1 Background and Significance

> 1.1 Problem Statement

Apoptosis is a programmed method of 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. Determining levels of apoptosis and DNA damage in a given sample of cells is an important diagnostic for genotoxicity and health status. Many methods of apoptosis detection take advantage of a property of apoptosis in which DNA is cleaved in predictable multiples of 180 kilobases. This 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 [1]. This so-called DNA laddering can be seen in Figure 1 in Appendix A.

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. Many of these issues can be immediately resolved by analyzing apoptosis at the single cell level, with a trade-off being much longer and more tedious post-lab analysis required to individually assess each imaged cell.

This project will deliver a platform for the systematic analysis of apoptosis at the single-cell level. An assay, image algorithms, and an analytical software will be designed to work seamlessly together to meet this end. Users will have the ability to analyze a large volume of cellular imagery with good confidence in results. A benefit of the proposed approach will be the ability to evaluate both apoptosis and DNA damage levels using differing image algorithms on images of cells from the same assay. In addition, the assay will run at a lower price than many current techniques, as enzymes and antibodies will not be required.

> 1.2 Previous Approaches

The following is a compilation of common methods in apoptosis and DNA damage assessment that are representative of the vast array of methods currently described in literature.

1.2.1 DNA Ladder Assay

DNA laddering as a technique to evaluate apoptosis originated with the canonical study mentioned earlier by AH Wyllie [1]. This technique is a very common form of apoptotic analysis,

and has led to the development of many of the assays described below. Of particular interest is a version of the DNA ladder assay described in a 2012 paper by S Suman, A Pandey, and S Chandna [2]. 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 this has significantly improved the DNA ladder assay, it still requires several millions of cells to perform, which is many fold more than is ideal for studies where samples are small in number and do not grow well. In addition, DNA ladder assay results are not easily quantifiable compared to other methods.

1.2.2 TUNEL Assay

The terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay is a widely used method for detecting DNA fragmentation caused by apoptosis. The method was first described in 1992, and works by labelling “nicks” in the terminal ends of DNA with the enzyme terminal deoxynucleotidyl transferase [3]. The accuracy of this method has been called into question with regard to its inability to differentiate between apoptotic, necrotic, and healthy cells [4], as well as its propensity to issue false positive results [5]. Methods to improve the TUNEL assay sensitivity have been put forward [6], but it remains contentious insofar as its false positive generation and loss of frail apoptotic cells during processing.

1.2.3 Nicoletti + Annexin V Assays

The propidium iodide flow cytometry assay, commonly referred to as the Nicoletti assay, was developed in 1991 as a way of measuring apoptosis by quantifying an increase in DNA fluorescence in the pre-G1 cell growth phase of apoptotic cells compared to healthy cells [7]. The authors later revisited and optimized the protocol in 2006 [8]. A related technique is the annexin V assay, which takes advantage of the fact that condensed chromatin strongly stains with annexin V [9]. While relatively rapid and sensitive, these techniques suffer from the requirement for flow cytometry, which is expensive and not worth any increase in throughput for those without access to such a device.

1.2.4 DNA Diffusion Assay

The DNA diffusion assay is a technique developed in 2000 to directly quantify apoptosis, necrosis, and healthy cells at the single cell level [10]. Cells are stained with the intense fluorescent dye YOYO-1 in order to maximize sensitivity to DNA which has diffused radially around apoptotic cells in agar gel. Representative images of apoptotic, necrotic, and healthy cells are provided in Figure 2 in Appendix A. This assay has been criticized for its use of the YOYO-1

dye rather than the much less expensive ethidium bromide (EB), as well as potential issues in defining necrotic cells [11]. In addition, the assay suffers from the need to individually analyze thousands of cells, which can be tedious and considered not worth the fast and simple protocol.

1.2.5 EB/AO Staining Assay

EB and acridine orange staining is the most popular form of DNA staining for the purposes of apoptosis evaluation. A recent method was developed to quantify apoptosis, necrosis, and healthy cells in a 96-well format using EB/AO staining protocols [12]. This method is one the best thus far for assay throughput, but suffers from poor imaging visibility due to the well plate wall and potential issues with proper mixing of cells with agar gel in the small wells. Though these issues arise due to the 96-well format, we believe that it is a viable method that could be improved upon for increased sensitivity.

1.2.5 Comet Assay

The single cell gel electrophoresis assay, known popularly as the comet assay, is a widely used method of quantifying DNA damage at the single cell level. This technique was first described by Östling and Johanson in 1984 [13], and was later modified to enhance sensitivity by Singh *et al.* in 1988 [14]. The alkaline version of this technique is beneficial due to its ability to assess low levels of both single and double stranded DNA breaks. Drawbacks of the comet assay include a long procedure that spans roughly three hours and the need for relatively expensive and specialized microgel electrophoresis units.

1.2.6 Fast Halo Assay

In 2009, the Fast Halo Assay (FHA) was implemented for the detection of DNA damage [15], and was recently modified to become even simpler [16]. This technique works similarly to the DNA diffusion assay, with subtle changes in aimed at quantifying DNA damage rather than apoptosis, as well as the use of EB staining. This assay is a simple, fast, and accurate assessment of DNA damage at the single cell level. A variation of the FHA and the DNA diffusion assay could be useful in simultaneous quantification of DNA damage and apoptosis.

> 1.3 Preliminary Data

Several years ago, our lab collaborated with Dr. Sayan Pathak to develop algorithms for automated quantification of apoptosis in DNA diffusion assay images. The algorithms devised were found to be effective in quantifying apoptotis relative to manual scoring techniques. This preliminary work showed a 95% confidence level of no significant difference between the

automated and manual image analyses. In addition, only marginal errors in misdetection and false positives were found. This approach has shown the efficacy of using automated image algorithms to quantify apoptosis, which we aim to expand to healthy and necrotic cell types. In addition, this work and similar algorithms commonly used for comet assay analysis suggest that we will be able to analyze DNA damage along with quantification of cell types.

Our lab is responsible for the development of the comet and DNA diffusion assays, which have shown great use as tools for apoptosis and DNA damage evaluation, respectfully. It has been shown by others that use of the DNA diffusion assay simultaneously with the comet assay is a strong method for total evaluation of cell temperament with respect to apoptosis and DNA damage [17]. This demonstrates that there is potential for an updated DNA diffusion assay with DNA damage quantification ability and high throughput image analysis software to be an effective tool for complete individual cell assessment.

> 1.4 Consequences of Success

Our complete system for apoptosis and DNA damage evaluation will allow researchers and clinicians to rapidly measure apoptosis, necrosis, healthy cells, and DNA damage with a high degree of sensitivity. This will aid in genotoxical, developmental, drug design, and aging research in particular. Clinicians will benefit when analyzing cell samples from patients for apoptosis and DNA damage, which is a common facet of many disease evaluations. Importantly, this system will allow for inexpensive quantification even with small samples of cells, such that research can still be performed with little available or poorly growing cells in environments ranging from first-class facilities to low wealth, isolated areas. In all, we will aid in quickening and lowering the cost of research and clinical evaluations with a simple assay and analytical software for image algorithm implementation.

2 References

[1] Wyllie AH (1980). "Glucocorticoid-induced thymocyte apoptosis is associated with endogenous endonuclease activation". *Nature* 284(5756): 555–6. doi: 10.1038/284555a0.

[2] Suman S, Pandey A, & Chandna S (2012): “An improved non-enzymatic “DNA ladder assay” for more sensitive and early detection of apoptosis”. *Cytotechnology* 64(1): 9-14. doi: 10.1007/s10616-011-9395-0.

[3] Gavrieli Y, Sherman Y, & Ben-Sasson SA (1992). "Identification of programmed cell death in situ via specific labeling of nuclear DNA fragmentation". *J. Cell Biol.* 119(3): 493–501. doi:10.1083/jcb.119.3.493.

[4] Kraupp, BG *et al.* (1995): "*In situ* detection of fragmented DNA (TUNEL assay) fails to discriminate among apoptosis, necrosis, and autolytic cell death: A cautionary note". *Hepatology* 21: 1465–8. doi: 10.1002/hep.1840210534.

[5] Stähelin BJ et al. (1998): "False positive staining in the TUNEL assay to detect apoptosis in liver and intestine is caused by endogenous nucleases and inhibited by diethyl pyrocarbonate". Mol. Path. 51: 204-8. doi: 10.1136/mp.51.4.204.

[6] Negoescu A *et al.* (1998): "Importance of DNA fragmentation in apoptosis with regard to TUNEL specificity". *Biomed Pharmacother.* 52(6): 252-8.

[7] Nicoletti I *et al.* (1991). "A rapid and simple method for measuring thymocyte apoptosis by propidium iodide staining and flow cytometry". *Journal of Immunological Methods* 139(2): 271–9. doi: 10.1016/0022-1759(91)90198-O.

[8] Riccardi C, Nicoletti I (2006). "Analysis of apoptosis by propidium iodide staining and flow cytometry". *Nature Protocols* 1(3): 1458–61. doi:10.1038/nprot.2006.238.

[9] Koopman G *et al.* (1994): "Annexin V for flow cytometric detection of phosphatidylserine expression on B cells undergoing apoptosis". *Blood.* 84(5): 1415-20.

[10] Singh NP (2000). "A simple method for accurate estimation of apoptotic cells". *Exp. Cell Res.* 256(1): 328-37.

[11] Gichner T *et al.* (2005): "Evaluation of the nuclear DNA Diffusion Assay to detect apoptosis and necrosis". *Mut. Res./Gen. Toxic. And Envir. Mutagen.* 586(1): 38-46. doi: 10.1016/j.mrgentox.2005.05.010.

[12] Ribble D *et al.* (2005): "A simple technique for quantifying apoptosis in 96-well plates". *BMC Biotechnol.* 5: 12. doi: 10.1186/1472-6750-5-12.

[13] Östling, O, & Johanson, KJ (1984): "Microelectrophoretic study of radiation-induced DNA damages in individual mammalian cells". *Biochem. Biophys. Res. Commun.* 123, 291-8 (1984)

[14] Singh, NP *et al.* (1988): "A simple technique for quantitation of low levels of DNA damage in individual cells". *Experimental Cell Research* 175(1): 184–91. doi: 10.1016/0014-4827(88)90265-0.

[15] Sestili P (2009). "The fast-halo assay for the assessment of DNA damage at the single-cell level". *Meth. Mol. Biol.* 521: 517-33. doi: 10.1007/978-1-60327-815-7_30.

[16] S. Galaz-Leiva *et al.* (2012). "A simplified chromatin dispersion (nuclear halo) assay for detecting DNA breakage induced by ionizing radiation and chemical agents". *Biotech. & Histochem.* 87(3): 208-17. doi: 10.3109/10520295.2011.604163.

[17] Díaz A *et al.* (2009): "Estimates of DNA strand breakage in bottlenose dolphin (Tursiops truncatus) leukocytes measured with the Comet and DNA diffusion assays". *Genet. Mol. Biol.* 32(2): 367-72. doi: 10.1590/S1415-47572009005000030.

APPENDIX A: Figures, Tables, etc

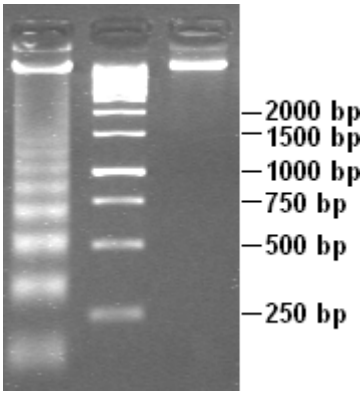

Figure 1: Apoptotic DNA Laddering (Wikimedia Commons). A cell sample is lysed and DNA is electrophoresed in a gel column. The left channel is DNA from a sample of apoptotic cells, the center channel is known sized DNA, and the right channel is DNA from control cells. This effect is caused by excision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

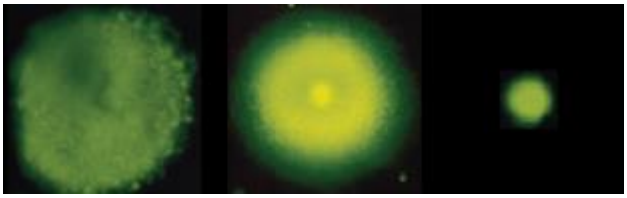

Figure 2: DNA Diffusion Assay Images [10]. Cells are lysed and DNA is allowed to diffuse radially in agar gel on a frosted microscope slide. From left to right, a necrotic, apoptotic, and health cell. Necrotic cells show random DNA distributions, no nuclear focal point, and a defined edge. Apoptotic cells show a gradient of decreasing intensity DNA around a condensed nucleus. Healthy cells appear small with bright, evenly distributed DNA.