Image formats accepted | PNG, JPEG, GIF, TIF | Feature testing |
| Analysis time per image | 15 sec | Internal timer |
| Algorithm sensitivity | 98% | Manual scoring |
| False positives | 1-2% | Manual scoring |
| DNA Damage Comparison | P > .05 | Manual scoring |
| DNA Damage Correlation | Linear relationship | Comet assay + VisComet |
| Software OS compatibility | Windows XP - 8 | Feature testing |

### 2.5.3 Statistical Basis for Design of Experiments

In order to ensure that representative samples of cells were studied when capturing images from the DDA and FHA, we imaged at least 1000 cells per slide with both YOYO-1 and EB stains. We also used at least two slides per sample to ensure that the images were representative of what is obtained during repeated trials. When performing the comet assay, at least 100 cells are scored for DNA damage propensity.

# 3 Results

## 3.1 Final Timeline (Gantt Chart)

## 3.2 Data, chronological narrative, tables, figures, and statistical analysis

## 3.3 Design Decisions Made During Project, Including Discussion of Design Iterations

# 4 Analysis and Conclusions

# 5 Suggestions for Future Work

# 6 Acknowledgements

# 7 References

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# 8 Appendices (optional)