Prospectus (2014)

Quantitative Detection and Colocalization of Protein Markers for Prognostic Use

(Technical Report)

A Comparative Study of Physician-Assisted Suicide and Its Abuse

(STS Topic)

A Thesis Prospectus in STS 4500
Presented to the Faculty of the School of Engineering and Applied Science
University of Virginia • Charlottesville, Virginia
In Partial Fulfillment of the Requirements of the Degree
Bachelor of Science in Biomedical Engineering

Author
Pai Li
November 05, 2014

Technical Project Team Members
Pai Li

On my honor as a University Student, I have neither given nor received unauthorized aid on this assignment as defined by the Honor Guidelines for Thesis-Related Assignments

Signature _____ Date _____

Approved _____ Date _____
Jongmin Lee, Department of Engineering and Society

Introduction

The presence and relative locations of different types of cells within a section of tissue can provide a wealth of prognostic and statistical information for clinical research and treatment. Protein markers are a common means in order to differentiate cell types in a tissue sample. For example, CD44 expression is indicative of effector-memory T-cells. As a result, colocalizing proteins, or determining which cells are positive for multiple proteins of interest, becomes an important step in prognostic and statistical analysis. However, current methods of protein detection and colocalization rely on three distinct RGB channels for separate stains. These methods are insufficient for colocalization of four or more proteins. The current project aims to provide a platform for protein colocalization without a protein limit, providing for a more powerful and flexible method of protein colocalization and, therefore, more accurate prognosis.

However, with accurate prognosis come a number of social and ethical consequences. In a hypothetical world where prognosis is 100% accurate, a patient with a terminal illness will have full knowledge of the suffering that awaits him at the end of his life. Patients with amyotrophic lateral sclerosis (ALS), for example, essentially become prisoners of their own bodies. Most voluntary muscles are paralyzed, and even involuntary muscles have difficulties functioning properly. ALS patients cannot move, eat or drink, or even breathe properly without aid ("Stages of ALS"). They are doomed to be trapped in their own bodies, fully conscious and aware, until they eventually die of respiratory failure. In such cases, should the patient have the right to decide to surrender the remainder of their life of agony in exchange for a peaceful death? If so, under what circumstances should mercy killings occur so as to prevent abuse? Indeed, physician- assisted suicide (PAS) is a complex issue with a number of social, political, economic, and

ethical realms to consider. However, with improved understanding of disease and more refined prognoses and diagnoses, the discussion on PAS becomes increasingly relevant.

Technical Topic: Quantitative Detection and Colocalization of Protein Markers for Prognostic Use

Cancer is the second most common cause of death in the United States, surpassed only by heart disease. The American Cancer Society estimates that roughly 1.6 million new cancer patients will be diagnosed in 2014. It is also estimated that roughly 600,000 people will die from cancer in 2014, averaging out to almost 1600 people each day. In addition, the National Institute of Health estimated that the monetary cost of cancer in 2009 to be 216.6 billion: $86.6 billion for direct health expenditures and $130 billion in loss of productivity due to premature death. (American Cancer Society, 2014) Thus, it is clear that cancer poses a significant health issue in the United States, both as a leading cause of death and as a source of monetary loss.

A key concept in cancer research is the idea of prognosis. What can one determine about patient outcome based on his or her current state and can this information be used to better develop treatment? To this end, prognostic protein markers have been researched fairly thoroughly. For example, at least 14 prognostic indicators for pancreatic cancer have been identified and found to correlate with significantly worse survival rate ($p < 0.05$), and expression of Ki67, p21, and nS has been found to correlate with mortality and increased recurrence in clear cell renal cell carcinomas. (Takadate et al., 2013; Weber et al., 2013) By understanding the requisite conditions for disease-related mortality, and subsequently the requisite conditions for long-term survival, a direction for research and treatment can be found.

However, it is often insufficient to simply look at the expression of various proteins independently. In addition to the simple presence or absence of singular protein indicators, co-

expression of multiple proteins can provide not only deeper insight into disease mechanisms, but also a more refined prognosis. It has been shown that CD8+ T cells lose CD27 expression and proliferative capacity once terminally differentiated. (Hamann, Roos, & van Lier, 1999) Since CD8+ T cells are activated via CD70 (CD27 ligand) induction, it has been suggested that retention of CD27 expression in CD8+ T cells could be a potential biomarker for fitter T cells. (Bullock & Yagita, 2005) In such cases, looking only at individual expression of CD8 and CD27 would be insufficient. As a result, proper protein colocalization and identification is essential.

Historically, multiple immunohistochemical staining (IHC) has relied on distinct primary antibodies that allow for specificity during labeling. However, this method is somewhat unreliable due to issues with antibody specificity and availability. (Pirici et al., 2009) Multiple staining has thus been limited typically to three proteins at most with the use of fluorophores for fluorescent microscopy. As a result, most protein colocalization methods only account for two or three distinct proteins. Recent antibody stripping methods, however, have been shown feasible for IHC and has allowed for, theoretically, any number of protein stains on a single sample of tissue. (Pirici et al., 2009) Thus, the limiting factor in analysis of multiple protein co-expression is no longer the feasibility of the stains, but the software and tools used in analysis. The current project therefore seeks to provide the tools necessary for multiple stain analysis.

For immunostained tissues, manual counting of double positives is subjective and questionable at best, and simply impossible for triple positives and beyond. Thus, a programmatic alternative is required. Certainly, various methods for identifying protein colocalization have been developed. (Cordelieres & Bolte, 2010; Costes et al., 2004; “inForm Advanced Image Analysis,” 2012) However, these methods may not be sufficient for all purposes in that they do not provide a clear count of cells that co-express specific proteins or, in

the case of Perkin Elmer, are ill-suited for analysis of a large data set due to resource requirements. In addition, many of the current programs currently in use are limited to double stains or three channel RGB filters. (Casavan, Gaidoukevitch, Murphy, Claxton, & Davidson; Zinchuk & Zinchuk, 2001)

The current project therefore attempts to provide a means to programmatically count cells expressing multiple proteins. The fundamental concept behind this project is nothing new. A binary mask is produced from each protein stain, based on a predetermined threshold and corresponding cell sizes (see Fig. 1). A separate counterstain, typically hematoxylin or heamtoxylin/eosin, is included to identify cells within conglomerates. These masks are then overlaid to determine relative locations, and cells expressing multiple proteins can be found and automated counted (see Fig. 2). What the current project focuses on is the development of the framework that extends this concept to an arbitrary number of proteins. With an arbitrary number of distinct images, there needs to be a method not only to determine the possible combinations of co-expression, but also to properly identify and colocalize these images. The current project makes use of string identifiers based on an image's filename that are used to

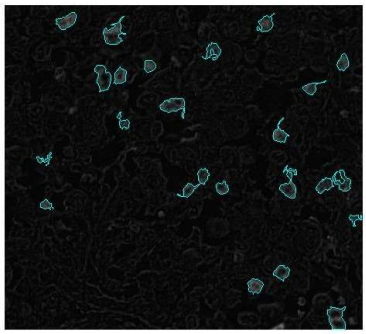

Figure 1: Overlay of raw image with generated mask. Note that the mask has been transformed from a binary mask to an outline for the overlay.

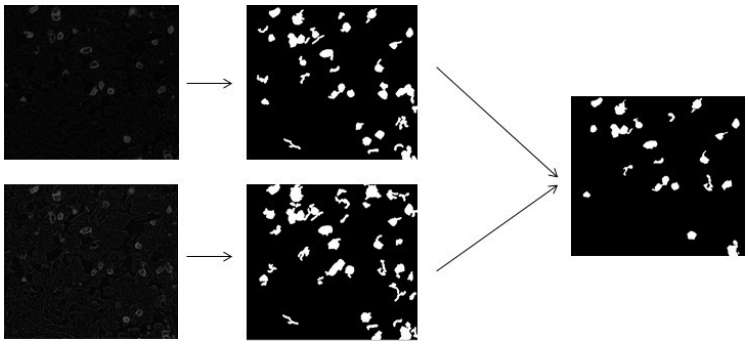

Figure 2: General process behind cell colocalization. Binary masks are generated from the source image. These masks are then overlaid to create a tertiary mask to locate cells expressing multiple proteins. This tertiary mask is then used to determine cell counts.

determine all possible protein combinations. In addition, these string identifiers are also the keys to a series of map objects containing related information used in colocalization, such as the intensity threshold and even the image itself. Thus, by organizing all the relevant data into key- value pairs, the current project provides a way to systematically organize image data, determine protein combinations, and count protein co-expression that applies for any number of proteins.

With the inclusion of more proteins comes the issue of data visualization. Sixteen distinct combinations are possible with 4 proteins, 32 with 5 proteins, and so on. These numbers are also only for one sample of tissue. For a typical tissue microarray with 20 cores, staining for even just 4 proteins would produce over 300 distinct data points for comparison. The current project seeks to alleviate this issue with the development of either a selection module for viewing relevant data, a novel data visualization technique such as those used by Baitsch et al., or both.(Baitsch et al., 2011)

In addition, while antibody stripping methods such as those described by Pirici et al. provide for a relatively reliable way to stain for multiple proteins, these methods are far from perfect.(Pirici et al., 2009) Most notably, the stripping process has been observed to cause random tissue movements on the order of a few micrometers. While not terribly significant for visual and manual analysis, this movement does mean that two subsequent images of the same tissue cannot be perfectly aligned. Although total alignment is not possible, local alignment is. The current project seeks to rectify this issue by summing smaller samples of locally aligned tissue. Once again, the underlying concept is nothing particularly novel. However, application and development of such a concept for multiple staining has yet to be done since multiple staining via antibody stripping is still relatively new.

Thus, the current project seeks to extend the use of binary masks to protein colocalization, allowing for analysis of any arbitrary number of proteins. The 14 protein prognostic indicators described by Takadate et al., for example, could be analyzed for co- expression, allowing for a more refined prognosis. Not only would prognoses be based on the presence or absence of these indicators, but also on the indicators' relations to one another. In line with this aim, the current project also attempts to develop a method for visualization and organization of the big data that comes with multiple protein colocalization. In addition, an attempt is made to compensate for random tissue movement in sequential antibody stripped stains. In doing so, the current project hopes to provide a novel method for prognostic and statistical analysis of protein co-expression that is not limited to traditional double costains or triple stains using fluorophores, allowing for more flexible and refined prognosis.

STS Topic: A Comparative Study of Physician-Assisted Suicide and Its Abuse

As prognostic estimates become more accurate and reliable, and eventual patient outcomes clearer, it becomes increasingly necessary to reconsider the discussion on PAS. For a patient with knowledge of his future outcome, it may initially seem humane and reasonable to draw out conditions for PAS for the patient's eventual suffering. However, PAS also brings about concerns for potential abuse. PAS guidelines in areas where it is legal, such as Washington, Oregon, Holland, and Belgium, typically follow a set of core beliefs. Specifically, PAS should be allowed only when a voluntary request is made, when the patient is in unbearable pain and suffering, when death is imminent, and when multiple, typically unrelated, healthcare professionals approve of PAS. ("Background about Euthanasia…", "The Washington Death with Dignity Act," 2008)

However, PAS is a complex subject with a large number of sociotechnical, religious, economic, and ethical factors. Some claim that legalization of PAS sets a precedent that can lead to unwanted practices, such as involuntary euthanasia or PAS for people with disabilities (Pappas, 1996). Most recently in the U.K., Charlotte Fitzmaurice won a court case in 2014 in which the High Court of Justice allowed Great Ormand Street Hospital to euthanize Fitzmaurice's 12 year old daughter, Nancy. ("Charlotte Fitzmaurice Wise wins…"; McDonald- Gibson, 2014) This case holds a great deal of significance in that it is the first ever recorded in which mercy killing was allowed for a non-terminal patient. In addition, it was also the first case in which euthanasia was allowed at the request of another, though it did involve a mother and her dependent. Even in areas where PAS is legal for minors, consent must be given by the individual in question and not by his or her guardians. (McDonald-Gibson, 2014) In light of Nancy Fitzmaurice's euthanasia, Dr. Andrew Fergusson, chair of anti-euthanasia group Care Not Killing, echoes Pappas' view, stating that "…what starts as a law to help people can be used to terminate disabled babies as happened in Holland and Belgium…" ("Why I begged…") Interestingly enough, this case occurred in the U.K., where PAS and euthanasia are generally illegal. (Choices, 2014) It could be that the High Court wants to set a precedent in which euthanasia and PAS are treated on a case-to-case basis; although without a formal statement, this is little more than a hypothesis.

Legalization of PAS also brings about a slew of moral, ethical, and possibly religious issues for both the patient and the physician. From a utilitarian perspective, PAS not only offers patients a relatively painless and dignified end, but also provides a variety of benefits such as monetary savings and the possibility to save more lives through the patients' organs. It is no secret that the demand for organs far outstrips the supply. On the flip side, as Pappas and

Fergusson theorize, legalization of PAS could set a precedent in which individuals are euthanized based on disabilities or other discriminatory factors. (Pappas, 1996; "Why I begged…") The Patients' Rights Council also points to potential abuse of PAS and euthanasia, though reports in Washington may suggest otherwise. ("Background about Euthanasia…", Washington State Department of Health, 2013)

In addition, abuse of PAS and euthanasia is a fairly subjective term. The Patients' Rights Council sees involuntary euthanasia as abuse, even though the High Court of Justice in the U.K. ruled in favor of such a case. The other side of the spectrum, however, is also often underappreciated. Only a fraction of physicians would willingly prescribe PAS in Oregon under any circumstance. (Lee et al., 1996) This brings to question as well the ethical implications of not prescribing PAS to patients in cases where PAS is voluntarily requested by a well-informed patient. In these cases, does not prescribing PAS constitute abuse?

The current study will attempt to focus on sociotechnical and economic pros and cons of PAS from a utilitarian perspective. First, outlines and definitions of PAS and abuse of PAS will be drawn based on historical precedents. Using these definitions, an attempt will be made to outline and quantify abuse of PAS and its guidelines in Washington, Oregon, Holland, and Belgium. It is understood that the value of a life and it relation to utilitarian measures are high subjective matters. As a result, no attempt will be made to evaluate whether or not PAS should be a legal option based on the level of abuse, or not, in these areas.

Conclusion

The current technical project hopes to satisfy a need in the field of medical prognostics by providing a method for automated detection and colocalization of prognostic protein markers

extending beyond simple RGB color channels. Hopefully, not only will this enable more refined prognosis, but also provide a tool for mechanistic research into diseases such as cancer.

The current STS project hopes to add to the discussion on PAS by providing insight into abuse of PAS guidelines in Washington, Oregon, Holland, and Belgium. To what extent is abuse prevalent in these areas and how does this affect outlook on proper PAS? Although the current STS project will attempt to answer such questions, the project only hopes to provide resources and insight for the reader into a highly controversial and subjective topic. No attempt will be made to evaluate whether or not PAS *should* be legal or if the benefits of PAS outweigh the risks and costs of its abuse.

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