Spectral Autofluorescence: Towards Quantitative Pathology

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Abstract

Cancer is the result of cells that have undergone abnormal changes in their programming, leading to uncontrolled growth and invasion into surrounding tissues. These cells propagate between healthy tissues and are characterized by subtle shape and color. Surgeons and pathologists train for years in recognizing these features, utilizing standard color cameras, white light and trained human eyes. Scientists at the Translational Imaging Center, developed technologies that probe the intrinsic signals in tissues utilizing modalities of imaging that overcome the limits of human vision: hyperspectral imaging and fluorescence microscopy. In previous work I applied these technologies to image cancer in fixed human tissue samples. Utilizing advanced microscopes I imaged fixed esophageal cancer biopsies from patients, mosaicking single-cell images across millimeter-wide samples and utilizing multiple laser illuminations. The product is a large, complex and information-rich database that spans across the dimensions of fluorescence emission and excitation, with corresponding datasets from the gold standard of pathology, labeled by expert pathologists.

This year explored this multi-dimensional dataset with analytical approaches to quantify the spectral signature of cancer with respect to the one of healthy tissues, referencing to the gold standard of pathology. I worked towards summarizing the results in a scientific publication.

Objective

- The objective of this project is to investigate a faster, quantitative method to distinguish cancerous tissue from non-cancerous tissue using autofluorescence microscopy.

Background

The current gold standard of pathology requires H&E staining of pathology slides, then technicians then perform white light imaging of the sample after it is stained. After, a pathologists uses previous experience and training to look at the morphology, stain color, and cell pattern to determine the possible presence of cancer. Limitations to this process include the price of the process, the time-consuming protocol, and the subjective clinical observations.

Pathology is a $311.2 billion industry where staining and imaging a slide costs $70, and hiring a pathologist costs $150/hr. The process is time-intensive, requiring 30-40 minutes for staining, and up to an hour or more for imaging and analyzing, depending on sample complexity. Importantly, a pathologist's view, subject to their training, isn't quantitative, presenting potential for error and bias.

Fig 1a: Esophageal Cancer Slide, Stained with H&E

Fig 1b: Esophageal Cancer Slide, Unstained

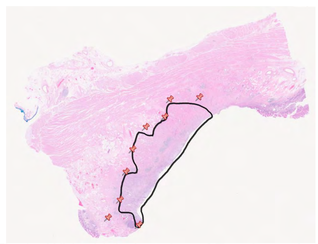


Fig 1c: White Light Imaging of Esophageal Cancer stained tissue with pathologist's annotation

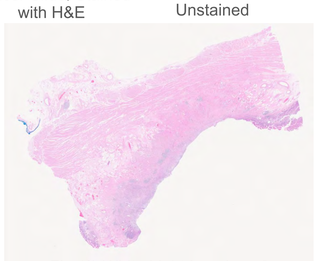
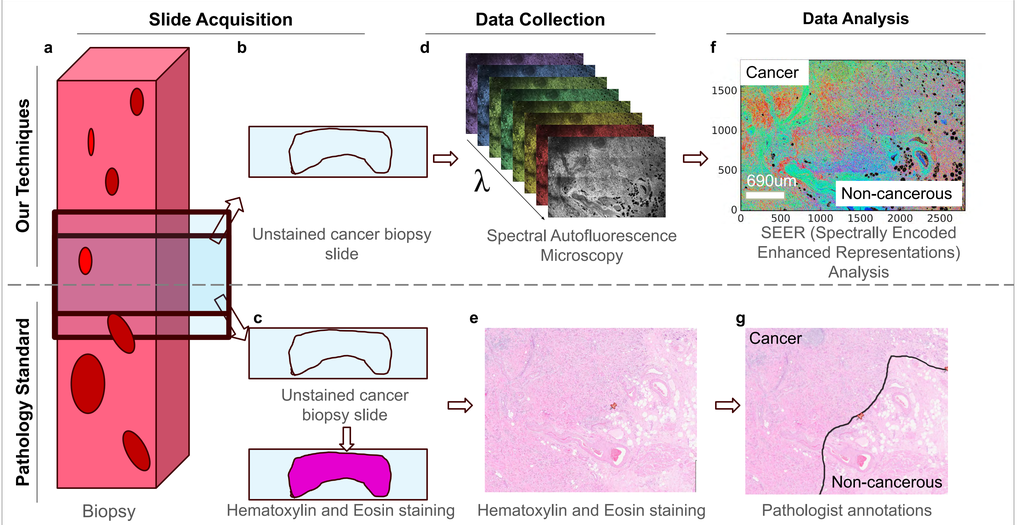


Fig 1d: White Light Imaging of Esophageal Cancer stained tissue with no annotation

Approach



Results

- Collected 23 hyperspectral datasets of 7 different samples all from different esophageal cancer patients.
- Excited each dataset at 7 different wavelengths (405nm,458nm,488nm, 514nm, 561nm, 594nm, 633nm) detected wavelengths in the spectral range 410nm-692nm.
- Used **SEER (Spectrally Encoded Enhanced Representations), which plots spectra onto a plot.** The sine **Fourier transform(S) is the y-axis and the cosine Fourier transform(G).** The sine and cosine Fourier transforms are compressed mathematical representation of a spectrum.
- Applied 5 different color maps to each image(contour, gradient descent, gradient descent mass morph, radius phasor, and radius phasor mass morph).
- Used Fiji to mask the cancerous area and non-cancerous area to find the barycentric center of mass for the phasor plot, which was represented by the g and s coordinates.
- Found the averages of all center of masses and used it to find where the average cancerous and non-cancerous area on the phasor plot for each wavelength.
- Used the average area for the cancerous and non cancerous area to plot a region of interest on the phasor plot, having the radius be the variance of the region.

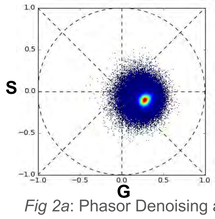


Fig 2a: Phasor Denoising and thresholding to remove the undesired signals

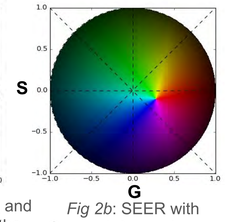


Fig 2b: SEER with Gradient Descent Mass Morph map.

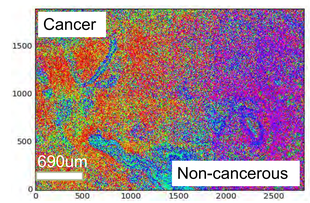


Fig 2c:Image after color map was applied

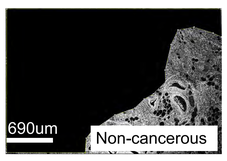


Fig 3a: Masked data for cancerous and non-cancerous areas

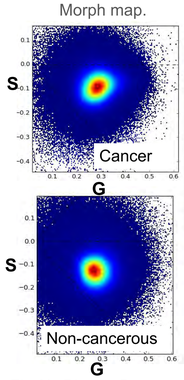


Fig 3b: Phasor Barycentric center of mass

Excitation[nm]	Average Non-cancerous G	Average Non-cancerous S	Average Cancer G	Average Cancer S
405	-0.15	0.085	-0.165	0.079
458	0.019	-0.201	0.002	-0.206
488	0.351	-0.084	0.3169	-0.095
514	0.442	0.045	0.422	0.052
561	-0.053	0.193	-0.12	0.158
594	-0.325	-0.295	-0.355	-0.271
633	0.11	-0.658	0.103	-0.658

Excitation[nm]	Variance Non-cancerous G	Variance Non-cancerous S	Variance Cancer G	Variance Cancer S
405	0.001	0	0	0
458	0.002	0	0.001	0
488	0.006	0.001	0.002	0
514	0.442	0.045	0.422	0.052
561	0.015	0.001	0.013	0.015
594	0.013	0.011	0	0
633	0	0	0	0

Fig 3c: Average and variance for each Barycentric center of mass coordinates

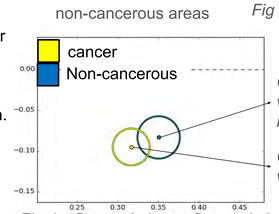


Fig 4a: Spectral phasor fingerprinting for cancer and non-cancerous regions for slides of 10 patients

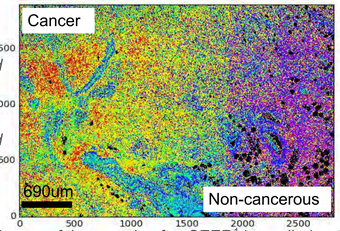
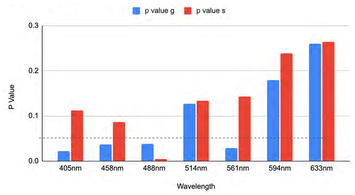


Fig 4b: Image of the sample after SEER[1] is applied

- Plot of the p values representing statistical difference between the phaso coordinates of cancer and non-cancero tissues for each wavelength. This is generally true for p < 0.05(dashed line) The p values of G at 405nm, 458nm, 488nm, and 561nm excitation, show good statistical difference. However, tha is true only the p value of S at 488nm excitation.



Excitation[nm]	P value G	P value S
405	0.022	0.112
458	0.038	0.086
488	0.038	0.005
514	0.126	0.134
561	0.029	0.144
594	0.18	0.239
633	0.26	0.264

Fig5: Table reporting the p values for G and S phasor coordinates of cancer/non-cancerous areas at different excitation values

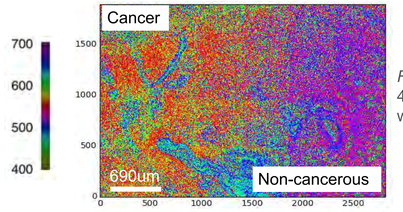


Fig 6a: SEER representation at 488nm excitation, the parameter with the lowest p value,

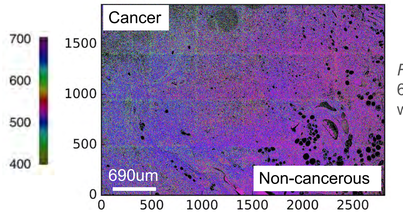


Fig 6b: SEER representation at 633nm excitation, the parameter with the highest p value,

Conclusion

- SEER analysis shows cancer and non-cancerous tissue have different spectra and can be visualized with different color.
- SEER images allow visual differentiation of cancerous and non cancerous regions.
- Created a database of 10 samples of Esophageal Cancer with 7 separate EX wavelengths and each EX wavelength has 32 EM wavelengths
- Tested multiple SEER maps and visually, Gradient Descent Mass Morph showed the most difference in color

Summary

Our research work centers on the potential use of autofluorescence spectral imaging to address the inherent subjectivity and potential for error in traditional pathology. This technology offers a faster, less costly alternative, providing a quantitative reference for pathologists. The quantitative analysis can expose features possibly missed using conventional methods, without necessitating slide staining. This compares favorably to the more time-consuming and expensive H&E staining process. In addition, our work with the SEER[1] technology has shown promising results, enabling rapid identification and differentiation between healthy and cancerous tissue. As we continue our research, we look forward to testing this technique on a broader range of cancers, such as skin cancer. We also aim to evaluate the efficacy of this approach on unstained slides without pathologist annotation, with the ultimate goal of generalizing the technology and identifying cancerous areas without a pre-existing ground truth.

[1] Shi, W., Koo, D.E.S., Kitano, M. *et al.* Pre-processing visualization of hyperspectral fluorescent data with Spectrally Encoded Enhanced Representations. *Nat Commun* **11,** 726 (2020). https://doi.org/10.1038/s41467-020-14486-8

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