Automated Quantification of Cellular Apoptosis and DNA Damage

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Design Proposal (402)

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 scoring 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 Background and Significance

> 1.1 Problem Statement

Apoptosis is a type of programmed cellular death which is generally non-inflammatory and naturally occurring in the cell cycle. Apoptosis is characterized by reduced cellular volume, chromatin condensation, and regularly fragmented DNA. Apoptosis is known to be a major factor in tissue remodeling, cancer elimination, aging, and age-related diseases [1, 2, 3]. Determining the apoptotic index, the ratio of apoptotic to live cells in a sample, along with the DNA damage level of a given cell sample is an important diagnostic for genotoxicity and health status. In many evaluations, apoptosis is contrasted with necrosis, an irregular and premature method of cell death. Many techniques of apoptosis detection take advantage of a property of apoptosis in which DNA is cleaved in predictable multiples of 180 kilobases. This property 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 [4]. This so-called DNA laddering effect can be seen in Figure 1 below.

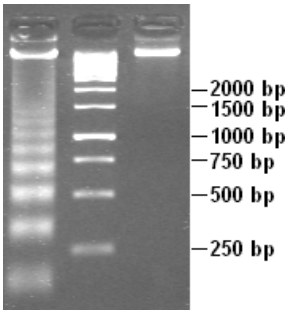

Figure 1: Apoptotic DNA Laddering (Wikimedia Commons). A cell sample is lysed and DNA is electrophoresed in a gel column. The left channel is DNA from a sample of apoptotic cells, the center channel is known sized DNA, and the right channel is DNA from control cells. This effect is caused by excision 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.

Current techniques in apoptosis evaluation suffer from a variety of functional issues, such as poor sensitivity, requiring large numbers of cells, artifact labeling, loss of detection of some cell types, false positive results, and relatively expensive procedures. 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, live, or some sort of artifact. Of particular interest to this project are the DNA diffusion assay (DDA) [5] and fast halo assay (FHA) [6], two single-cell techniques for quantifying apoptosis and DNA damage, respectively. While these assays are fast, sensitive, inexpensive, and can analyze small cell samples, there are two major drawbacks: 1) current protocols are not effective in measuring apoptosis of adherent cell lines and 2) a much longer and more labor intensive post-lab analysis is required to assess each cell image individually.

This project will 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 will first research methods of adherent cell detachment that preserve the apoptotic index of the cell sample. Additionally, we will evaluate and modify the procedures of the DDA and FHA to optimize assay efficiency. We will use the knowledge gained to design

software for the automatic quantification of apoptosis. This software will include automatic image analysis algorithms and a program to control the algorithms and perform data analysis with the goal of rapidly and accurately assessing apoptosis. Our modified assay will involve lysing the cells in an alkaline solution, which will allow us to evaluate both apoptosis and DNA damage levels using differing image algorithms on the same cell images. In addition, the assay will run at a lower price than many current techniques, as enzymes, antibodies and electrophoresis will not be required.

> 1.2 Prior Work: Apoptosis Analysis Methods

The following are the most common methods of apoptotic analysis, each having one or more of the following issues: (1) Poor sensitivity, (2) requiring large numbers of cells, (3) artifact labeling / false positives, (4) poor detection of some cell types, and (5) relatively expensive procedures. The DDA solves these issues by individually assessing cells with an inexpensive and rapid protocol, and thus will be expanded upon in this project.

1.2.2 DNA Ladder Assay (1, 2)

DNA laddering originated with the canonical study mentioned earlier by AH Wyllie [4]. This technique works by lysing and staining cells with a DNA binding dye, then electrophoresing the sample to look for DNA gel bands. Of particular interest is a version of the DNA ladder assay described by Suman *et al.* [7]. The authors devised a non-enzymatic version of the assay that allowed them to address many of the shortcomings of the DNA ladder assay, such as loss of DNA fragments, many procedural steps, and expensive reagents. While these improvements have significantly benefitted the DNA ladder assay, it still requires a million or more cells and an electrophoresis stage to be performed. In addition, its apoptotic index evaluation is less quantifiable than other techniques, as gel DNA fragmentation bands are insensitive to small changes in apoptosis.

1.2.3 TUNEL Assay (3, 4)

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 [8]. The accuracy of this method has been called into question with regard to its inability to differentiate between apoptotic, necrotic, and live cells [9], as well as its propensity to issue false positive results [10]. Methods to improve the TUNEL assay sensitivity have been put forward [11], but it remains contentious as to its false positive generation and loss of frail apoptotic cells during processing.

1.2.4 Annexin V Assay (4, 5)

The Annexin V assay is a 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 [12]. While relatively rapid and sensitive, this technique suffers from false positives incurred when live cells incur membrane damage, as phosphatidylserine is a common intracellular protein [13]. Additionally, it requires flow cytometry, which is not widely available in low-resource settings.

> 1.3 Relevant Techniques and Processes

The following methods and platforms will be relevant for the research and design portions of the project. The only known attempt at automatic single-cell apoptosis image analysis comes from collaboration within our lab, and thus its discussion is provided in Section 1.4 – Preliminary Data.

1.3.1 DNA Staining Consideration

Ethidium bromide (EB) staining is the most popular form of DNA staining for the purposes of single-cell apoptosis evaluation. While relatively inexpensive, EB is thought to be mutagenic, and is much less sensitive than intense dyes such as YOYO-1. Though YOYO-1 is more expensive than EB, it is not known to be mutagenic. That aside, any high affinity DNA binding molecule has the potential for mutagenicity.

1.3.2 DNA Diffusion Assay

The DDA is a simple, relatively rapid technique for direct quantification of apoptosis, necrosis, and live cells [5]. Cells are lysed and stained with YOYO-1 to detect DNA which has radially diffused in agar gel around apoptotic cells. Representative images of apoptotic, necrotic, and live cells are provided in Figure 2 below. This assay has found criticism for its use of YOYO-1 rather than EB, as well as potential issues in defining necrotic cells [14]. This assay and others like it cannot currently measure apoptosis of adherent cells, due to a lack of cell detachment protocols that will not lyse frail dying cells.

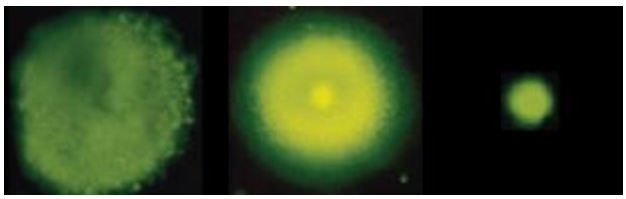

Figure 2: DNA Diffusion Assay Images [5]. Cells are lysed and DNA is allowed to diffuse radially in agar gel on a frosted microscope slide. From left to right: a necrotic, apoptotic, and live cell. Necrotic cells show DNA breaks at random sites, no nuclear focal point, and a defined edge. Apoptotic cells show a gradient of decreasing intensity DNA around a condensed nucleus. Live cells appear small with bright, evenly distributed DNA.

1.3.3 Fast Halo Assay

In 2009, the FHA was implemented as a simple, fast, and accurate method for the detection of DNA damage [15], and was recently modified to become even simpler [6]. This technique works similarly to the DDA with some procedural differences, including the use of EB for staining. A modified variation of the FHA and the DDA could be useful for simultaneous quantification of DNA damage and apoptosis.

1.3.4 Cellular Image Analysis Fundamentals

Cellular image analysis is achieved by staining cell samples, capturing photos, and transferring the images to an image manipulation capable program. Once loaded, images are first converted to gray scale, where an increase in pixel intensity correlates with an increase in staining brightness. For the purposes of this project, the specific tasks involved will include filtering background noise, thresholding the images, distinguishing between overlapping cell objects, automating cell scoring, and differentiating between apoptotic, necrotic, and live cells based on DNA diffusion morphology.

Noise reduction is generally achieved by convolving images with smoothing filters, which reduces variability between pixels. When choosing a noise reduction method, it is important to assess the nature of the noise with respect to the desired image characteristics in order to evaluate tradeoffs in the time of analysis and sacrifice of fine details due to blurring. Thresholding is employed to normalize and maintain desired features of an imaged sample. Sezgin and Sankur provide a thorough assessment of many thresholding techniques [16], which will be a useful resource for designing algorithms in this project. Separating overlapping cells has commonly been achieved by segmentation algorithms, which work by creating a negative of the image and "filling in" the darker "craters" where cells are located [17]. When fills from two cavities meet, the boundary between two cells is established. A segmentation example can be seen in Figure 3 below. Another more recent innovation is the regularized centroid transform (RCT), which collapses normally circular cells to their centers [18]. This method is targeted towards cells that are completely overlapping in an image.

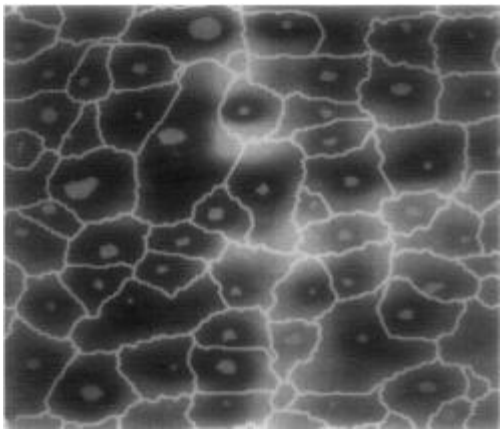

Figure 3: Image Segmentation Algorithm [17]. The image of cells is inverted such that cells are represented by the darker regions. These darker regions, which can be thought of as craters, are "filled" as if water was uniformly being added to the image, causing the lowest pixel values to increase. When the fill from two craters meets, the algorithm establishes a boundary between the cells, allowing for an accurate method of discerning between cells that are overlapped. Shown here are the boundaries found between cells.

1.3.5 Cell Profiler

Cell profiler is an open-source project with the aim of providing image analysis modules for biologists [19]. 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. In all, Cell Profiler contains many automated cell analysis algorithms which will be useful references for this project.

> 1.4 Preliminary Data

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 [20] algorithm (A-MRF) and a hierarchical thresholding (H-Thresh) technique. The A-MRF algorithm worked by localizing cell regions using a k-means algorithm [21] 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 [18] 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, the algorithms themselves have gone missing over the years, and currently we have not been able to make contact with Dr. Pathak regarding guidance for the project.

Dr. Singh is responsible for modifying, to great success, the highly popular single cell gel electrophoresis (comet) assay [22] as well as devising the DDA [5]. Others have shown that use of the DDA simultaneously with the comet assay is a strong method for total evaluation of cell status with respect to apoptosis and DNA damage levels [23]. This knowledge demonstrates the potential for an improved DDA with automatic image analysis software to quantify both DNA damage and apoptosis as an effective approach for more complete analysis of a sample's genotoxic health status.

> 1.5 Consequences of Success

Our research aims to find methods of cell detachment which will permit apoptotic evaluation of adherent cells which otherwise must be studied using different methods than the DDA or FHA. The results of this research will not be limited to our own lab, and will find use in other such cases where adherent cells must be detached for analysis but are in a necessarily sensitive state.

Our 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, stem cell, cancer, drug design, and aging 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 quantification even with small

samples of cells, such that research can be performed when resources are limited. In all, we will help to reduce the cost and time required for research and clinical evaluations with a simple, robust assay protocol and analytical software for image algorithm implementation.

> 1.6 Ethical and Social 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 a hot topic for ethical debate. By benefitting stem cell research, this platform could also fall under scrutiny. In addition, the program could find use in DNA damage and cancer drug studies. If these studies are performed on large populations on people, it will be very important for the algorithms to be highly accurate. Poor sensitivity could lead to misplaced conclusions and any ill consequences involved.

> 1.7 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.

> 1.8 Legal and Regulatory Issues

While this project is relatively benign in terms of regulatory issues, it will need to comply to the FDA's CDRH Laboratory of Image Analysis' guidelines for computer-aided diagnostics if it hopes to be applicable in clinical evaluations. The program and GUI will be written with Python and Kivy, two free and open languages that any person can use to develop and distribute applications. MATLAB is not free, but is commonly available and can be freely implemented as a part of applications.

2 Plan of Work

> 2.1 Overview

Methods of apoptosis and DNA damage assessment are lacking in the key factors of speed, cell concentration efficiency, and sensitivity in particular. The overarching goal of this project is to assess apoptosis and DNA damage at low cost, high speed, and reliable sensitivity. This project first aims to research methods of cell detachment which will preserve states of cell death. We will then directly compare the DDA and FHA to find

the optimal procedure for a modified alkaline gel diffusion assay to prepare cells of all types for fluorescent microscopy image capture and subsequent software evaluation. Image algorithms will be 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. A program will be written to implement the algorithms, store experimental data, compute statistics, and present data will aid researchers and clinicians in handling assay results and drawing conclusions regarding their samples.

The design goals of this project are related to the development of MATLAB automatic image algorithms and a Python-based program. These designs will progress through iterative changes in content, quality evaluations, optimization of code and output, and piecewise-developed builds and architectures.

2.1.1 Phase I: Assay Research

The purpose of the first project phase is to research procedures relevant to single cell apoptosis assays. The two major aims are 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, will be analyzed with the goal of finding procedures that best preserve frail apoptotic and necrotic cells. The primary interests will be combinations of trypsin, EDTA, and citric saline. The DDA and FHA will be compared based on image clarity, artifact propensity, and cell morphology given by each. Combinations of each assay will then be tested to best optimize an alkaline gel diffusion assay.

2.1.2 Phase II: Software Development

The purpose of the second project phase is to design automatic image analysis tools for apoptosis and DNA damage evaluation. There are two major constituents to this process: 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 will implement the algorithms and collect results for analysis. The image algorithms will be written using MATLAB and will implement thresholding and segmentation techniques to discern between cell morphologies and quantify DNA spread from cell nuclei. The program will 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 will 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 is to provide optional features which will improve the program and user interaction. This phase will 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 be time to develop a Python

based apoptosis module for Cell Profiler by translating from the MATLAB algorithms. The output of this phase will be program features that improve clinicians' and researchers' workflow and analyses.

> 2.2 Design Strategy

The following is a thorough description of the project phases. Appendix C contains supporting information for these descriptions, including the timeline, milestones, and deliverables for each phase in Table 3, a design concept for the image analysis algorithms in Figure 5, and a Gantt chart in Figure 6.

2.2.1 Phase I: Assay Research

> *Approach*: The first project phase aims to research methods of adherent cell detachment conducive to retaining apoptotic and necrotic cell types, as well to directly compare and evaluate the DDA and the FHA. Results from experiments will be compared with analysis of variance (ANOVA) using Prism software. Experiments will be performed in triplicates, with a p value less than .05 indicating significant differences between means. Using the DDA and FHA as a baseline, a modified alkaline gel diffusion assay will be optimized for clarity of image acquisition, number of cells required per sample, and the level of throughput. The reworked assay will tentatively be referred to as the Saturn assay for the purposes herein.

The first experimental aim of this phase will involve measuring the apoptotic index of a MOLT-4 suspension human acute lymphoblastic leukemia cell sample as different detachment reagents are encountered. To induce apoptosis, 8.82 uM H_2O_2 will be administered for one hour. Cells will be then be centrifuged at 5000 RPM for 5 minutes, media discarded, and administered treatments of trypsin, ethylenediaminetetraacetic acid (EDTA), and citric saline to simulate detachment protocols. Control cells will be given phosphate buffer solution (PBS) to normalize the apoptotic index. Detachment reagent solutions will be prepared as such:

- Trypsin & EDTA: Dissolve .25 g of each into 100 mL of PBS, respectively.
- Citric saline: Dissolve 1.0 g of KCl and .45 g of sodium citrate into 100 mL of PBS.

The second experimental aim will test multi-treatment modalities for cell detachment. Such modalities will be 1) treatment with citric saline or EDTA alone for 5 minutes, removal of detached cells, and then treatment with trypsin for 5 minutes; 2) Treatment with EDTA and citric saline together for 10 minutes and in series 5 minutes each; and 3) citric saline for 5 minutes followed by 1:1 trypsin-EDTA for 5 minutes. These procedures will first be used on BT-474 adherent human breast ductal carcinoma cells to quantify the level of cell detachment. Once this is established, the apoptotic index will be evaluated with MOLT-4 cells. If no satisfactory protocols are found using the reagents proposed, we will look into alternative methods of cell detachment, such as cell scraping and Poly N-isopropylacrylamide. Treatment protocols for the two experimentation phases are presented in Table 1 below.

		PBS	Trypsin	EDTA	Citric Saline	Time
Experiment 1	**Control**	2 mL				5 min
	Test 1	1 mL	1 mL			5 min
	Test 2			2 mL		5 min
	Test 3				2 mL	5 min
Experiment 2	**Control**	4 mL				10 min
	Test 1			2 mL		5 min
		1 mL	1 mL			5 min
	Test 2				2 mL	5 min
		1 mL	1 mL			5 min
	Test 3				2 mL	5 min
			1 mL	1 mL		5 min

Table 1: Detachment Solution Treatment Protocol. MOLT-4 suspension cells will be centrifuged, washed, and then treated with the solutions above for the duration indicated. These cells will then be analyzed using the DDA for their relative apoptotic index, normalized to that of the control sample. This information will be used as a model for adherent cell dissociation for the purposes of apoptosis and necrosis evaluation.

With a cell detachment procedure worked out which maximizes the normalized apoptotic index, we will begin comparisons of the DDA and FHA procedures. Initially, experiments will evaluate differences in image clarity, artifacting, and cell morphologies produced by each assay. MDA-MB-231 adherent human breast adenocarcinoma cells, HL-60 suspension human promyelocytic leukemia cells, MOLT-4 cells, and BT-474 cells will be tested for assay robustness. Subsequently, procedure combinations will be tested to maximize assay speed while keeping the previously mentioned parameters in mind. The resulting Saturn assay will be satisfactory when all properties have been assessed and all cell lines are found to be clear, and morphologically similar under microscopic inspection.

> *Deliverables*: The first project phase will deliver a solution to the problem of evaluating apoptosis for adherent cells in an alkaline gel diffusion assay. Additionally, the first phase will see the optimization of the so-called Saturn assay, which will be used subsequently as a means of preparing cell samples for apoptosis, necrosis, and DNA damage analysis with automatic image analysis algorithms.

> *Anticipated outcomes*: Anticipated problems could involve protocol errors where slides and samples are prepared incorrectly, or steps are carried out with poor timing or in incorrect order. This could lead to issues with image clarity through the agar gel, which could throw off analyses of assay protocols if not foreseen. A record of potential procedural errors will be kept in order to evaluate the resulting effects on experimental outcomes, should they arise. Running plenty of trials will ensure data outliers are discovered and ignored.

2.2.2 Phase II: Software Development

> *Approach*: During the second phase of the project, software development will begin with the aim of creating an array of automated image analysis algorithms and a program to implement the algorithms, collect and present data, and facilitate statistical comparisons pertinent to research and clinical evaluations. The

algorithms will be evaluated in terms of speed, sensitivity, and false-positive generation rate. The program will be evaluated based on speed, compatibility, image acceptance and organization, and data presentation.

Automated image analysis algorithms will be designed in MATLAB with the aid of imaging resources available in publications, technical tutorials, and MATLAB documentation. To count and analyze cells, the algorithms will first distinguish between the background noise and the smallest diffused DNA molecules. Then they will employ thresholding techniques to normalize intensity levels across the image and a method to segment adjacent and overlapping cells. Algorithms measuring apoptosis and necrosis will base counts on morphological differences between cell types. A preliminary analysis of the cells from Figure 2 can be found below in Figure 4. Algorithms measuring DNA damage will primarily compare the relative intensity of DNA outside of the nucleus compared to that inside of the nucleus, which will be defined by a threshold value. Normalized DNA single and double strand break levels will be decided by evaluating control samples prepared without treatment for both the comet and Saturn assays.

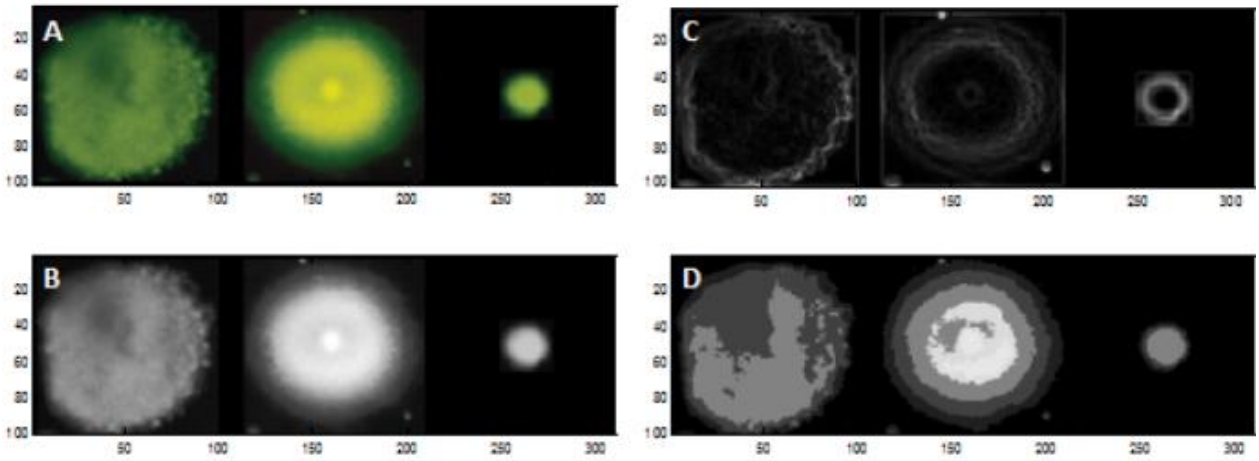

Figure 4: Preliminary Cell Image Analysis. Cell images from Figure 2 [5] were analyzed using MATLAB. The original image (A) was imported and converted to grayscale (B). The grayscale image was then convolved with a filter to accentuate changes (C) and processed with a simple thresholding scheme (D) to demonstrate the morphological differences seen in image analysis.

The differences between created algorithms will be in their scanning methods, ability to thoroughly handle artifacts, and thresholding methods. Some algorithms will be made to be crude and fast, while others will perform more stringent analyses that will yield higher specificity. The algorithms will be tested against manual scoring techniques to evaluate sensitivity and false positive generation rate; those with the highest sensitivity which complete in the shortest amount of time will be further explored to optimize analysis time and ability. To directly compare algorithms, two sided t-tests of results will be performed. Significant differences in algorithm ability ($p < .05$) will help to drive specific implementation and development strategy.

The controlling program will be written in Python and will take advantage of the vast amount of Python tutorials and documentation available online. Code writing will commence in stages, with each part adding functionality in a manner conducive to debugging. The program will initially be written to accept image files as inputs and produce text and excel files containing output data. Once these functions are working properly, statistical analyses functions that output to separate files will be added.

With the core functionality of the program established and running without bugs, the GUI design will commence. The GUI will be designed in the Python-variant language Kivy, an ideal language for cross-platform app development [24]. The GUI will initially be designed with all core features on a basic pallet, and will increase in complexity as graphical representations, algorithm comparison tools, user customization and input controls, pseudo-color displays, and spreadsheets are sequentially included. Once these GUI features are established and bugs eliminated, useful navigational features will be added, including quick access to prior results and folder and file representation. The GUI will be tested for speed, fluidity of experience, and ease of use by various members of the lab.

> *Deliverables*: The deliverables of the second project phase will be automatic image analysis algorithms and a program that can implement them to study cell samples through statistics and visual data representations. A table of the acceptance criteria for all software is given in Table 2 below.

Design Specification	Target Values and Tolerances	Validation Tool
Image analyzing time	15 - 30 sec	Internal timer
Algorithm sensitivity	98%	Manual scoring
False positives	1-2%	Manual scoring
Mean normalized DNA damage	P > .05	Comet Assay + VisCOMET
Software OS compatibility	Windows XP - 8	Feature testing
Image formats accepted	PNG, JPEG, GIF, TIF	Feature testing

Table 2: Acceptance Criteria. These criteria will form the basis for what the algorithms and program must deliver in order to be considered successful. Individual images must be analyzed within 15-30 seconds with a 98% sensitivity and only 1-2% false positive generation when considering apoptosis. DNA damage algorithms must deliver a mean normalized level of DNA damage which is insignificantly different ($p < .05$) than that found using the comet assay and VisCOMET analytical software using a two sided t-test. The controlling program must be compatible with all Windows operating systems since XP, and at the very least handle PNG, JPEG, GIF, and TIF image formats for analysis.

> *Anticipated outcomes*: Design decisions in this phase will rely on finding a balance between speed, specificity, and complexity of both the algorithms and the program. Most important will be the specificity of the algorithms, as results must be reproducible and delivered at a high degree of certainty. Exceptionally fast algorithms will be included as options for users not requiring great accuracy.

Many problems are likely to arise during the software development. These issues can be with poor programming structure, incorrect method implementation, data type errors, and file communication problems, which will cause specific exceptions to be thrown by the program compiler, depending on the error. These issues

will be dealt with by general debugging, as well as studying MATLAB and Python literature for solutions. By building the software piece by piece, it will be much easier to isolate issues as they arise. More difficult issues will be taken to friends and advisors with relevant programming knowledge to help converge on a solution.

2.2.3 Phase III: Additional Program Features

> *Approach*: The third phase of the project will focus on adding program functionality that is not required for the core process to work. Such features will include instantaneous display of results in real time as images are analyzed, image drag-and-drop analysis, the ability to pop out any tab as a separate window and vice versa, and a completely optimized and fluid interface for user interaction. These features will be assessed by feedback from peers and supervisors. An advanced goal of the project is to translate the developed algorithms into one or more modules for Cell Profiler. Since Cell Profiler has moved to exclusively use Python in its newest form, this process will involve reading many tutorials on Python image analysis packages and translating algorithms from MATLAB to Python in order to successfully port the algorithms.

> *Deliverables*: This phase of the project will deliver a high quality program interface and tools which will benefit users in their cell sample analyses. If time permits, this phase will yield a module for Cell Profiler which will be easily implementable by users given knowledge of Cell Profiler and its pipeline creation.

> *Anticipated outcomes*: Design in this phase will rely largely on subjectivity when it comes to interface improvements; thus the larger the pool of feedback, the better. Any features added will not be allowed to interfere with processing time and the ability of the program to perform core functions. The Cell Profiler module will need to run as efficiently as possible while remaining within the style guidelines set forth by the Cell Profiler community.

Similarly to the second phase, problems that arise in this phase will largely have to do with errors in coding, and as such will be dealt with similarly. Issues with direct translation between MATLAB and Python are guaranteed to occur, and will be handled by reading through documentation and help texts, along with consulting knowledgeable peers and advisors as to find the best solutions.

> 2.3 Key Personnel

This project will be completed under the direction of the laboratory PI, Dr. Narendra Singh, who is an expert in apoptosis and DNA damage analysis, and developed the DNA diffusion and comet assays. Dr. Singh will be a resource for discussing results, troubleshooting experimental errors, and assessing the usefulness of experimental ideas that arise during the research phase of the project.

Dr. Chris Neils will be a resource for the software development phase of the project, as he is proficient in many programming languages, GUIs, and image analysis modalities. Dr. Neils will be able to provide help when

troubleshooting code, teach advanced MATLAB image analysis implementations, and offer advice on project direction should issues arise.

If Dr. Sayan Pathak can be contacted, he will hopefully be able to provide old algorithm code for comparison with the algorithms developed in this project and for general reference. Otherwise, Dr. Pathak could be a resource for helping to guide the software implementation, as he is proficient in imaging, automation, and many programming languages.

> 2.4 Equipment and Facilities

All work will be performed in the Singh Laboratory, which has all the equipment necessary for running assays and capturing images. The majority of the cost of this project will be required for the research phase, and will be covered by departmental RCR funding. Possible research scholarships could also provide additional funding for the project. MATLAB is available on laboratory computers in the UW bioengineering department for use, and the student version is available for $99.00 dollars for personal computing use. It may be necessary to request access to advanced MATLAB image analysis toolkits from the bioengineering department computing services manager if not currently available. Python and Kivy are both freely available for download and use.

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4 Appendix A: Request for Proposal

Ross Jones
Singh Lab
< Design >

Automated Quantification of DNA Damage and Apoptosis

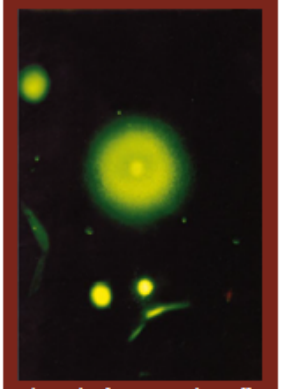
Evaluation of cellular apoptosis is an important caveat of various diseases. Apoptosis is also known to be a major factor in tissue remodeling, cell injury, cancer elimination, aging, and age-related diseases. Current techniques to evaluate apoptosis suffer from a variety of functional issues, such as requiring large numbers of cells, insensitivity to low levels of apoptosis, artifact labeling, loss of detection of some cell types, and false positive results. The DNA diffusion assay developed by NP Singh is a low cost, highly sensitive technique for quantifying apoptosis of cell populations at the single cell level. However, individually analyzing thousands of cells can be a time consuming and arduous process.

The Singh Lab seeks a solution to standardize and automate the DNA diffusion assay by development of an analytical software package to implement DNA diffusion assay image processing algorithms. The project should include the following: (1) a standardized, consistent protocol for the DNA diffusion assay that is optimal for automatic image analysis; (2) the ability to implement and compare different algorithm results; (3) built in statistical analyses functions; (4) histogram, spreadsheet, and custom graphical outputs; (5) at-a-glance results in the GUI; and (6) compatibility with Windows XP through Windows 8 operating systems.

5 Appendix B: Concept Sheet

BIOENGINEERING SOLUTIONS TO REAL WORLD PROBLEMS

Automated Quantification of Apoptosis and DNA Damage



A typical apoptotic cell (Singh 2000)

"In the recent past, extensive efforts have been made to develop simpler assay methodology for detection of apoptosis"

- Suman *et al.* (2012)

Clinical Need

Cellular apoptosis is known to be a major factor in tissue remodeling, cancer elimination, aging, and age-related diseases. Determining the level of apoptosis and DNA damage in a given sample of cells is an important diagnostic for genotoxicity and health status. Current techniques in apoptosis evaluation suffer from a variety of functional issues, such as requiring large numbers of cells, insensitivity to low levels of apoptosis, artifact labeling, loss of detection of some cell types, and false positive results. New techniques to analyze apoptosis and DNA damage need to be inexpensive, simple, fast, and give accurate, reliable results.

Bioengineering Solution

The DNA diffusion assay developed by Dr. Narendra Singh is a low cost, highly sensitive technique for quantifying apoptosis of cell populations at the single cell level[1]. However, long processing and analysis times are involved with the technique and others similar to it. Several years ago, Dr. Singh collaborated with Dr. Sayan Pathak to develop an algorithm for automatic analysis of DNA diffusion assay images. The Singh lab now looks to expand this algorithmic approach and use it to revamp and standardize a DNA diffusion assay optimized for image processing and quantifying both apoptosis and DNA damage at the individual cell level. A complete software program for analysis and interpretation of data will be created to implement the algorithms developed by Dr. Pathak, as well as new algorithms for DNA damage assessment. These aims will have the effect of reducing the time and cost involved in DNA damage and apoptosis evaluations, while maintaining sensitivity, requiring few cells, and achieving higher specificity for desired cell types. This project will be of value for various areas of medicine.

Current Status and Results

In 2006, the Fast Halo Assay (FHA) was implemented for the detection of DNA damage[2], and has recently been modified[3]. This technique has the potential to be one of the most sensitive simple, fast, and accurate assessment of DNA damage at the single cell level. The Singh Lab believes that this assay can be further modified and expanded upon to yield improved results.

Drs. Singh and Pathak found the automated image analysis algorithms then developed to be effective in quantifying the apoptotic index using the DNA diffusion assay. This preliminary work showed a 95% confidence level of no significant difference between the automated and manual image analyses. In addition, they found only marginal errors in misdetection and false positives.

Personnel

Ross Jones
Sayan Pathak
Narendra Singh

Funding

UW BIOE RCR

April 17th, 2013
Concept Sheet

6 Appendix C: Supplementary Material

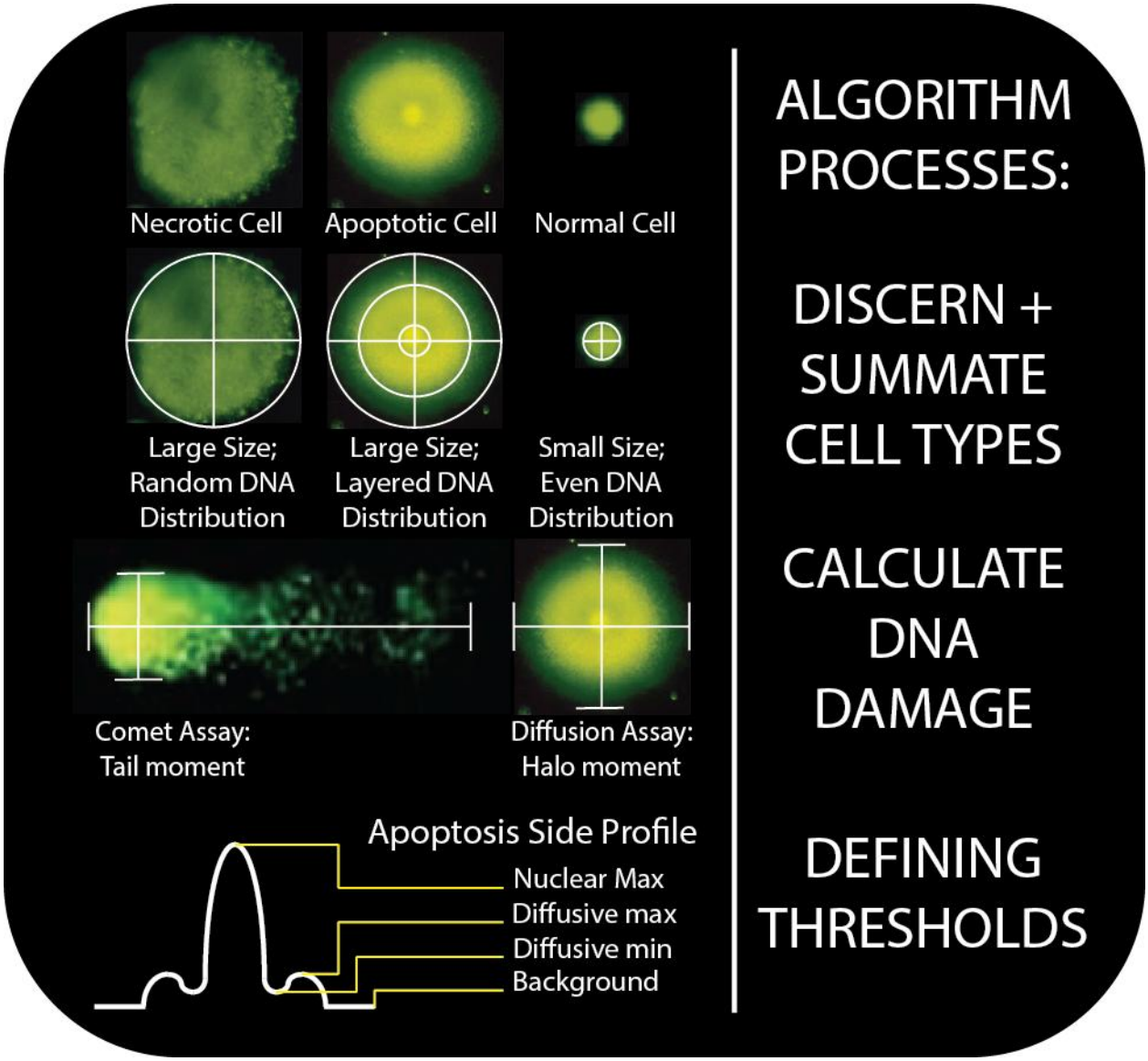


Figure 5: Algorithm Design Concept. As shown in Figure 2, necrotic, apoptotic, and live cells can be distinguished based on cell morphology. This morphology will be detected by the algorithms, which will employ thresholding techniques to find the layering and distribution of fluorescent intensity of cellular bodies. Necrotic cells will be large and have random DNA distribution; apoptotic cells are large and have intense nuclei with decreasing fluorescence further from the center, appearing as bands of different threshold intensities; live cells are small with concentrated and bright nuclei. To determine DNA damage levels, the halo moment, or the intensity of DNA fluorescence at a distance from the center of a cell will be measured. This is analogous to the tail moment assessment common to comet assay image analyses. Image thresholds will be established based on relative fluorescent intensities of peaks and valleys observed when apoptotic cells are viewed from the side profile. These thresholds will be especially relevant in locating apoptotic cells, as they will appear with rings around their structure, rather than being random or uniform like the other cell types.

Milestones, Timeline and Deliverables	Specific Activities
Phase 1: Assay Research **Timeline:** September, 2013 – December, 2013 **Major deliverable(s) or outcome(s):** - Evaluate methods of cell detachment for preservation of apoptotic cells. - Study and compare DNA diffusion and fast halo assays.	Test trypsin, EDTA, citric saline for effect on adherent cell apoptotic index using MOLT-4 suspension cells.
	Test order of detachment modality treatments for effect on apoptosis, can multiple be used effectively in series?
	Compare DNA diffusion and fast halo assay procedures to optimize an alkaline gel diffusion assay process. Compare images when different steps are taken. - Image clarity - Artifact propensity - Cell morphology
Phase 2: Software Development **Timeline:** November, 2013 – March, 2014 **Major deliverable(s) or outcome(s):** - Image analysis algorithms for DNA damage & apoptosis - Program to implement algorithms, organize data, build graphical representations, export to spreadsheets, and statistically analyze results.	Write and compare image scoring algorithms to count apoptotic, necrotic, and live cells, as well as assess DNA single and double strand break damage.
	Write program to call algorithms on cellular images.
	Iteratively add functionality to program, starting with file I/O, spreadsheet export, and statistical functions.
	Design GUI: add built-in graphical representations, pseudo-color image displays, and user customization.
	Optimize program speed and algorithm implementation.
Phase 3: Additional Program Features **Timeline:** April, 2014 – June, 2014 **Major deliverable(s) or outcome(s):** - Instant representation of results - Optimized interface for user interaction - Translate algorithms into Cell Profiler module	The software will be given features allowing the instantaneous display of results in real time as images are being analyzed. This will likely be in the form of histograms and bar charts.
	The interface will be optimized for simplicity and ease of use. This will involve finding volunteers to test and give feedback on the GUI.
	Cell Profiler is an open source toolkit for cell imaging. If time permits, the image analysis algorithms for apoptosis and DNA damage will be translated into modules which can be used in the Cell Profiler software.

Table 2: Milestones, Timeline, and Deliverables. This outline demonstrates the project sequence and design order. Phase I will largely be research oriented, with many tests evaluating the best methods for cell preparation in an alkaline gel electrophoresis assay. Phase II will focus on algorithm and program development to work with images yielded from the first phase. Phase III will add additional program features and expound upon the project if time permits.

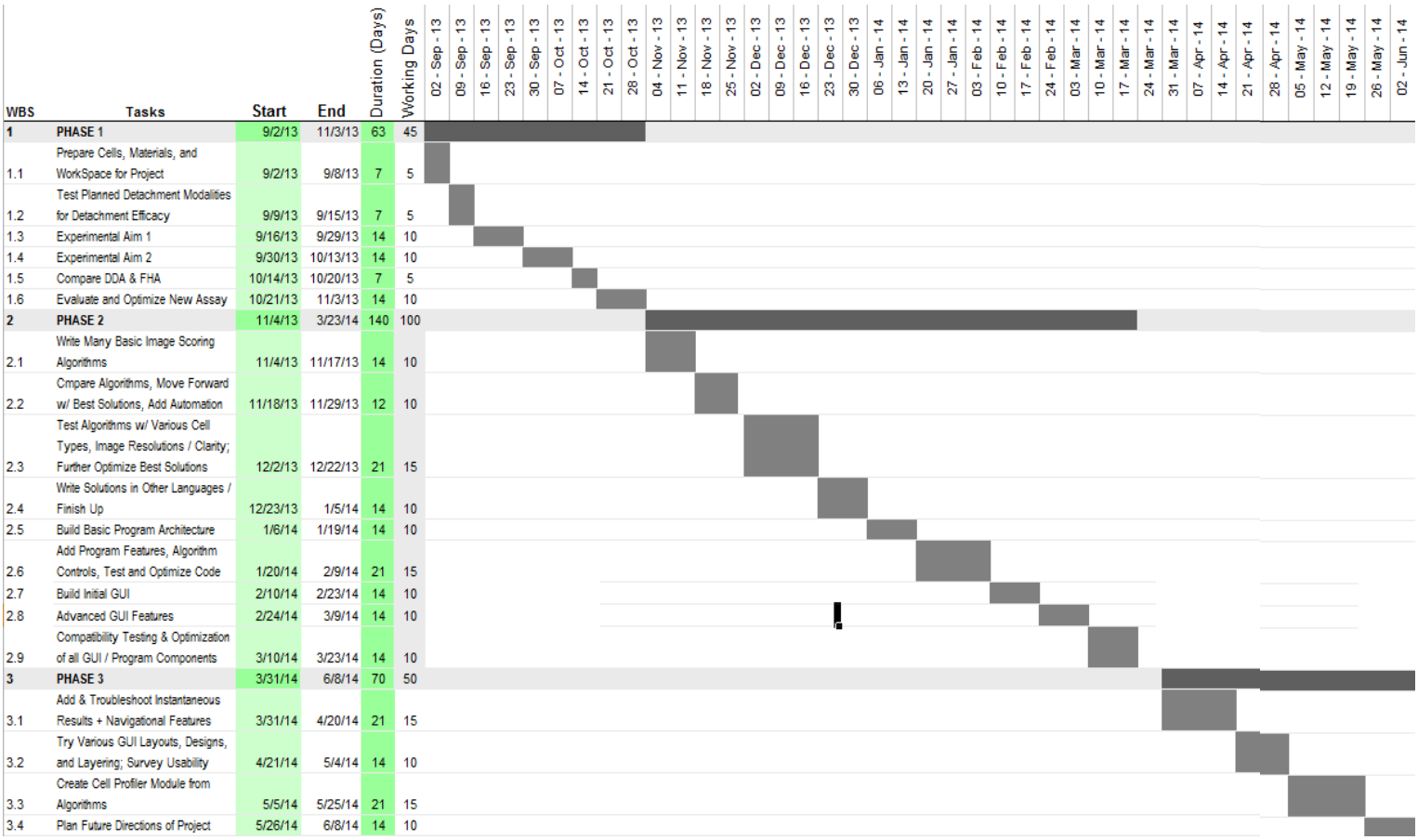


Figure 6: Project Gantt Chart. This chart demonstrates more explicitly the specific timeline for completion of project subtasks. Goals will be completed in a sequential order, as later milestones most often require the completion of earlier tasks. This timeline will demand approximately 16 hours of in-laboratory work per week, but will also require outside work in order to continue learning mathematical and programming methods pertinent to the algorithm and program.