Intra-operative Assessment of Breast Tumor Margins Using Diffuse Reflectance Spectroscopy

by

Torre Michelle Bydlon

Department of Biomedical Engineering
Duke University

Date: ______________________
Approved: ______________________

Nirmala Ramanujam, Supervisor

___________________________
William T. Barry

___________________________
Joseph Geradts

___________________________
Kathryn Nightingale

___________________________
Lee G. Wilke

___________________________
Tuan Vo-Dinh

Dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the Department of Biomedical Engineering in the Graduate School of Duke University

2012
ABSTRACT

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Abstract

Breast cancer is one of the leading causes of death every year in the United States for women. Breast conserving surgery (BCS) is one treatment option for these patients where achieving tumor-free surgical margins is desired to avoid local recurrence [1, 2]. Unfortunately, as many as 17.7-72% of patients undergoing BCS require repeat surgeries due to a close or positive surgical margin diagnosed post-operatively [3-11]. Histopathology is the current gold standard for determining surgical margin status; however, given the large volumes of resected breast tissue it is not feasible to section the entire specimen. High re-excision rates and limited histopathological sampling of the tissue represent a significant unmet clinical need for margin assessment for both the surgeon and pathologist. Quantitative diffuse reflectance (DR) spectral imaging has been shown to be a promising tool for interrogating tumor margins but patient and surgical factors have to be accounted for in order to fully exploit the discriminatory capability of the technology. The objective of this work was to characterize an instrument for margin assessment and to evaluate the effects of inter-patient variability and surgical and excisional factors on quantitative tissue optical properties, to devise strategies to exploit optical contrast for the detection of positive (<2mm) tumor margins. In addition, the performance of the spectral imaging platform was evaluated.
The DR spectral imaging device utilized in these studies consisted of a Xenon lamp, a multi-channel imaging fiber-optic probe, an imaging spectrograph, and a 2D charge-coupled device (CCD) [12]. The instrument was found to extract quantitative optical parameters related to tissue micro-morphology with <15% error. Cross-talk at the tissue surface was <1% when the spacing between adjacent channels was 10mm and the sensing depth of each channel was found to be 0.5-2.2mm, an appropriate depth for identifying close and positive tumor margins. Reproducibility of the imaging protocol was best when the probe was interfaced with lumpectomy specimens from the side; this methodology was maintained for all measurements from lumpectomies in this dissertation.

DR spectral images were acquired from lumpectomy margins and converted into composition maps of quantitative optical parameters. Mammographic breast density was found to have the greatest impact on the optical data with β-carotene concentration ([β-carotene]) and the ratio of [β-carotene] to the wavelength-averaged reduced scattering coefficient from 450-600nm (μs') being significantly higher in the negative margins of high-density patients (p=0.017 and p=0.038, respectively). We originally hypothesized that increased [β-carotene] would be associated with an increase in fat; however the significant increase in [β-carotene] cannot be attributed to differences in the percentage of adipose tissue since low-density patients should theoretically have higher percentages of this tissue type. Hematoxylin and eosin analysis of the adipose sites
vi

(n=25) showed increased \([\beta\text{-carotene}]\) (p=0.066), increased adipocyte density (p=0.034), and smaller adipocyte sizes (p=0.051) in the adipose tissues (where \(\beta\)-carotene is stored) of high-density patients. This analysis suggests that increased \([\beta\text{-carotene}]\) is associated with smaller adipocytes and that high-density breasts overall have smaller adipocytes, thus affecting optical contrast. This increase in \([\beta\text{-carotene}]\) actually served to increase contrast between negative and positive margins which resulted in better classification accuracy in the high-density patients with a conditional inference tree model (77% in low-density and 80% in high-density).

If the purpose of the spectroscopy tool is to provide a differential diagnosis of benign versus malignant tissue, there must be an understanding of how excision of the tissue affects the optical properties over time, and how differences in surgical techniques affect optical properties. DR spectra were acquired 17±4 minutes post-excision from 12 incised mastectomies and from the surface of 10 lumpectomies 7±3 minutes post-excision. A linear longitudinal model was used to fit the data and obtain a rate of change for the tissue parameters. In lumpectomies, \([\beta\text{-carotene}]\), \(\mu'\), and \([\beta\text{-carotene}]/\mu'\) had the lowest percent change (<14%) over 30 minutes; total hemoglobin concentration ([THb]) and \([\text{THb}]/\mu'\) had higher percent changes (>40%) over 30 minutes; hemoglobin saturation (HbSat) showed non-linear changes making it a poor variable for ex vivo margin assessment; and Lymphazurin\textsuperscript{TM} concentration (denoted as \([\text{Lymphazurin}^{\text{TM}}]\)) changed more than 200% in 30 minutes. Although the percent error
in [Lymphazurin™] was high, all other tissue parameters could be quantified with <3.3% error even when Lymphazurin™ was 80µM. No significant difference was found between benign and malignant rates of change, and baseline values were not significantly correlated with elapsed time post-excision. Initial values from benign non-cauterized mastectomy (n=13) and cauterized lumpectomy (n=59) sites were compared to assess the effect of cautery. [THb] was the only parameter that was significantly higher in the cauterized lumpectomies (p=0.013) compared to non-cauterized mastectomies.

The work in this dissertation shows the feasibility of using an optical device for margin assessment and that [β-carotene] and [β-carotene]/<µ> emerge as important variables for differentiating negative and close/positive margins. These two parameters were likely most important since they were least affected by kinetics, cautery, and the presence of Lymphazurin™.
To all that have lost their battle with breast cancer and to all that have survived.
Contents

Abstract ................................................................................................................................. iv

List of Tables ....................................................................................................................... xv

List of Figures ..................................................................................................................... xviii

Symbols/Abbreviations ....................................................................................................... xxiv

Acknowledgements ............................................................................................................ xxvii

1 Background and Significance .......................................................................................... 1

1.1 Normal Breast Anatomy ............................................................................................... 1

1.2 Optical Sources of Contrast in the Breast ................................................................. 2

1.3 The Benign Breast and How It Changes Throughout a Woman’s Lifetime .......... 3

1.4 Effects of Patient Characteristics on the Optical Signatures of Benign Breast Tissue .............................................................................................................. 5

1.5 Breast Cancer and the Tumor Micro-environment .................................................... 6

1.6 Risk Factors for Breast Cancer.................................................................................... 8

1.7 Mammographic Breast Density .................................................................................. 10

1.8 Management of Breast Cancer..................................................................................... 12

1.9 Breast Conserving Surgery (BCS) ............................................................................. 13

1.10 Current Intra-operative Margin Assessment Techniques ........................................ 14

1.11 Intra-operative Margin Assessment Device Needs ................................................ 16

1.12 Emerging Optical Tools for Breast Margin Assessment ......................................... 17

1.13 Diffuse Reflectance Spectral Imaging ................................................................ ...... 19

1.14 Monte Carlo Model [4, 5, 100] ................................................................................. 20
1.15 Initial Clinical Breast Studies With Diffuse Reflectance Spectral Imaging...23
1.16 Specific Aims and Organization of Chapters...........................................25

2 Characterization of a Diffuse Reflectance Spectral Imaging Platform for Breast Tumor Margin Assessment [12]........................................................................................................30

2.1 Introduction........................................................................................................30

2.2 Instrumentation..................................................................................................31

2.2.1 8 Channel System – Version 1 (8CH-1).........................................................31

2.2.2 8 Channel System – Version 2 (8CH-2)..........................................................33

2.3 Software ..............................................................................................................34

2.4 Methodology for Imaging Partial Mastectomy Tumor Margins .....................35

2.4.1 Clinical Procedure............................................................................................35

2.4.2 Pathological Evaluation.....................................................................................37

2.5 Analyzing the Diffuse Reflectance Data.............................................................38

2.6 Signal to Noise Ratio (SNR) of the Imaging Platform........................................44

2.6.1 Methods ..........................................................................................................44

2.6.2 Results ..............................................................................................................44

2.7 Accuracy of the Imaging Platform.........................................................................45

2.7.1 Methods ..........................................................................................................45

2.7.2 Results ..............................................................................................................48

2.8 Sensing Depth of the Imaging Platform.............................................................50

2.8.1 Methods ..........................................................................................................50

2.8.2 Results ..............................................................................................................54
2.8.2.1  Monte Carlo Simulations of Sensing Depth ............................................ 54
2.8.2.2  Sensing Depth of Tissue Data ............................................................ 57
2.9   Evaluation of Cross-talk at the Tissue Surface ........................................... 59
  2.9.1  Methods .............................................................................................. 59
  2.9.2  Results ............................................................................................... 61
2.10  Reproducibility of the Imaging Platform .................................................. 62
  2.10.1  Methods ............................................................................................ 62
  2.10.2  Results .............................................................................................. 63
2.11  Discussion and Conclusions ................................................................... 64
3   Effects of Inter-patient Variability on Optical Data and the Ability to Predict Surgical Margin Status ................................................................. 70
  3.1   Introduction ........................................................................................... 70
  3.2   Methods ............................................................................................... 74
    3.2.1  Patient Population ............................................................................ 74
    3.2.2  Instrumentation ............................................................................... 74
    3.2.3  Measurement Procedure .................................................................. 75
    3.2.4  Margin-level Histology ..................................................................... 76
    3.2.5  Site-level H&E Image Analysis ......................................................... 77
    3.2.6  Spectral Data Analysis ..................................................................... 79
    3.2.7  Empirical Cumulative Distribution Functions .................................... 79
    3.2.8  Conditional Inference Tree Models .................................................. 79
  3.3   Results and Discussion .......................................................................... 82
3.3.1 Spectral imaging surveys tissue composition and micro-morphology in the normal breast .................................................................................................................. 84

3.3.2 Spectral imaging tracks shifts in histological landscapes associated with breast density ............................................................................................................. 86

3.3.3 Adipocytes in high density breasts are smaller and have a higher baseline β-carotene concentration ........................................................................................................ 88

3.3.4 Spectral surveying of histologic landscapes can be leveraged in detection of residual cancer on a tumor margin ......................................................................................... 91

3.4 Conclusions ........................................................................................................ 102

4 Effects of Surgical and Excisional Factors on Optical Data ........................................ 106

4.1 Introduction ........................................................................................................ 106

4.2 Methods ............................................................................................................ 113

4.2.1 Clinical Protocol .......................................................................................... 113

4.2.2 Instrumentation and Measurements ............................................................ 114

4.2.3 Histopathology ........................................................................................... 116

4.2.4 Data Analysis of Mastectomies and Lumpectomies .................................... 117

4.2.4.1 Lumpectomy Kinetics ............................................................................. 117

4.2.4.2 Cautery ................................................................................................... 118

4.2.4.3 Effects of Tissue Histology .................................................................... 118

4.2.5 Lymphazurin™ Simulations and Phantom Studies ....................................... 119

4.3 Results ............................................................................................................. 122

4.3.1 Sample Sizes ............................................................................................... 122

4.3.2 Which parameters are least affected by excision in lumpectomies? .... 123
4.3.3  Does the addition of Lymphazurin™ affect the optical absorbers and scatterers in the breast? ......................... ................................................... ................................................... 129

4.3.4  Are there differences between cauterized and non-cauterized tissue? ...... 131

4.3.5  Are kinetics different between benign and malignant tissue? ............. 133

4.3.6  What happens to optical contrast for margin assessment? ................ 136

4.4  Discussion and Conclusions ........................................................................... .... 139

5  Conclusions and Future Directions ........................................................................... ................. 144

5.1  Conclusions .............................................................................................................. 144

5.2  Future Work .............................................................................................................. 149

5.2.1  Microscopic Scale: Assessing the Tumor Micro-environment................. 149

5.2.1.1  β-carotene, Adipocyte Size, and Breast Density .............................................. 149

5.2.1.2  Surveillance of the Tumor Micro-environment Using Quantitative Spectral Imaging .............................................................................................................. 151

5.2.1.3  β-carotene and Tumor Aggressiveness .......................................................... 153

5.2.2  Macroscopic Scale: Additional Studies for Margin Assessment .......... 154

5.2.2.1  Benign Abnormalities and the Less Common Breast Cancers .......... 154

5.2.2.2  Neoadjuvant Therapy ...................................................................................... 155

5.2.3  Technological Improvements .......................................................................... 156

5.2.3.1  Footprint Reduction ......................................................................................... 156

5.2.3.2  Microscopic Imaging ......................................................................................... 156

5.2.3.3  Modeling Collagen and Cells in Breast Tissue Using Mie Versus Rayleigh Light Scattering Models .............................................................................................................. 157

5.2.3.4  Fluorescence Imaging and Contrast Agents .................................................. 158
References.................................................................................................................. 162

Biography ...................................................................................................................... 173
List of Tables

Table 1: Optical sources of contrast in breast tissue. DRS = diffuse reflectance spectroscopy; ESS = elastic-scattering spectroscopy; NIR = near-infrared; FL = fluorescence; SPX = spectroscopy; OCT = optical coherence tomography. .................................3

Table 2: Reported trends in optical properties versus patient characteristics. All arrows indicate the direction of change in the optical property with an increase in the patient characteristic. For example, hemoglobin concentration decreases with increasing BMI. θ = no significant finding, StO₂ = oxygen saturation, a = scatter amplitude, b = scatter power. ............................................................................................... ........................................ 5

Table 3: Reported sensitivities and specificities for frozen section and touch-prep analysis. .................................................................................................................................................................................. 16

Table 4: Patient sample sizes for all of the analyses in this dissertation................................ 29

Table 5: Listing of which instruments were used for each study in this dissertation, the type of breast tissue the instrument was used on, and corresponding chapter with the discussion of that study. .......................................................................................................................................................... 34

Table 6: A list of the different extracted parameters used to create the descriptive variables. Mean scattering refers to the wavelength-averaged μ′ from 450-600nm. ...........................41

Table 7: Signal to noise ratio of each channel at 450 and 600 nm. .............................................. 45

Table 8: Values of the expected optical properties for the phantom study. β-carotene concentrations were converted into Cr concentrations by matching μa. .........................47

Table 9: Mean difference (bias) and standard deviation of differences (precision) between the expected and extracted phantom data. ..................................................... ...... 50

Table 10: Median values of the extracted absorption and reduced scattering coefficients at specific wavelengths for various tissue types from the site-level data. FA = fibro-adipose, FG = fibro-glandular. ....................................................................... ....................... 53

Table 11: The simulated 90% sensing depth of the 8-channel probe for various tissues in a single layer model and two-layer model. In the two-layer model, the first layer was simulated with a thickness of 1 mm and the second layer with a 29 mm thickness. FG = fibro-glandular. ......................................................................................57
Table 12: SNR due to cross-talk calculated using MC simulation. Simulations are based on radius=0.515 mm for illumination core and 200 µm collection fibers..........................62

Table 13: Comparison of the percent error in accuracy versus percent differences between tissue types for the extracted parameters. .................................................................66

Table 14: Patient and tumor demographics. BMI – body mass index, IDC – invasive ductal carcinoma, DCIS – ductal carcinoma in situ.........................................................83

Table 15: Sensitivity (Se), specificity (Sp), positive predictive value (PPV), negative predictive value (NPV), and classification accuracy (A) of the device and the surgeon. Performance within each MBD subgroup is given. The surgeon’s performance is based on the primary specimen (and no additional shavings) taken during the first operation. Fisher’s empirical $P$ value is provided for each CIT model..............................101

Table 16: Number of false negative (FN) and false positive (FP) margins (margin histology and surgical margin status) and patients (MBD) calculated from the surgeon performance, as well as, the performance of the device. ....................................................102

Table 17: Planned and actual absorber and scatter levels for the tissue mimicking phantom. Scattering levels were calculated for a wavelength range of 450-600nm. .....120

Table 18: Sample sizes of histologically-confirmed sites. .....................................................123

Table 19: Correlations of [Lymphazurin™] with each optical variable for the initial measurements in the lumpectomy sites (n=61)..................................................................131

Table 20: Rate of change per minute (fitted values from the model) for each tissue parameter of the benign (n=13) and malignant (n=7) sites measured in mastectomy specimens and benign (n=59) sites measured in the lumpectomy specimens. Reported values indicate the average ± standard deviation. P-values indicate the statistical differences in the rate of change between the benign and malignant mastectomy sites.135

Table 21: Comparison of the percent change in each optical parameter at 10, 20, and 30 minutes post-excision to the percent differences between 1) adipose (A) and positive (P) sites, 2) fibroadipose (FA) and positive sites, and 3) fibroglandular (FG) and positive sites from our initial site-level study [12, 25]. A negative value in the percent difference indicates that positive sites were greater; a positive value means the benign tissue was greater. ..............................................................................139

xvi
Table 22: Comparison of diffuse reflectance spectral imaging to other optical techniques for breast margin assessment. DRSI – diffuse reflectance spectral imaging; FL – fluorescence; SPX – spectroscopy; OCT – optical coherence tomography; HRME – high resolution micro-endoscopy.
List of Figures

Figure 1: Anatomy of the benign breast [14] showing the network of ducts surrounded by fatty tissue and lymph nodes. ................................................................. 1

Figure 2: Molar extinction coefficients of the 4 primary breast absorbers over the visible wavelength range; deoxygenated hemoglobin (HbH), oxygenated hemoglobin (HbO2), β-carotene, and Lymphazurin™ [113]. ...................................................................... 20

Figure 3: Block diagram of the inverse Monte Carlo developed by our group [4, 5, 100]. A – absorber concentration, \( \mu_a(\lambda) \) - the wavelength-dependent absorption coefficient, \( \mu_s(\lambda) \) - the wavelength-dependent scattering coefficient, \( R(\lambda) \) - reflectance. ........................................ 21

Figure 4: Block diagram of the clinical instrument and the fiber arrangement of the multi-channel probe. Each channel has 4, 200 µm collection fibers and a central bundle of 19, 200 µm illumination fibers. All 8 channels are arranged in a 4x2 array with a separation distance of 10 mm (center to center). ...................................................... 32

Figure 5: A) Picture of the clinical instrument. B) Picture of the fiber optic probe in an aluminum adaptor to space each of the 8 probes 10 mm apart in a 4x2 array and the two pieces of the plexi-glass box that slide together to hold the specimen in place. C) A specimen in the plexi-glass box with light gray dots (green ink) indicating the margin border and black-white striped dots (orange ink) indicating specific sites for pathologic co-registration. ................................................................. 33

Figure 6: Distribution of imaged margin sizes from 91 lumpectomy patients. ............... 36

Figure 7: Examples of the diffuse reflectance, absorption and reduced scattering coefficient spectra from a benign site (fat) and a malignant site (IDC – invasive ductal carcinoma). ................................................................. 40

Figure 8: A) Photograph of the imaging probe interfaced with mock tissue inside the specimen imaging container, B) Sample hyperspectral reflectance cube from a margin sample positive for residual invasive ductal carcinoma, C) Hyperspectral absorption \( \mu(x,y,\lambda) \) and reduced scattering coefficient cubes \( \mu'(x,y,\lambda) \), extracted from the reflectance cube \( R(x,y,\lambda) \), D) 2-dimensional β-carotene concentration map, total hemoglobin (oxy- plus deoxy-hemoglobin) concentration map, and wavelength-averaged reduced scattering coefficient \( \langle \mu' \rangle \) map extracted from the absorption and scattering spectral data cubes. ............................................... 42
Figure 9: A) Parameter map of the ratio of \([\beta\text{-carotene}]/<\mu'>\) in a pathologically positive margin. The black boxes are sites with ductal carcinoma in situ (DCIS) and the dotted gray box is a site with fibro-adipose tissue. B) Histogram of the pixels in the parameter map.

Figure 10: A) Parameter map of the ratio of \([\beta\text{-carotene}]/<\mu'>\) in a pathologically negative margin. The dotted gray boxes are sites with adipose tissue. B) Histogram of the pixels in the parameter map.

Figure 11: Box and whisker plots of \([THb]\) (µM), \([\beta\text{-carotene}]\) (µM), \(<\mu'>$\) (cm⁻¹), \([\beta\text{-carotene}]/<\mu'>$\) (µM-cm), \([THb]/<\mu'>$\) (µM-cm), and \([\beta\text{-carotene}]/[THb]\) (a.u.) for the site-level data (n=854). The two dark gray boxplots use the y-axis on the left and the light gray boxplots use the y-axis on the right. The box represents the median, 25th percentile, and 75th percentile; the whisker represents values within 1.5 times the interquartile range.

Figure 12: Average percent error and standard deviation of expected versus extracted plots of Hb concentration (A), Cr concentration (B), \(<\mu'>$\) (C), the ratio of Hb concentration to \(<\mu'>$\) (D), the ratio of Cr concentration to \(<\mu'>$\) (E), and the ratio of Cr to Hb concentration (F). Each circle refers to one phantom extracted from the best reference phantom. The solid line shows the line of perfect agreement between the expected and extracted values and the dashed lines represent the 95% prediction interval.

Figure 13: Example images of the weighted visiting frequency in each grid separated by depth versus radial distance from the center of the illumination fiber. White lines represent the depth where a percentage (50% or 90%) of the detected photon weight distribution is contained within.

Figure 14: Simulated weighted visiting frequency as a function of depth for various tissue types (P=positive, A=adipose, FG=fibro-glandular) in a single layer model. Black circles correspond to the depth at which 90% of the collected photons reach.

Figure 15: Simulated weighted visiting frequency as a function of depth for a two-layer tissue model. The first layer is either adipose (A) or fibro-glandular (FG) tissue with a 1 mm thickness and the second layer is positive (P) with a 29 mm thickness. Black circles correspond to the depth at which 90% of the collected photons reach.

Figure 16: Box and whisker plots of site-level data. µₐ(A,B) and µₛ'(C,D) coefficients at 450 nm and 600 nm as a function of depth. Positive sites correspond to a depth of 0 mm.
Close sites are 0-1 mm and 1-2 mm. Negative sites are greater than 2 mm. P-values are only shown for categories with significant differences. The box represents the median, 25th percentile, and 75th percentile; the whisker represents values within 1.5 times the interquartile range.

Figure 17: The 4x2 arrangement of the fibers in the multi-channel probe to show how SNR arising from cross-talk was calculated for a channel with varying separation distances (S.D.) between channels. Light gray circles represent the illumination (I) fibers, black circles represent collection fibers for channels that contribute to the noise, and dark gray circles represent the collection (C) fibers for the channel that contributes to the signal.

Figure 18: Various 8CH probe orientations with the plexi-glass box for reproducibility testing.

Figure 19: This plot shows the median coefficient of variation for the 32 sites which was calculated from 10 measurements on 4 separate lumpectomy specimens. $\beta = \beta$-carotene.

Figure 20: A) 50x bicubic interpolated image of $[\beta$-carotene]$/<\mu'>$ from a negative margin. Sites with corresponding histopathology are highlighted with diagnoses of adipose (A) or fibroglandular plus adipose (FG+A). B) Absorption ($\mu_s$) and scattering ($\mu'_s$) spectra for representative adipose (A), fibroadipose (FA), and fibroglandular (FG) sites from a negative margin. C) Representative H&E images of a predominately collagen site and another predominately fat. D) Empirical cumulative distribution functions (eCDFs) of the site-level data for fibroglandular (FG), fibroadipose (FA), and adipose (A) sites.

Figure 21: A) Parameter maps from a low density margin; B) Parameter maps from a high density margin; blue indicates higher values of the corresponding variable. C) eCDFs of all measured sites from negative, neoadjuvant naïve margins, separated by mammographic breast density. P-values were calculated with modified Kolmogorov-Smirnov statistics.

Figure 22: Analysis of adipose tissue between low and high density breast tissue. A) Representative H&E micrographs (100x) from all 4 MBDs. Cell area and cell density were calculated from an automated image analysis algorithm. $[\beta$-carotene] and $<\mu'>$ were measured via quantitative spectral imaging. The adipose sites are from B) the negative margins of neoadjuvant-naïve patients and C) the negative margins of...
neoadjuvant-naïve patients with a BMI restricted to 25-30. P-values were calculated with a Wilcoxon rank-sum.

Figure 23: A) 50x bicubic interpolated images of \([\beta-\text{carotene}]/<\mu_s'>\) from a negative and positive margin in 2 different patients with MBD-3. Sites with corresponding histopathology are highlighted with diagnoses of adipose (A), fibroglandular (FG), ductal carcinoma in situ (DCIS). B) Absorption (\(\mu_\alpha\)) and scattering (\(\mu_s'\)) spectra for representative adipose (A), fibroadipose (FA), fibroglandular (FG), and malignant (M) sites. C) Representative H&E images of a fibroadipose and DCIS site. D) Empirical cumulative distribution functions (eCDFs) of the site-level data for A, FA, FG, and M sites. E) Scatterplots of the wavelength-averaged reduced scattering coefficient (<\(\mu_s'\)> versus the percent of glands to the percent of collagen in benign sites containing glands.

Figure 24: A-B) eCDFs of all measured sites from negative and positive, neoadjuvant naïve margins, separated by radiographic breast density. The p-values indicated correspond to modified Kolmogorov-Smirnov tests between: 1) negative versus positive margins in low density patients and 2) negative versus positive margins in high density patients. Cartoon showing \(\beta\)-carotene contrast between tumor and the surrounding adipocytes; little contrast is observed when adipocytes are large, more contrast is observed when adipocytes are smaller. C) Delta eCDFs between the positive and negative margins for both low and high density patients. Negative values indicate the negative distribution has higher values.

Figure 25: Conditional inference tree predictive model for the 88 margin (70 patient) dataset, with mammographic breast density as the first decision node. The bar plots at each terminal node indicate the relative fraction of \(n\) margins classified into that node that are negative or positive.

Figure 26: eCDFs for samples classified into each terminal node of the MBD-specific CIT model, separated by the corresponding decision node listed in each figure panel. In B and C, the <\(\mu_s'\)>CDFs for the margin samples classified into those nodes are shown along with the eCDF for all pixels from all positive margin samples in the study, which served as a reference distribution for two-sided Kolmogorov-Smirnov testing. Red curves indicate distributions composed of majority positive margin samples, and blue curves indicate distributions of majority negative margin samples.

Figure 27: A) Cross-sectional view of a mastectomy sliced open to reveal solid tumor. Two channels of the 8CH-1 probe were placed on the tissue for measuring kinetics in mastectomy specimens (\(n=12\)). Measured sites were inked for histopathology. B)
Lumpectomy specimens (n=10) were placed in a plexi-glass box and interfaced with the 8CH probe, secured in an aluminum adaptor, via the holes in the box. 116

Figure 28: A) Histogram of the extracted [Lymphazurin™] for the site-level data (854 sites). Maximum extracted [Lymphazurin™] was 72.7 µM. B) Extracted absorption coefficient spectra for 3 different fat sites with varying [Lymphazurin™]. C) Extinction coefficients for oxy-hemoglobin (HbO2), deoxy-hemoglobin (HbH), and β-carotene measured by Prahl [117]; and Lymphazurin™ measured by our group. 120

Figure 29: Example data acquired from 2 lumpectomy margins in our previous study [115]. A) 50x bicubic interpolated images of β-carotene/µs from a negative margin and a positive margin. Benign (fat and fibro-adipose tissue) and malignant (ductal carcinoma in situ – DCIS) sites are highlighted. B) Images of THb/µs with the same sites highlighted. C) Cumulative distribution functions of the pixels in the negative (N) and positive (P) margins. 125

Figure 30: Example plots of the tissue parameters versus time for three histologically known sites (adipose-A, fibroadipose-FA, fibroglandular-FG) from three different lumpectomy specimens. Symbols are the measured data and lines are the fitted data. 127

Figure 31: Percent change in each tissue parameter versus time from excision. Percent change is calculated as the fitted rate of change divided by the absolute value of the fitted intercept, multiplied by time from excision. Data is from histologically-confirmed lumpectomy sites (not all outliers are shown). The median percent change at 30 minutes is shown for each parameter. 129

Figure 32: Average percent errors for Monte Carlo simulated data and phantom data. Data is shown for a single reference “phantom” (µs=3.85cm⁻¹, µa=6.79cm⁻¹ for the simulated data; µs=3.02cm⁻¹, µa=5.81cm⁻¹ for the phantom data). Simulated: 216 diffuse reflectance spectra were created consisting of 3 levels of scattering (4.85, 6.68, 9.15 cm⁻¹), THb (16.97, 31.03, 55.09 µM), and β-carotene (10.29, 16.29, 24.37 µM) and 8 levels of Lymphazurin™ (0:10:70 µM). Phantom: Lymphazurin™ was titrated 12 times (0-79µM) into a phantom consisting of 5.81cm⁻¹ (µa), 16.69 µM (THb), and 11.23µM (β-carotene). 131

Figure 33: Optical parameters of the first time point from the histologically-confirmed benign sites of mastectomies (M) and lumpectomies (L). Statistical significance (*) indicates p<0.05) calculated with a Wilcoxon rank-sum test. 132
Figure 34: Rate of change (fitted values from the model) in the tissue parameters from the histologically-confirmed benign sites of mastectomies (M) and lumpectomies (L) constrained to a time window of 10 min for all sites. Statistical significance (* indicates p<0.05) calculated with a Wilcoxon rank-sum test.................................................. 133

Figure 35: Example plots of the tissue parameters versus time for four histologically known sites from two mastectomy patients. Symbols indicate the measured data, lines are the model fits for the benign and malignant tissues............................................. 134

Figure 36: 50x bicubic interpolated images of a negative and positive margin from different patients, where the “initial” images of the margins were imaged at approximately the same time points post-excision. The “initial” images represent the actual data measured. The median percent change at 10, 20, and 30 minutes was applied to either the negative or positive image to show how an image would change if measured at various time points beyond the “initial” image. A) For β-carotene, THb, and THb/<µ¿>, the negative margins have higher values and the kinetics decrease over time. Therefore, the percent change is applied to the negative margin to show decreasing contrast (worst case scenario). B) For β-carotene/<µ¿> the negative margins have higher values and the kinetics increase over time. C) For <µ¿> the positive margins have higher values but the kinetics decrease over time.................................... 138
Symbols/Abbreviations

ACPPs - activatable cell-penetrating peptides
BMI – body mass index
CAA – cancer-associated adipocytes
CCD – charge coupled device
Cr – crocin
DCIS – ductal carcinoma in situ
DR – diffuse reflectance
DUMC – Duke University Medical Center
eCDF – empirical cumulative distribution function
ECM – extracellular matrix
Hb – hemoglobin
HbH – deoxy-hemoglobin
HbO2 – oxy-hemoglobin
HbSat – hemoglobin saturation
H&E – hematoxylin and eosin
HRME – high resolution microendoscope
IDC – invasive ductal carcinoma
ILC – invasive lobular carcinoma
LCIS – lobular carcinoma in situ
LOX - lysyl oxidase

LZ - Lymphazurin™

MBD – mammographic breast density

n – sample size

n – index of refraction

NA – numerical aperture

Q-DRI – Quantitative diffuse reflectance imaging

R - reflectance

SNR – signal to noise ratio

THb – total hemoglobin

UV – ultra-violet

VIS – visible

λ - wavelength

μ – absorption coefficient

μs – scattering coefficient

μ′ – reduced scattering coefficient

<μ> - wavelength averaged absorption coefficient

<μ′> - wavelength averaged reduced scattering coefficient

2-NBDG - 2-[N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino]-2-deoxy-D-glucose

8CH-1 – the first 8-channel instrument used to collect phantom and clinical data
8CH-2 – the second 8-channel instrument used to collect clinical data

[] - concentration
Acknowledgements

I’d first like to thank my family and friends for always believing in me and supporting me throughout my time in grad school. I’d also like to thank my advisor, Nimmi Ramanujam, for her scientific guidance, for always pushing me to ask more questions, and not letting me give up when the science didn’t make sense. And to Quincy Brown for being an incredible mentor who was always there to bounce ideas off of and provide advice when needed. To the rest of the TOpS lab, thank you for all your help with running experiments, analyzing data, and making the lab an exciting place to work. I’d also like to thank our collaborators and my committee members, Joseph G eradts, Lee Wilke, and Bill Barry, for their scientific input and for all the time I’ve spent with them learning about medicine and statistics. My other committee members, Tuan Vo-Dinh and Kathy Nightingale, I’m grateful for your unbiased perspectives of the research. This work couldn’t have been completed without the help of many other people, including the Surgical Pathology staff, the Ambulatory Surgery Center nurses, and all the other breast surgeons at Duke. Most importantly, I’d like to thank all of our patients who consented to research; without you, none of this would have been possible.
1 Background and Significance

1.1 Normal Breast Anatomy

The benign breast is composed of a specialized epithelium and stroma. The epithelium is organized into ducts that originate at the nipple; branching of the large ducts leads to a terminal duct lobular unit which then branches into a cluster of small acini to form a lobule (Figure 1). The stroma, interspersed with the ducts and lobules, consists of dense fibrous tissue (made up of collagen) and adipose tissue (or adipocytes) [13]. The composition of the human breast is highly variable from person to person; although the organ is composed of the same tissue types, the specific arrangements and relative contributions of these tissue types are patient specific.

Figure 1: Anatomy of the benign breast [14] showing the network of ducts surrounded by fatty tissue and lymph nodes.
1.2 Optical Sources of Contrast in the Breast

Optical imaging of tissue is attractive because it is relatively fast and non-destructive to the tissue. Optical techniques can also measure tissue composition endogenously in a variety of ways and do not necessarily require contrast agents. These optical techniques utilize information about how light is scattered, absorbed, or fluoresces in the tissue to determine tissue composition. For example, blood and fat both absorb light, while cells and collagen scatter light; and certain molecules such as NADH or FAD fluoresce when illuminated with particular wavelengths of light. Table 1 provides a breakdown of the different optical tools that have been used to specifically measure breast composition; this table also provides the measurement parameter (i.e. the source of contrast for differentiating tissues). Although this is not an exhaustive list, this table shows the main technologies that have been explored to date. What is important to note in this table is that the main sources of contrast for all of these technologies are related to the measurement of 1) blood which comes from the vasculature in the breast, 2) fat, 3) collagen, and 4) cells (i.e. epithelial cells that line the ducts of the breast).
Table 1: Optical sources of contrast in breast tissue. DRS = diffuse reflectance spectroscopy; ESS = elastic-scattering spectroscopy; NIR = near-infrared; FL = fluorescence; SPX = spectroscopy; OCT = optical coherence tomography.

<table>
<thead>
<tr>
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<td></td>
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1.3 The Benign Breast and How It Changes Throughout a Woman’s Lifetime

It is well known that the composition of breast tissue can change significantly within a woman as a function of factors such as menstrual cycle stage, pregnancy, lactation, menopausal status, body weight, and age. For example, at the beginning of the menstrual cycle the lobules are relatively quiescent. At puberty and with the onset of estrogen secretion the lobules grow and the amount of adipose tissue increases. After ovulation, cell proliferation increases, as does the number of acini per lobule, and the stroma becomes edematous. With menstruation the epithelial cells undergo apoptosis, stromal edema disappears, and the size of the lobules decreases. At the onset of
pregnancy, lobules increase in size and number which alters the ratio of stroma to epithelium. By the end of pregnancy the breast consists almost entirely of lobules separated by a small amount of stroma. After birth the breast produces colostrum and then milk. Once lactation has stopped, the lobules regress and atrophy, however, the breast never returns to its pre-pregnancy state. There is a permanent increase in the number and size of lobules. Finally, as women age, the lobules and stroma start to involute and the fibrous stroma is replaced by adipose tissue. [13, 34]

Adipose tissue is a major storage site for both lipids, retinol and carotenoids, with the main carotenoids being β-carotene and lycopene. It is believed that more than 80% of β-carotene reserves are found in adipocytes [35]. Under normal conditions β-carotene and retinol are absorbed through the intestines and circulate in the blood before being transported to the liver and adipocytes [35-37]. Inside the adipocyte, β-carotene is converted into retinaldehyde via central cleavage by the enzyme β-carotene monooxygenase type I (Bcmo1) [38]. Retinol, a byproduct of β-carotene is also converted to retinaldehyde with aldehyde dehydrogenase [38]. Retinaldehyde can then be converted into retinoic acid with retinaldehyde dehydrogenase [38]. This β-carotene/retinoic acid pathway is important in normal adipocyte differentiation and has also been shown to play an important role in modulating adipocyte size [38, 39].
1.4 Effects of Patient Characteristics on the Optical Signatures of Benign Breast Tissue

As was just discussed, it is well known that the composition of the human breast is highly variable from person to person; although the organ is composed of the same tissue types, the specific arrangements and relative contributions of these tissue types are patient specific. In order to identify optical parameters which may be robust predictors of cancer in patients of varying demographics, it is important to understand how these optical parameters are modulated by normal changes in breast composition. Several groups have looked at the effects of patient characteristics on the optical data of breast tissue measured from large volumes, predominantly within the near-infrared (NIR) region. A summary of these findings can be seen in Table 2.

Table 2: Reported trends in optical properties versus patient characteristics. All arrows indicate the direction of change in the optical property with an increase in the patient characteristic. For example, hemoglobin concentration decreases with increasing BMI. θ = no significant finding, StO$_2$ = oxygen saturation, a = scatter amplitude, b = scatter power.

<table>
<thead>
<tr>
<th></th>
<th>[Hb]</th>
<th>Blood Volume</th>
<th>StO$_2$</th>
<th>$\mu_s'$</th>
<th>a</th>
<th>b</th>
<th>Lipid Content</th>
<th>Water Content</th>
</tr>
</thead>
<tbody>
<tr>
<td>BMI</td>
<td>↓[40-43]</td>
<td>↓[44]</td>
<td>$\theta$[41]</td>
<td>↓[42, 44]</td>
<td>↓[43]</td>
<td>↓[43, 45]</td>
<td>↑[41, 45]</td>
<td>↓[41]</td>
</tr>
<tr>
<td>Breast Thickness</td>
<td>↓[41, 43]</td>
<td>↑[41]</td>
<td>↓[43]</td>
<td>↓[43]</td>
<td>↑[41]</td>
<td>↓[41]</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Breast Diameter</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Age</td>
<td>↑[41, 46]</td>
<td>$\theta$[40, 41]</td>
<td>↓[47]</td>
<td>↓[40, 43, 46]</td>
<td>↑[41]</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Menopausal Status (Pre→Post)</td>
<td>↑[24, 46, 47]</td>
<td>↑[24, 46]</td>
<td>↓[47]</td>
<td>↓[46]</td>
<td>↑[46]</td>
<td>↓[46, 47]</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Breast Density (low → high)</td>
<td>↑[42, 48]</td>
<td>↑[48]</td>
<td>↑[40, 48]</td>
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</tr>
</tbody>
</table>
These studies have shown that optical technologies are capable of sensing the morphologic changes associated with age, BMI, menopausal status, breast size, and breast density and that the trends are in the biologically expected direction. However, few groups have quantified how these changes in the normal tissue affect the contrast observed between the benign and malignant portions of the breast. Pogue et al [48] did not quantify how the variations in the normal tissue affected contrast between benign and malignant areas, however, it was noted that to compare absolute values among patients would not be possible due to the large variations in the normal tissue. In order to interpret the changes in the NIR parameters the values must be compared relative to the background properties. In the preliminary work by Intes et al [80] ratios were calculated for all of their optical variables between a suspicious region of interest and the background tissue. To our knowledge they are the only group that has normalized the optical data to the background tissue before observing differences between benign and malignant regions.

1.5 **Breast Cancer and the Tumor Micro-environment**

2011 statistics from the American Cancer Society estimate that there are 230,480 new cases of invasive breast cancer, 57,650 *in situ* cases, and 39,520 deaths from the disease in U.S. women [49]. Worldwide these numbers increase to approximately 1.3 million new cases of breast cancer each year and 464,854 deaths [50].
The majority of breast cancers originate out of the epithelial cells of the ducts or lobules and can be either in situ or invasive. In situ carcinomas are considered a pre-cancer as they are confined to the ducts or lobules and have not yet invaded into the surrounding stroma. In situ carcinomas are divided into two main categories, lobular carcinoma in situ (LCIS) and ductal carcinoma in situ (DCIS). DCIS is the most common form of in situ carcinoma where about 1 in 5 new breast cancers will be DCIS. Like in situ carcinomas, invasive carcinomas are also classified by their growth patterns. Invasive ductal carcinoma (IDC) is the most common infiltrating carcinoma and accounts for approximately 75% of the infiltrating carcinomas diagnosed in the United States. IDC starts in the ducts and then breaks through the ductal wall and grows into the stroma of the breast where it may then metastasize to other areas of the body through the lymphatic and vascular systems. Invasive lobular carcinoma (ILC) is another common infiltrating carcinoma that can also metastasize [51, 52].

Recently there has been a large focus on the tumor micro-environment and its role in breast cancer progression. The most recent research [53-55] has been focused on cancer associated adipocytes (CAA’s) and suggests that crosstalk between tumor cells and nearby adipocytes leads to a reduction in adipocyte size via lipolysis and a high fibroblast-like cell to adipocyte ratio in the stroma surrounding cancer cells. In these studies, CAA’s also showed decreased levels of PPAR-γ, aP2, C/EBPα, resistin, and hormone-sensitive lipase, all of which are important in the normal differentiation and
regulation of adipocytes. Increased levels of inflammatory markers, such as IL-6, were also seen in CAA’s co-cultured with cancer cells; increased IL-6 has been correlated with poor disease outcome and supports tumor growth and metastasis. These adipocytes essentially dedifferentiate and signal pathways that are crucial for cancer cell survival and growth.

Other research [56-58] has focused on the extracellular matrix (ECM) and its role in promoting tumor growth. Specifically, the way in which collagen organizes itself around the tumor can promote tumor invasion. Increased lysyl oxidase (LOX), an enzyme that cross-links collagen, essentially linearizes collagen to the tumor promoting the tumor cells to migrate. Stiffness of the collagen matrix may also prompt tumor growth by altering the integrins and their adhesion interactions allowing tumor cells to migrate easier [59]. These studies suggest that the surrounding micro-environment plays a significant role in tumor invasion and that differences in the “normal” tissue from patient to patient may impact the tumor micro-environment which in turn could influence the tumor’s propensity for growth.

1.6 Risk Factors for Breast Cancer

There are a number of factors associated with increased breast cancer risk. Age, a genetic predisposition (including personal and family history), race and ethnicity, and some lifestyle factors all contribute to breast cancer risk. Older women are at higher risk of developing breast cancer; 1 out of every 8 invasive breast cancers are found in women
under the age of 45, while 2 out of 3 invasive breast cancers are found in women over age 55. It is believed that roughly 5-10% of breast cancers are hereditary and related to mutations in the BRCA1 and BRCA2 genes, which are responsible for producing proteins that keep cells from growing abnormally. Overall, white women are at higher risk of developing breast cancer, however, African-American women are more likely to die of the disease. In women under the age of 45, breast cancer is more likely to be seen in African-American women. In terms of lifestyle factors, the following are thought to increase breast cancer risk: nulli-parous women or those who had their first child after the age of 30, hormone replacement therapy, alcohol, and obesity. [60]

A number of epidemiological studies have looked at breast cancer risk and dietary intake of carotenoids, such as β-carotene. The majority of these studies have been done by measuring serum concentrations of the carotenoids which may be more reflective of a persons’ current dietary status, whereas tissue concentrations of the carotenoids may be more reflective of long-term dietary status. Yeum et al [36] found that breast tissue adipose concentrations of 9-cis β-carotene were significantly higher in breast cancer patients compared to benign breast tumor patients. Palan et al [61] found increased β-carotene levels in grossly malignant biopsies when compared to grossly benign breast tissue. And Lunetta et al [35] found that β-carotene concentrations in the adipose tissue of cancer patients were higher than in the adipose tissue of non-cancer patients. These three studies all measured tissue concentrations of β-carotene and report
similar findings that β-carotene is higher in cancer patients. Interestingly, in a review article [62] of other epidemiological studies of β-carotene and breast cancer risk, negative associations were found in 16 out of 42 studies, and no positive associations were found. Although, more recent reports point out the issues with epidemiological studies and suggest that there is no association with breast cancer risk and β-carotene intake/storage [63, 64]. Taken all together, this may indicate that serum levels of β-carotene may not be associated with breast cancer risk, however, tissue levels of β-carotene may be more important in breast cancer risk.

1.7 **Mammographic Breast Density**

The relative contributions of the predominant tissue types within the breast (i.e. adipose, collagen, and glands) are constantly changing throughout a woman’s lifetime. One way of measuring the relative contributions is to determine the density of the breast with a mammogram. Mammograms are a recommended screening tool for women over the age of 40 to monitor normal and abnormal changes in the breast [65]. Radiographic breast density is defined as the percentage of the image with light-gray/white space (fibrous/glandular tissue) to the percentage of the image with dark-gray/black space (fatty tissue) [66]. Although a percentage could theoretically be calculated, radiographic breast density is typically given a semi-quantitative ordinal value. The BI-RADS scale is a 4-point scale where a breast density of 1 is almost entirely fatty, 2 is scattered fibroglandular, 3 is heterogeneously dense, and 4 is extremely dense.
Women with very fatty breasts therefore are considered to have low-density breast tissue while women with more fibrous/glandular tissue are considered to have high-density breast tissue. Given these associations, as a woman ages and the breasts become fattier, her breast density will decrease. [60]

Breast density is also considered to be a risk factor for developing breast cancer and the BI-RAD score can be used to define a woman’s relative risk. Although it is unknown why, women with denser breast tissue are at an increased risk of developing breast cancer [67, 68]. Unfortunately, this is a problem with mammographic screening because the denser tissue makes spotting abnormalities more difficult for radiologists since both cancer and fibroglandular tissue appear more white/light-gray on the images [60, 67].

Knowledge of the underlying biological mechanisms of breast density and breast cancer risk are severely understudied but recent work is beginning to shed light on this link, including the research on collagen organization and stiffness of the ECM [68]. Provenzano et al [57, 68, 69] found that in a mouse model bread to have more collagen in the mammary gland (associated with increase breast density), that increased stromal collagen led to increased tumor formation and lung metastases by facilitating local invasion through organization of the collagen fibers. In addition, stiffness of the ECM has been shown to increase formation of abnormal cells [68]. High levels of LOX have been shown to increase collagen cross-linking, thus making tissue stiffer, and it is
believed that this cross-linking of the collagen fibers is the link between breast density and breast cancer risk [68].

To our knowledge, only two studies have been completed that looked at breast density, serum levels of β-carotene, and breast cancer risk. The first epidemiological study by Vachon et al [70] found no associations between increasing breast density and carotene intake. The other study by Tamimi et al [71] found that women in the highest quintile of total circulating carotenoids had 4.1 percentage points greater mammographic breast density compared to those in the lowest quintile (p=0.02). Although not significantly greater, this trend was also observed when just looking at β-carotene concentrations. This study also found a 40% reduction in breast cancer risk with β-carotene suggesting that there may be some relationship between β-carotene, breast density, and cancer risk.

1.8 Management of Breast Cancer

Careful monitoring of breast tissue is important for early detection of breast cancers. Women in their 20’s and 30’s are advised to have regular clinical breast exams and can also do monthly self-exams. Starting at the age of 40, women are recommended to have a screening mammogram once a year to monitor changes in breast composition. Women at high risk of developing breast cancer are advised to get an MRI in addition to their yearly mammogram. On a mammogram, radiologists look for calcifications that may or may not be associated with cancer, cysts, and masses (cancerous and non-
Mammograms can be compared with earlier images to monitor changes in the breast composition. If a suspicious mass is identified, the tissue can be biopsied to determine if it is cancerous or not. [72]

Treatment options for breast cancer include chemotherapy, endocrine therapy, radiation therapy, and surgery for the removal of the cancerous lesion(s). Almost all patients will have to undergo either mastectomy or lumpectomy (partial mastectomy) in order to remove the lesion. Mastectomy involves the removal of the entire breast, while a lumpectomy is the surgical removal of the tumor only and is also called breast conserving surgery (BCS). Radiation therapy is a type of treatment where high-energy rays destroy cancer cells and is typically given after BCS. Endocrine and chemotherapy treatments can either be given prior to surgery (referred to as neoadjuvant therapy) to help shrink the tumor or after surgery (adjuvant therapy) to help kill any cancer cells that may have been left behind. [60]

1.9 Breast Conserving Surgery (BCS)

Breast conserving surgery (BCS) is a recommended treatment for early-stage breast cancer and for breast cancers that have been reduced in size by neoadjuvant therapy. In BCS (also known as a partial mastectomy or lumpectomy), the surgeon attempts to excise the tumor along with a margin of normal tissue, while preserving as much of the normal breast tissue as possible. Approximately 60-75% of patients are eligible for breast conserving therapy each year and as many as 17.7-72% of patients
undergoing BCS require repeat surgeries due to a close or positive surgical margin diagnosed post-operatively [3-11, 73].

Histopathology is the current gold standard for determining surgical margin status. At Duke University Medical Center, the standard of care is to grossly section the specimen into 3 mm slices perpendicular to the long axis of the specimen. The tissue slices are then further sectioned to fit into histological cassettes for automated processing. From each of the paraffin blocks, a 5 µm thick section is taken for staining and histological review. If any of those sections contain cancer within 2 mm of the specimen surface, then the entire margin is deemed “close” or “positive” for residual cancer, regardless of the location of the residual cancer on the specimen surface. Margin classification is as follows: negative - malignant cells > 2 mm from tissue surface, close - malignant cells ≤ 2 mm from tissue surface, or positive - malignant cells at surface.

The pathologic margin status is an important predictor of local recurrence of an invasive or in situ cancer after BCS [74, 75]. Thus, complete excision of the tumor is essential to reduce the risk of local recurrence [76]. However, there is much debate in the literature over the definition of a close margin with a range of 0mm – 10mm [8, 10, 74, 77-84].

1.10 Current Intra-operative Margin Assessment Techniques

Currently, surgeons generally do not have adequate intra-operative assessment tools to ensure that the cancer has been completely removed at the time of first surgery.
The lack of this capability represents a significant unmet clinical need. Only a small number of hospitals that perform BCS (less than 5%, including the Moffitt Cancer Center in Tampa, FL and the MD Anderson Cancer Center in Houston, TX) currently utilize intra-operative cytologic or pathologic analysis of tumor margins. Touch-preparation (touch-prep) cytology is a technique in which cells on the surface of the tissue are transferred to glass slides by touching the specimen to the glass, and are then stained for pathologic observation. Touch-prep cytology allows for evaluation of the whole lumpectomy surface, albeit with a wide range of sensitivities (38-100%) and specificities (83-100%) reported in the literature [85-92]. Furthermore, this technique is time consuming, requires special expertise by a cytopathologist, and does not detect tumor cells close to the lumpectomy surface. Frozen section analysis, in which the tissue is frozen and select microscopically thin sections are cut from the specimen for pathologic observation, is a technically challenging procedure due to the significant amount of fatty tissue found in breast specimens. Sensitivity ranges in the literature from 59-91% and specificity ranges from 86-100% [88, 93-99]. Table 3 shows a list of reported sensitivities and specificities in the literature for frozen section and touch-prep cytology.
Table 3: Reported sensitivities and specificities for frozen section and touch-prep analysis.

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<th>Author</th>
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<th>Touch-Prep (%)</th>
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<tr>
<td></td>
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1.11 Intra-operative Margin Assessment Device Needs

A fast, non-destructive device that could image breast tumor margins in the operating room would be highly desirable to ensure complete removal of the cancer and thus reduce the risk of local recurrence. The device needs to 1) be capable of surveying multiple margins in an acceptable amount of time (within 20 minutes which is the amount of time for a frozen section) [94], 2) have a sensing depth of 0-2 mm (the accepted criterion for clear margins) [82, 103-106], 3) cover a large area (the majority of margin areas range from ~10-20 cm² in our study), 4) image with a resolution on the order of millimeters (comparable to the thickness of bread loafed slices evaluated by
pathology), and 5) effectively detect differences between benign and malignant tissues and to do this without the need for immediate pathologic evaluation, or tissue processing.

The group that is furthest along clinically with regards to a margin assessment device is an Israeli company, Dune Medical. They have developed a pen-like probe called the MarginProbe, which uses radio waves to measure the electromagnetic properties of breast tissue over a 7 mm diameter area and 1 mm deep volume. A prospective trial with their device was reported to have a sensitivity and specificity of 71% and 68%, respectively and they completed a randomized multi-center clinical trial in the U.S. in late 2009 [107, 108].

### 1.12 Emerging Optical Tools for Breast Margin Assessment

Optical imaging of tumor margins is attractive because it can quickly sample an entire tumor margin intra-operatively without damaging the tissue and optical technologies provide a sensing depth that is acceptable for detecting clear margins (1-2mm). Several groups are working on optical techniques for breast tumor margin assessment. In a preliminary study, Bigio et al used reflectance spectroscopy to look at \textit{in vivo} sites on the tumor bed in 24 patients (13 cancer and 59 normal sites). They showed using hierarchical cluster analysis, that cancer and normal sites could be separated with a sensitivity of 67% and a specificity of 79% [109]. Haka et al recently published on Raman spectroscopy to prospectively examine freshly excised lumpectomy
specimens, which were sliced to expose tumor sites in 21 patients (123 benign and 6 malignant tissue sites) and reported a sensitivity of 83% and a specificity of 93% [28]. Their previous retrospective study showed 94% sensitivity and 96% specificity for ex vivo measurements of frozen samples [94]. Volynskaya et al demonstrated the ability of diffuse reflectance spectroscopy and intrinsic fluorescence spectroscopy to differentiate various benign and malignant tissues in breast biopsies from 17 patients (95 benign and 9 malignant sites), resulting in a sensitivity of 100% and a specificity of 96% [95]. Nguyen et al demonstrated that optical coherence tomography can detect tumor margin positivity in 20 patients (9 positive and 11 negative margins) with a sensitivity and specificity of 100% and 82%, respectively [31]. Keller et al recently published work on diffuse reflectance and fluorescence spectroscopy to detect cancerous sites on breast tumor margins in 32 patients (145 normal and 34 individual tumor sites), and reported a sensitivity and specificity of 85% and 96%, respectively, for classifying individual sites [22]. Nachabe et al [110] used diffuse reflectance spectroscopy to acquire spectra from 102 ex vivo samples that consisted of adipose, glandular, fibroadenoma, invasive carcinoma, and DCIS. Using a K-nearest neighbor algorithm, malignant and non-malignant samples were separated with a sensitivity of 94±4% and a specificity of 98±2%.

These works support the fact that optical techniques can be used to differentiate benign from malignant breast tissue. However, it is important to note that all of the
techniques described above used a point based approach and cannot cover full tumor margins, especially ones that are as large as 10-20 cm². In addition, the study by Nguyen et al [31] was the only one that measured all tissue sites on the margin surface and did not bread-loaf their specimens prior to measuring the tissue.

1.13 Diffuse Reflectance Spectral Imaging

Our group has developed a first-generation optical spectral imaging platform that operates in the visible spectral range (450-600 nm) to rapidly and non-destructively create molecular composition maps of the tumor margin. The optical spectral imaging platform is based on diffuse reflectance spectroscopy. In diffuse reflectance spectroscopy a light source illuminates the tissue, usually through a fiber optic probe, and the remitted light is collected as a function of wavelength. The magnitude and shape of the spectrum is reflective of the absorption and scattering properties of the tissue. Our group has also developed a fast, scalable Monte Carlo model [111, 112] to reliably and quantitatively determine the wavelength dependent absorption and reduced scattering coefficients of the tissue (µa and µ' respectively) from the diffuse reflectance spectra measured with the optical spectral imaging system. The concentration of the absorbers can be easily derived from the absorption coefficient spectra using the Beer-Lambert equation (Equation 1), where ε is the extinction coefficient, A is the absorber concentration, and l is the path length.
\[ \mu_a(\lambda) = 2.303 \varepsilon(\lambda) A l \]

**Equation 1**: Beer-Lambert equation, \( \varepsilon \) – extinction coefficient, A – absorber concentration, l – path length, \( \mu_a \) – absorption coefficient, and \( \lambda \) – wavelength.

The primary absorbers in the breast over the visible spectral range are oxygenated hemoglobin, deoxygenated hemoglobin, \( \beta \)-carotene, and Lymphazurin™ (a blue dye used in sentinel lymph node mapping procedures). The molar extinction coefficients of these absorbers are shown in Figure 2. The primary scatterers reflected by the scattering coefficient are cells, sub-cellular organelles, and collagen.

![Figure 2](image.png)

**Figure 2**: Molar extinction coefficients of the 4 primary breast absorbers over the visible wavelength range; deoxygenated hemoglobin (HbH), oxygenated hemoglobin (HbO2), \( \beta \)-carotene, and Lymphazurin™. [113]

**1.14 Monte Carlo Model [4, 5, 100]**

In order to convert a measured diffuse reflectance spectrum into concentrations of the breast absorbers and scattering, an inverse Monte Carlo model developed previously by our group was utilized. This Monte Carlo model was based off of an earlier model of light transport in multi-layered tissues described by Wang et al [114].
Figure 3 helps to describe how this model works. The adjustable, or free, parameters in the model are the absorber concentrations, scatterer size, and scatterer density. The fixed parameters include the extinction coefficients ($\epsilon$) of the absorbers and the refractive indices ($n$) of the medium and scatterer. The diffuse reflectance spectrum is a function of the wavelength-dependent absorption and scattering coefficients. The absorption coefficient ($\mu_a(\lambda)$) is calculated, using Equation 1, from the concentrations of the various absorbers ($A$) and their extinction coefficients. The scattering coefficient ($\mu_s(\lambda)$) is given by Mie theory for a specific scatterer size and scatterer density. $\mu_a(\lambda)$ and $\mu_s(\lambda)$ are then used to create a diffuse reflectance spectrum ($R(\lambda)$) by modeling light transport through tissue.

![Block diagram of the inverse Monte Carlo developed by our group [4, 5, 100]. A – absorber concentration, $\mu_a(\lambda)$ - the wavelength-dependent absorption coefficient, $\mu_s(\lambda)$ - the wavelength-dependent scattering coefficient, $R(\lambda)$ - reflectance.](image-url)

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21
In a simplified manner, photon propagation is modeled as follows [114]:

- Step 1: A photon is launched into tissue with an initial weight of 1
- Step 2: A random number is drawn to determine how far the photon will travel for one path-length
- Step 3: The weight of the photon is adjusted by how likely the photon is to be absorbed
  \[ \text{weight}_{\text{new}} = \text{weight}_{\text{old}} \times \frac{\mu_a}{\mu_a + \mu_s} \]
- Step 4: A 0 or 1 is randomly drawn to determine if the photon will scatter
  - If a 0 is drawn, the photon will be absorbed and this process stops
  - If a 1 is drawn, the photon scatters and the direction of deflection is randomly sampled
- Step 5: If the weight of the photon is below a specified threshold, the photon is given a chance of surviving
  - If the photon survives or the weight is above the threshold, this process is repeated starting at Step 2

Millions of photons are typically launched and tracked. The path of each photon is recorded and the photons that exit tissue near a collection fiber of a fiber optic probe will make up the modeled diffuse reflectance spectrum. This process is very computationally intensive. Therefore, to speed up this process, the diffuse reflectance spectrum from a baseline Monte Carlo simulation for a given set of absorption and
scattering coefficients is scaled to predict the diffuse reflectance for any combination of absorption and scattering coefficients which are then compiled into a look-up table.

In order to correct for system throughput and wavelength dependence, the measured diffuse reflectance spectrum is calibrated to the diffuse reflectance spectrum from a reference phantom which has known optical properties. The reference phantom allows the measured diffuse reflectance spectrum to be put on the same scale as the modeled diffuse reflectance spectrum so the two can be compared. Day-to-day variations in optical throughput are accounted for by dividing the measured diffuse reflectance by a diffuse reflectance standard measurement. The inverse Monte Carlo model then works in an iterative manner. An initial guess is made for the free parameters to obtain estimates for the absorption and scattering coefficients and a modeled diffuse reflectance spectrum is created from the look-up table. The modeled spectrum is compared to the measured spectrum and the error is calculated as the sum across wavelengths of the squared difference between the measured and modeled spectra. The free parameters are iteratively updated until this error is minimized at which point the concentrations of the absorbers and the scattering coefficient are then extracted.

1.15 Initial Clinical Breast Studies With Diffuse Reflectance Spectral Imaging

Our group completed a clinical study on 120 patients undergoing BCS from 2007 to 2009 using this first generation optical device [3, 101-103]. The goal of this study was
to determine the feasibility of using the device for intra-operative margin assessment and the ability of the device to detect residual cancer at the tumor surgical margin. Diffuse reflectance spectra were collected from up to 128 sites on any given margin (up to 5 margins per patient), inverted with the Monte Carlo model (section 1.14) to extract the concentrations of the various breast absorbers (section 1.13) and scattering information, and converted into tissue composition maps (or images). Wilke et al [115] reported on an initial subset of patients (n = 48) where parameters involving the ratio of \([\beta\text{-carotene}]\) to scattering and total hemoglobin concentration ([THb]) to scattering were able to detect margin positivity with a sensitivity of 79.4% and a specificity of 66.7%; these numbers were based on a leave-one-out cross-validated tree-based model. The close/positive margins included several types of malignancies, mainly ductal carcinoma \textit{in situ} and invasive ductal carcinoma but also lobular cancer, lobular carcinoma \textit{in situ}, and tubular cancer. This initial study showed that the sensitivity of the technology is comparable (Table 3) to currently available intra-operative margin assessment tools such as frozen section but has the benefits of not requiring any type of tissue cutting, preparation, or a pathologist in the operating room.

In another publication by our multi-disciplinary group [25], individual sites (referred to as site-level analysis) from lumpectomy specimens were separated by different categories of normal (adipose, fibroglandular - FG, and fibroadipose - FA) and malignant (invasive ductal carcinoma - IDC and ductal carcinoma \textit{in situ} - DCIS) tissues,
as well as by depth of disease from the margin (0mm, 0-1mm, 1-2mm). The results showed that the single site optical signatures of \textit{ex vivo} breast tissue were affected by the variations in normal and malignant tissue types. Within normal sites, FG tissues showed increased scattering while adipose tissues showed increased [\(\beta\)-carotene]. Scattering differentiated adipose sites from IDC and DCIS, [\(\beta\)-carotene] showed marginal differences between FG and DCIS, and [THb] separated malignant sites at the margin from FG, FA, and adipose sites. These results were consistent with the earlier work by Zhu et al [73, 104]. The results also showed that, as expected, pre-menopausal women had a higher percentage of fibrous sites while post-menopausal women had a higher percentage of adipose sites. Our initial 48 patient study [115] utilized \([\beta\text{-carotene}}]/\langle\mu_s'\rangle\) and \([\text{THb}}]/\langle\mu_s'\rangle\) as the primary sources of contrast; however, in regions of fibrous tissue, the \(\langle\mu_s'\rangle\) and \([\beta\text{-carotene}}]\) levels are similar to that of IDC, making it difficult to differentiate IDC from fibrous tissues. Conversely, in regions of adipose tissue, \(\mu_s'\) would be expected to decrease and \([\beta\text{-carotene}}]\) to increase resulting in a greater degree of contrast from IDC. These earlier site-level studies showed that the local micro-environment greatly affects the optical signatures of both benign and malignant sites.

### 1.16 Specific Aims and Organization of Chapters

There is a significant unmet clinical need for intra-operative assessment tools to ensure that all of the cancer has been removed during the first operation. Optical
spectroscopy offers a fast, non-destructive method to probe tissue physiology and morphology from which absorption and scattering information can be quantitatively extracted. In our initial study [115], we showed that diffuse reflectance optical spectral imaging can be used to differentiate negative from close/positive margins with a sensitivity of 79.4% and a specificity of 66.7%. The main goal of this work was to determine the various instrumentation, surgical, excisional, and inter-patient factors that affect the quantitative optical parameters of breast tissue margins. An understanding of these effects can provide insight into areas that can be improved for future generations of diffuse reflectance spectral imaging devices and/or ways to leverage inherent optical contrast between benign and malignant tissue, for the purposes of breast tumor margin assessment. The work in this dissertation tested the following hypotheses:

**Hypothesis 1:** Diffuse reflectance spectral imaging can accurately and reproducibly probe breast tissue within 2mm of the surgical margin.

**Hypothesis 2:** Patient characteristics (such as mammographic breast density) impact the optical properties of benign breast tissue and thus alter the optical contrast between benign and malignant tissue. This optical contrast can be leveraged to predict margin positivity intra-operatively.

**Hypothesis 3:** The use of cauterization to remove the tissue, injection of blue dye for sentinel lymph node mapping, and tissue degradation associated with *ex vivo* analysis are uncontrollable surgical factors that impact optical properties.
The following chapters are organized to address these hypotheses. Chapter 2 describes in detail the instruments and software used to collect data. It also explains how data was collected from ex vivo breast specimens (specifically lumpectomy specimens), the histological co-registration with the optical data, and how the diffuse reflectance data was analyzed. In addition, it describes the characterization of the diffuse reflectance imaging platform for breast tumor margin assessment. Specifically, the accuracy, signal to noise ratio (SNR), cross-talk at the tissue surface, sensing depth, and reproducibility of the system are described.

Inter-patient variability, specifically radiographic breast density, is discussed in Chapter 3 to shed light on how different patient populations impact the ability to diagnose surgical margin status. Data from the negative margins of lumpectomy specimens was used to determine the effect of breast density on the optical contrast between benign and malignant tissue. This chapter also describes the use of conditional inference tree models to diagnose surgical margin status by using the inter-patient variability to leverage the inherent optical contrast.

The effects of surgical and excision factors on the optical data are described in Chapter 4. In this chapter, we discuss the effects of excision time, the presence of Lymphazurin™, and cauterization of the tissue. To determine the effect of Lymphazurin™ usage, simulations and tissue mimicking phantom studies with hemoglobin, crocin (a substitute for β-carotene), polystyrene spheres, and
Lymphazurin™ were completed to determine how accurate the system is at extracting [THb], [β-carotene], and scattering for different concentrations of Lymphazurin™. To determine the effect of tissue kinetics, diffuse reflectance spectra were measured from non-cauterized incised mastectomy and cauterized lumpectomy specimens for as long as possible without interfering with the surgical team. Initial values and rates of change between the two specimen types were used to test for the effect of cautery. The results in this dissertation will ultimately help to create a more robust device and identify areas for improvement for future margin assessment devices.

The majority of the data in this dissertation were acquired from lumpectomy specimens that were imaged with our spectral imaging device over a ~5 year period. The clinical protocol and measurement procedure for these specimens are described in detail in the following chapters. One thing to note is that sample sizes are not identical throughout the chapters due to the analyses being completed at various time points within this ~5 year period. Information learned from the initial 48-patient study [115], the site-level study [25], and the analyses in Chapter 2, influenced which lumpectomy specimens were used for the analyses in Chapter 3. The data in Chapter 4 were acquired from a completely different cohort of patients and were from patients undergoing both lumpectomy and mastectomy procedures. Table 4 provides a breakdown of number of patients used for each type of analysis. The studies listed under “Lumpectomy Margin Imaging” were all from the same cohort of patients.
Table 4: Patient sample sizes for all of the analyses in this dissertation.

<table>
<thead>
<tr>
<th>Study</th>
<th># of Patients</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lumpectomy Margin Imaging</td>
<td></td>
</tr>
<tr>
<td>Size of margin areas</td>
<td>91</td>
</tr>
<tr>
<td>Time from excision to imaging</td>
<td>55</td>
</tr>
<tr>
<td>Initial 48-patient study</td>
<td>48</td>
</tr>
<tr>
<td>Accuracy of system</td>
<td>104</td>
</tr>
<tr>
<td>Sensing depth of sites</td>
<td>99</td>
</tr>
<tr>
<td>Reproducibility</td>
<td>4</td>
</tr>
<tr>
<td>Inter-patient variation</td>
<td>70</td>
</tr>
<tr>
<td>Predicting margin status</td>
<td>70</td>
</tr>
<tr>
<td>Lumpectomy Kinetics/Cautery</td>
<td>10</td>
</tr>
<tr>
<td>Mastectomy Kinetics/Cautery</td>
<td>12</td>
</tr>
</tbody>
</table>
2 Characterization of a Diffuse Reflectance Spectral Imaging Platform for Breast Tumor Margin Assessment

[12]

2.1 Introduction

This chapter describes the characterization of the instrumentation of the diffuse reflectance imaging platform that is used for the majority of the experiments in this dissertation. The first 4 sections describe how optical data is acquired from breast specimens, compared to the gold standard (post-operative histology), and how the optical data is analyzed. The performance metrics of the imaging platform are then discussed in a manner that is relevant to breast tumor margin assessment. Specifically, this chapter quantifies important sources of systematic and random errors that could arise when the system is used in a clinical setting. The endpoints characterized are, the signal to noise ratio (SNR) of the system, the accuracy with which the device characterizes the composition of tumor margins, the sensing depth, the amount of crosstalk between adjacent channels of the probe, and reproducibility. The optical properties of histologically normal and malignant breast tissues obtained from the randomly inked sites (site-level analysis described in Section 2.4.1) served as the basis for characterizing the instrument performance metrics.
2.2 Instrumentation

2.2.1 8 Channel System – Version 1 (8CH-1)

A block diagram of the 8CH-1 clinical instrument is shown in Figure 4 [12]. The instrument consists of a 450 Watt Xenon lamp coupled to a monochromator (Gemini 180 - Jobin Yvon Horiba) set for zero-order diffraction, a multi-channel fiber-optic imaging probe (custom built by RoMack Inc.) interfaced to a plexi-glass tissue specimen box, an imaging spectrograph (Triax 320 – Jobin Yvon Horiba), and a 2D CCD camera (CCD-1024x256-OPEN-STE – Jobin Yvon Horiba). There are 8 channels on the multi-channel probe. Each channel has a core of 19, 200 µm (NA=0.22) illumination fibers surrounded by 4, 200 µm (NA=0.22) collection fibers with source-detector separations spanning 0.23-1.10 mm. The typical power output at the probe tips is ~3 µW and 25 µW within a 10 nm band pass at 450 and 600 nm, respectively. The probe tips of the 8 channels are arranged in a 4x2 array with a 10 mm spacing (center to center) between each channel. The illumination fibers within each channel are continuously illuminated regardless of whether data collection is taking place.
Figure 4: Block diagram of the clinical instrument and the fiber arrangement of the multi-channel probe. Each channel has 4, 200 µm collection fibers and a central bundle of 19, 200 µm illumination fibers. All 8 channels are arranged in a 4x2 array with a separation distance of 10 mm (center to center).

A photo of the instrument along with a computer for instrument control and data analysis is shown in Figure 5A. A tumor specimen can be placed inside the plexi-glass box and interfaced with the fiber optic probe from the side (Figure 5B). Each hole of the plexi-glass box is 5 mm apart (center to center) and has a diameter of 3.75 mm. The imaging probe placement can be shifted by 5 mm to sample inter-leaving holes between the 10 mm channel to channel spacing. The probe covers an area of approximately 2 cm x 4 cm in 4 consecutive placements of the probe. The plexi-glass slides in 1-dimension to conform to different sized specimens.
2.2.2 8 Channel System – Version 2 (8CH-2)

A second 8 channel system was also used to collect clinical data. The 8CH-2 system is nearly identical to the 8CH-1 system, with the individual components of the system replaced with newer versions from the manufacturer. This system consists of a 450 Watt Xenon lamp coupled to a monochromator (Gemini 180 - Jobin Yvon Horiba), a second multi-channel fiber-optic imaging probe with the same fiber geometry (custom built by RoMack Inc.), an imaging spectrograph (iHR 320 – Jobin Yvon Horiba), and a 2D CCD camera (Synapse – Jobin Yvon Horiba). Table 5 shows which instrument was used for the various studies in the following chapters and the type of tissue the instrument measured.
Table 5: Listing of which instruments were used for each study in this dissertation, the type of breast tissue the instrument was used on, and corresponding chapter with the discussion of that study.

<table>
<thead>
<tr>
<th>Instrument</th>
<th>Study</th>
<th>Tissue Type</th>
<th>Corresponding Chapter</th>
</tr>
</thead>
<tbody>
<tr>
<td>8CH-1</td>
<td>Initial 48-patient</td>
<td>Lumpectomies</td>
<td>1, 2</td>
</tr>
<tr>
<td>8CH-1</td>
<td>SNR</td>
<td>n/a</td>
<td>2</td>
</tr>
<tr>
<td>8CH-1</td>
<td>Accuracy</td>
<td>Phantoms</td>
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<tr>
<td>8CH-1</td>
<td>Sensing depth</td>
<td>Simulations,</td>
<td>2</td>
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<tr>
<td></td>
<td></td>
<td>Lumpectomies</td>
<td></td>
</tr>
<tr>
<td>8CH-1</td>
<td>Reproducibility</td>
<td>Lumpectomies</td>
<td>2</td>
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<tr>
<td>8CH-1</td>
<td>Inter-patient variation</td>
<td>Lumpectomies</td>
<td>3</td>
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<tr>
<td>8CH-1</td>
<td>Predicting margin status</td>
<td>Lumpectomies</td>
<td>3</td>
</tr>
<tr>
<td>8CH-1, 8CH-2</td>
<td>Lymphazurin™</td>
<td>Phantoms, Simulations, Mastectomies, Lumpectomies</td>
<td>4</td>
</tr>
<tr>
<td>8CH-1, 8CH-2</td>
<td>Kinetics</td>
<td>Mastectomies,</td>
<td>4</td>
</tr>
<tr>
<td>8CH-1, 8CH-2</td>
<td>Cautery</td>
<td>Mastectomies,</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Lumpectomies</td>
<td></td>
</tr>
</tbody>
</table>

### 2.3 Software

The 8CH system was originally controlled by the Synergy software supplied by Jobin Yvon Horiba. To speed up the data acquisition process, a custom LabVIEW (National Instruments) and MATLAB based program was developed by our lab [116]. The application provided features to control the flow of information from instrument initialization to image display, to provide automated selection of data acquisition parameters, to enable data post-processing and the output of results to the operator.
2.4 Methodology for Imaging Partial Mastectomy Tumor Margins

2.4.1 Clinical Procedure

A Duke Institutional Review Board approved clinical study (protocol ID – Pro00007857 and Pro00017428) to image breast tumor margins was performed on patients undergoing BCS. Partial mastectomy specimens were excised and oriented by the surgeon. For the purposes of specimen orientation, the partial mastectomy specimen was viewed as a cube. The surgeon oriented the specimen by putting sutures and/or surgical clips at the center of 4 of the 6 margins (anterior, posterior, inferior, superior, medial, and lateral). The specimen was then sent to mammography as part of the routine standard of care. After x-ray examination, the specimen was placed in the plexiglass box 16±5 minutes post-excision with the clip or suture at the center of the box face to maintain surgical orientation for each margin of the specimen. The imaging probe was interfaced to the lumpectomy specimen via holes in the plexi-glass box. Diffuse reflectance measurements were collected from sites 5 mm apart until the entire margin had been scanned, taking 8 measurements simultaneously with each placement. The average size of each measured margin was 14.7cm². The histogram in Figure 6 shows the distribution of margin areas imaged.
Figure 6: Distribution of imaged margin sizes from 91 lumpectomy patients.

The CCD integration time was automatically adjusted to maximize the signal’s use of the detector’s dynamic range. For any given patient, between 1 and 5 margins were measured depending on how much time was available without interfering with the surgeon’s time. To increase the yield of positive margins, the surgeon indicated if any margin on the primary specimen was likely to be positive, based on palpation of the specimen and specimen radiography. The first margin imaged, was the one most likely to be positive based on the surgeon’s assessment. If additional time was available, the next most likely margin was imaged, and so forth. After all measurements were completed, the four corners of the measured margin were inked green (light gray dots in Figure 5C) for pathological correlation with the imaged area to obtain a margin-level diagnosis by a board-certified pathologist. This margin-level diagnosis was either: negative, close, or positive. In addition, up to 10 individual sites were inked orange (black-white striped dots in Figure 5C) for separate pathological evaluation for a site-level diagnosis for use in the retrospective characterization of the instrument
performance metrics (Chapter 2), adipocyte analysis (Chapter 3), and kinetics/cautery analysis (Chapter 4). To preserve the inked areas for accurate pathological coregistration, acetic acid or acetone was applied to the specimen after inking and the specimen was wrapped in gauze or paper towel to maintain the integrity of the inked areas.

2.4.2 Pathological Evaluation

Post-operative pathology was used as the gold standard to classify each margin as negative (malignant tissue > 2 mm from tissue surface), close (malignant tissue < 2 mm from tissue surface), or positive (malignant tissue at surface). The pathologic margin status is an important predictor of local recurrence of an invasive or in situ cancer after BCS. From a patient management perspective, both a positive and a close margin require re-excision. This approach is sufficiently effective to ensure complete excision of the positive margin in the second surgery in the majority of cases [41, 58, 105].

After the specimen was marked with ink, it was sent to surgical pathology where it was fixed overnight in 10% buffered formalin and processed in standard fashion. At Duke University Medical Center, the standard of care is to grossly section the specimen into 3 mm slices perpendicular to the long axis of the specimen. The tissue slices are then further sectioned to fit into histological cassettes for automated processing. Then, from each of the paraffin blocks, a 5 µm thick section is taken for staining with
hematoxylin and eosin (H&E) and histological review. If any one of those sections contained cancer within 2 mm of or at the specimen surface, then the entire margin was deemed “close” or “positive” for residual cancer, regardless of the location of the residual cancer on the specimen surface. Therefore, for our purposes, an entire parameter map of the specimen surface was only paired with the overall pathologic diagnosis of that surface (i.e., negative, close, or positive), as no spatial information about the location of the malignancy was available from pathology. For the purposes of our margin imaging study, the close and positive margins are lumped together as “positive” since, clinically, both require re-excision.

In addition, the study pathologist also reviewed the inked sites microscopically to provide a histologic assessment of the underlying tissue composition of the individual sites. A qualitative assessment of the various types of tissues (fat, stroma, benign glandular tissue, carcinoma in situ, invasive carcinoma) was given for each site.

### 2.5 Analyzing the Diffuse Reflectance Data

In the 8CH system, diffuse reflectance spectra were collected for two separate wavelength ranges (381-511 nm and 500-630 nm) and spliced together by averaging an 11 nm overlap to cover the entire 381-630 nm wavelength range. A shorter wavelength range of 450-600 nm was used for data analysis. Diffuse reflectance spectra (450-600 nm) were corrected by CCD integration time and for daily variations in optical throughput using a Spectralon reflectance standard (LabSphere). Examples of the calibrated diffuse
reflectance spectra collected from breast tissues are shown in Figure 7. The spectra from a benign (fat) and malignant (invasive ductal carcinoma – IDC) site are plotted and show clear differences in shape. Collection of throughput-calibrated diffuse reflectance spectra from each site on the surface of each specimen allowed creation of a spectral reflectance cube \( R(x, y, \lambda) \), where \( R \) is calibrated reflectance, \( x \) and \( y \) are spatial coordinates of the tissue surface, and \( \lambda \) is wavelength (Figure 8B).

The inverse Monte Carlo model (section 1.13-1.14), was used to extract the optical properties (absorption and scattering) of the tissues from the diffuse reflectance spectra. The free parameters related to absorption were the concentrations of the intrinsic absorbers in this wavelength range, namely, oxygenated hemoglobin (HbO\(_2\)), deoxygenated hemoglobin (Hb), and \([\beta\text{-carotene}]. \) Lymphazurin\(^{\text{TM}}\) (Tyco Healthcare), a blue dye injected peri-tumorally prior to surgery and used to locate the sentinel node during surgery, was also included as an extrinsic absorber since it was present in many of the samples. The extinction coefficients of oxyhemoglobin, deoxyhemoglobin, and \( \beta \)-carotene were taken from an online spectra database [117], whereas the extinction coefficient of Lymphazurin was measured in our laboratory. A Gaussian-shaped absorber with a center wavelength of 515 nm and a full width half maximum (FWHM) of 10 nm was used as well, to be consistent with our previous methods [21]. For scattering, the fixed parameters were the refractive indices of the scatterers (1.4) and the surrounding medium (1.36), and the anisotropy factor (0.8), whereas the free parameters
were the size and density of the spherical scatterers. The inverse Monte Carlo model used the concentrations of \( n \) assumed absorbers, along with their extinction coefficients, to generate \( \mu_a(\lambda) \) which along with \( \mu'(\lambda) \) gives the modeled reflectance \( R(\lambda) \) at each \((x, y)\) location, which is used in an optimization algorithm as described previously in Section 1.14. Example spectra for a benign and malignant site are shown in Figure 7. For an entire tumor margin, spectral cubes \( \mu(x, y, \lambda) \) and \( \mu'(x, y, \lambda) \) were created from the diffuse reflectance spectral cube (Figure 8C).

![Figure 7: Examples of the diffuse reflectance, absorption and reduced scattering coefficient spectra from a benign site (fat) and a malignant site (IDC – invasive ductal carcinoma).](image)

Using the extracted optical properties (\( \mu_a(\lambda) \) and \( \mu'(\lambda) \)), important tissue parameters including \([\text{THb}]\), \([\beta\text{-carotene}]\) and \(\langle \mu' \rangle\), were calculated for each measured site on the specimen surface (this is described in more detail in section 1.13-1.14). It took approximately 15 seconds to image and 10 seconds to process the data per placement (8 simultaneous measurements) of the probe with the 8CH system. A full list of these extracted parameters can be seen in Table 6.
Table 6: A list of the different extracted parameters used to create the descriptive variables. Mean scattering refers to the wavelength-averaged $\mu'$ from 450-600nm.

<table>
<thead>
<tr>
<th>Extracted Parameters</th>
<th>Units</th>
</tr>
</thead>
<tbody>
<tr>
<td>Oxy-hemoglobin</td>
<td>$\mu$M</td>
</tr>
<tr>
<td>Deoxy-hemoglobin</td>
<td>$\mu$M</td>
</tr>
<tr>
<td>Total hemoglobin (THb)</td>
<td>$\mu$M</td>
</tr>
<tr>
<td>$\beta$-carotene</td>
<td>$\mu$M</td>
</tr>
<tr>
<td>$&lt;\mu',&gt;\text{cm}^{-1}$</td>
<td></td>
</tr>
<tr>
<td>THb/$&lt;\mu',&gt;$</td>
<td>$\mu$M-cm</td>
</tr>
<tr>
<td>$\beta$-carotene/$&lt;\mu',&gt;$</td>
<td>$\mu$M-cm</td>
</tr>
<tr>
<td>Hemoglobin Saturation (HbSat)</td>
<td>a.u.</td>
</tr>
<tr>
<td>Lymphazurin™</td>
<td>$\mu$M</td>
</tr>
</tbody>
</table>

These extracted parameters can be used to create 2-D surface maps $A_o(x,y)$, which are maps of the various absorbers $A$ at each $(x,y)$ location. Likewise, the reduced scattering coefficient cube was reduced to $<\mu'(x,y)$, where $<\mu'>$ is the wavelength-averaged reduced scattering coefficient (Figure 8D). Although all of the various spectral formats for displaying and analyzing the data are potentially useful without any further processing, for instance by using empirical pattern recognition or spectral analysis techniques, in this work we chose to focus on the quantitative 2-D parameter maps generated via use of the Monte Carlo inversion algorithm. This serves to reduce the number of variables which must be considered, but more importantly because of the morphological insight and ease of interpretation that the quantitative parameter maps provide.
Figure 8: A) Photograph of the imaging probe interfaced with mock tissue inside the specimen imaging container, B) Sample hyperspectral reflectance cube from a margin sample positive for residual invasive ductal carcinoma, C) Hyperspectral absorption $\mu_a(x,y,\lambda)$ and reduced scattering coefficient cubes $\mu'_s(x,y,\lambda)$, extracted from the reflectance cube $R(x,y,\lambda)$, D) 2-dimensional $\beta$-carotene concentration map, total hemoglobin (oxy- plus deoxy-hemoglobin) concentration map, and wavelength-averaged reduced scattering coefficient ($<\mu'_s>$) map extracted from the absorption and scattering spectral data cubes.
Figure 9 and Figure 10 show example parameter maps of the extracted parameter $[\beta\text{-carotene}/\mu_s']$ along with histograms of the pixels from each parameter map. Figure 9A shows a pathologically-positive margin while Figure 10A shows a pathologically-negative margin. Visually these two parameter maps are very different; the positive margin contains a greater number of pixels with low values of $[\beta\text{-carotene}/\mu_s']$ while the negative margin has higher values. Different sites are also highlighted on the parameter maps that show the specific type of tissue present at those locations. These individual sites show that the malignant tissue has lower $[\beta\text{-carotene}/\mu_s']$ values than the benign tissue. Looking at histograms, of these margins it is clear that they have very different distributions and are the basis for differentiating negative and close/positive margins in Chapters 3 and 4.

Figure 9: A) Parameter map of the ratio of $[\beta\text{-carotene}/\mu_s']$ in a pathologically positive margin. The black boxes are sites with ductal carcinoma in situ (DCIS) and the dotted gray box is a site with fibro-adipose tissue. B) Histogram of the pixels in the parameter map.
2.6 Signal to Noise Ratio (SNR) of the Imaging Platform

2.6.1 Methods

The signal-to-noise-ratio (SNR) of the system was calculated by taking repeated measurements (n=6) of a Spectralon reflectance standard (LabSphere). Diffuse reflectance spectra were collected for all 8 channels. The signal level was comparable to the signal levels of the diffuse reflectance spectra collected from tissue. Spectra were corrected by integration time. The signal was calculated as the mean of the 6 measurements and noise was calculated as the standard deviation of the measurements. For each of the 8 channels, the SNR was calculated at 450 and 600 nm.

2.6.2 Results

The SNR of the clinical system is seen in Table 3. Average SNR across all channels is >100 at both 450 nm and 600 nm. The large range of SNRs across channels is

Figure 10: A) Parameter map of the ratio of $[\beta$-carotene]/$<\mu'_s>$ in a pathologically negative margin. The dotted gray boxes are sites with adipose tissue. B) Histogram of the pixels in the parameter map.
likely due to varying efficiencies of the CCD where each channel is binned (each channel is binned separately on the CCD with no overlapping pixels).

Table 7: Signal to noise ratio of each channel at 450 and 600 nm.

<table>
<thead>
<tr>
<th>Channel #</th>
<th>SNR @ 450 nm</th>
<th>SNR @ 600 nm</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>340.97</td>
<td>45.12</td>
</tr>
<tr>
<td>2</td>
<td>238.46</td>
<td>44.58</td>
</tr>
<tr>
<td>3</td>
<td>162.81</td>
<td>113.82</td>
</tr>
<tr>
<td>4</td>
<td>292.61</td>
<td>133.56</td>
</tr>
<tr>
<td>5</td>
<td>167.14</td>
<td>97.08</td>
</tr>
<tr>
<td>6</td>
<td>137.08</td>
<td>68.29</td>
</tr>
<tr>
<td>7</td>
<td>131.95</td>
<td>248.83</td>
</tr>
<tr>
<td>8</td>
<td>87.62</td>
<td>85.75</td>
</tr>
<tr>
<td>Mean</td>
<td>194.83</td>
<td>104.63</td>
</tr>
</tbody>
</table>

2.7 Accuracy of the Imaging Platform

2.7.1 Methods

The site-level data and corresponding pathology served as the basis for the retrospective characterization of the 8CH optical spectral imaging device. The site-level data was used in two ways. First, the range of the extracted parameters (\(<\mu'_{s}>, [THb], [\beta-carotene], [THb]/<\mu'_{s}>, [\beta-carotene]/<\mu'_{s}>, \) and [\(\beta\)-carotene]/[THb]) was determined for all measured sites (854 sites from 104 patients). These ranges were used to make tissue mimicking phantoms to retrospectively determine the accuracy of the technology for the specific optical properties seen in partial mastectomy specimens.

The extracted values of [THb], \(<\mu'_{s}>, [\beta-carotene], [THb]/<\mu'_{s}>, [\beta-carotene]/<\mu'_{s}>, \) and [\(\beta\)-carotene]/[THb] from all measured sites (n=854) are shown in the boxplots of
Figure 11. These plots show the empirical distribution of the data for sites on breast tumor margins, regardless of pathology.

![Box and whisker plots](image)

To cover the range of data seen in the boxplots, three different levels were defined as shown in Table 8. These values were used to create the 36 phantoms with varying absorption and scattering properties which mimic the optical properties of the breast tissue. Since β-carotene is not soluble in water, crocin (Cr) was used as a substitute. The extinction coefficients of β-carotene and crocin are not identical; β-carotene is ~11.6 times larger than crocin. Therefore, the absorption coefficients rather than the concentrations of β-carotene and crocin were designed to match in this study. The 3 hemoglobin levels (42.09, 64.22, and 99.81 µM) are in the upper range of the boxplot. Bender et al [118] tested phantoms with hemoglobin concentrations ranging from 1 to 35 µM and showed
that the model can accurately extract absorption and scattering with accuracies of 9.8±8.2% and 7.68±6.3%, respectively [118]. Therefore, in this phantom study we wanted to look at higher concentrations of hemoglobin since previous studies have characterized the accuracy at lower concentrations.

Table 8: Values of the expected optical properties for the phantom study. β-carotene concentrations were converted into Cr concentrations by matching $\mu_s$.

<table>
<thead>
<tr>
<th>$&lt;\mu_s&gt;$ (cm$^{-1}$)</th>
<th>THb (µM)</th>
<th>β-carotene (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>4.85</td>
<td>42.09</td>
<td>0</td>
</tr>
<tr>
<td>6.68</td>
<td>64.22</td>
<td>10.29</td>
</tr>
<tr>
<td>9.15</td>
<td>99.81</td>
<td>16.29</td>
</tr>
</tbody>
</table>

A tissue phantom study, as detailed by Bender et al [118] and Palmer et al [111], was conducted to assess the Monte Carlo model accuracy for extracting $<\mu_s>$ and the concentrations of THb and β-carotene seen in the breast tumor margins. Liquid phantoms consisted of hemoglobin (H0267 Ferrous Hemoglobin, Sigma-Aldrich), 1.025µm diameter polystyrene spheres (Polysciences), and crocin (17304 Standard Fluka, Sigma-Aldrich) diluted with distilled water. A total of 36 phantoms were made that represented 3 scattering levels, 3 THb concentrations at each scattering level; and 4 levels of Cr (one with no Cr) at each THb and scattering level. Diffuse reflectance measurements were made with a single channel of the 8CH probe. The purpose of this experiment was to show that tissue parameters can be extracted with reasonable accuracy for a single site on the tissue, therefore, only a single channel was used to
demonstrate this, since all 8 channels have nearly identical probe geometries. The phantoms were mixed with a stir bar and plate between each measurement to maintain the homogeneity of the phantom. The data was normalized by CCD integration time, calibrated using the Spectralon standard and processed using the inverse Monte Carlo model described in the data analysis section on page 37. The parameters ([THb], <μs>, [Cr], [THb]/<μs>, [Cr]/<μs>, and [Cr]/[THb]) extracted using the inverse Monte Carlo model were compared to expected values of these parameters to assess model accuracy.

2.7.2 Results

The accuracy of the model at extracting Hb concentration (Figure 12A), crocin concentration (Figure 12B), and <μs> (Figure 12C), along with the concentrations divided by <μs> (Figure 12D and Figure 12E), and the ratio of crocin concentration to hemoglobin concentration (Figure 12F) were plotted as expected data versus extracted data. As described by Palmer et al [111], the inverse Monte Carlo model uses a reference phantom to put the experimental and simulated diffuse reflectance spectra on the same scale prior to the extraction of optical properties. The best reference phantom was the one that most accurately extracted all other phantoms across a range of optical properties from the set of 36 phantoms. In all of the plots, each circle corresponds to one phantom inverted against the best reference phantom (μ = 5.25, μs = 5.75) from the set of 36 phantoms. The solid line in each figure shows the line of perfect agreement between the expected and extracted values and the dashed lines represent the 95%
The average percent error and standard deviation for all 36 phantoms are also shown in each plot.

**Figure 12:** Average percent error and standard deviation of expected versus extracted plots of Hb concentration (A), Cr concentration (B), $\mu_s$ (C), the ratio of Hb concentration to $\mu_s$ (D), the ratio of Cr concentration to $\mu_s$ (E), and the ratio of Cr to Hb concentration (F). Each circle refers to one phantom extracted from the best reference phantom. The solid line shows the line of perfect agreement between the expected and extracted values and the dashed lines represent the 95% prediction interval.
Table 9 shows the bias (mean of the difference between expected and extracted data) and precision (standard deviation of the differences) for each parameter. Taking ratios of the parameters improves the precision of the model and also decreases the percent error.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Bias</th>
<th>Precision</th>
</tr>
</thead>
<tbody>
<tr>
<td>(&lt;\mu_a&gt;) (cm(^{-1}))</td>
<td>0.06</td>
<td>0.40</td>
</tr>
<tr>
<td>[Hb] (µM)</td>
<td>0.96</td>
<td>4.33</td>
</tr>
<tr>
<td>[Cr] (µM)</td>
<td>-2.80</td>
<td>37.95</td>
</tr>
<tr>
<td>(&lt;\mu_s'&gt;) (cm(^{-1}))</td>
<td>-0.36</td>
<td>0.71</td>
</tr>
<tr>
<td>[Hb]/(&lt;\mu_s'&gt;) (µM-cm)</td>
<td>0.31</td>
<td>0.75</td>
</tr>
<tr>
<td>[Cr]/(&lt;\mu_s'&gt;) (µM-cm)</td>
<td>1.55</td>
<td>6.26</td>
</tr>
<tr>
<td>[Cr]/[Hb] (a.u.)</td>
<td>0.03</td>
<td>0.57</td>
</tr>
</tbody>
</table>

2.8 Sensing Depth of the Imaging Platform

2.8.1 Methods

In the ex vivo margin assessment study, the sensing depth is a key factor in determining the feasibility of using this optical instrument as a surgical tool for detecting close and positive margins. At DUMC, a clear margin is one that has a 2 mm or greater rim of benign tissue between the margin and the cancerous cells. The 8CH probe was designed to evaluate tissue composition within 2 mm of the surface. The design was based on Monte Carlo simulations of a previous fiber optic probe designed by our group which was found to have a sensing depth of 0.5-2 mm within homogenous tissues having a range of 0.3-20 cm\(^{-1}\) for \(\mu_a\) and a range of 8.4-18 cm\(^{-1}\) for \(\mu_s'\) [100, 106]. The sensing depth of each channel of the 8CH probe was re-simulated with Monte Carlo
modeling for the specific tissue optical properties of benign and malignant tissues as well as for non-homogeneous, layered tissue sites that are representative of close margins, by simulating light propagation through a layered medium. These simulations were carried out using a weighted photon Monte Carlo model, previously described by Liu et al [107, 108] and Zhu et al [106]. The model records the visiting history, exit weight and maximum depth, of each photon that is detected by the collection fibers. The multi-channel probe geometry was modeled with a central illumination fiber (the composition of all the illumination fibers bundled together as one) of r=0.515 mm, an r=0.100 mm collection fiber, and a source-detector separation of 0.636 mm (based on the actual distance from the center of the illumination core to one of the collection fibers from a channel in the multi-channel probe). The single illumination fiber used in these simulations is equivalent in size to the 19 hexagonally packed illumination fibers of the actual multi-channel probe. An index of refraction \(n\) of 1.45 for the fibers and \(n=1.37\) for the tissue was used. The simulated homogenous medium had a thickness of 3 cm, radius of 3 cm, and was divided into grids of 0.01 cm \(r\) x 0.01 cm \(z\). This model works by tracking the path of photons through a medium. The volume is divided into a grid pattern of discrete voxels and the photons are tracked as a function of these voxels. Weighted visiting frequency as a function of depth was used to determine the theoretical sensing depth of the probe for the wavelengths of 450, 500, 550, and 600 nm. Visiting frequency refers to the number of times a photon visits a grid divided by the total
attenuation coefficient at that grid. In order to get weighted visiting frequency, the visiting frequency is multiplied by the survival weight of the photon. Weighted visiting frequency was further normalized by the peak and sensing depth was defined as the depth at which 90% of the photons visit before being collected by the detection fiber.

Two separate simulations were run; the first was for a single-layer tissue model to determine the range of sensing depths at various wavelengths for malignant, adipose, and fibro-glandular tissue types. The optical properties for the positive sites (malignant tissue), adipose, and fibro-glandular sites were used. Fibro-adipose tissue was not used due to its similarity to adipose tissue. Close sites were excluded due to their mix in tissue composition (non-malignant and malignant tissue within the region of interest).

A second simulation was carried out for a two-layer tissue model. This model was intended to model a “close” site where there is non-malignant tissue at the margin with underlying disease less than 2 mm from the margin. Two different non-homogenous tissues were modeled with either adipose or fibro-glandular tissue for the first 1 mm layer, and both with malignant tissue for the second layer (1 mm-3 cm). Although the definition of close is between 0 and 2 mm, an average of 1 mm was used to approximate a typical close margin.

Sites were separated by specific tissue type: positive malignant (n=10), adipose (n=323), fibro-glandular (n=24), and fibro-adipose (n=59), and the median $\mu_a$ and $\mu_s'$ at 450, 500, 550, and 600 nm were determined. This set of site-level data was used to
simulate the sensing depth of the multi-channel probe and the percentage cross-talk between adjacent channels. Positive malignant sites consisted of either ductal carcinoma \textit{in situ} (DCIS) or invasive ductal carcinoma (IDC). Close sites (disease < 2 mm from the margin) were not considered for sensing depth and cross-talk (discussed later) because of their mixture of both malignant and non-malignant tissue. Non-malignant tissue was divided into adipose, fibro-glandular, and fibro-adipose tissues since these three tissue types make up the majority of the sites measured. Table 10 shows the optical properties at various wavelengths for the different tissue types. The fibro-adipose category was excluded from the sensing depth and cross-talk calculations since the optical properties fall between the optical properties of adipose and fibro-glandular tissue and therefore, the sensing depth and cross-talk of fibro-adipose tissue would be between that of adipose and fibro-glandular tissue.

**Table 10:** Median values of the extracted absorption and reduced scattering coefficients at specific wavelengths for various tissue types from the site-level data.  
FA = fibro-adipose, FG = fibro-glandular.

<table>
<thead>
<tr>
<th></th>
<th>Malignant</th>
<th>Non-Malignant</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Positive</td>
<td>Adipose</td>
</tr>
<tr>
<td></td>
<td>(n=10)</td>
<td>(n=323)</td>
</tr>
<tr>
<td>Absorption</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Coefficient</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(cm$^{-1}$)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>450 nm</td>
<td>20.34</td>
<td>11.29</td>
</tr>
<tr>
<td>500 nm</td>
<td>5.54</td>
<td>3.33</td>
</tr>
<tr>
<td>550 nm</td>
<td>9.77</td>
<td>3.29</td>
</tr>
<tr>
<td>600 nm</td>
<td>1.42</td>
<td>0.55</td>
</tr>
<tr>
<td>Reduced</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Scattering</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Coefficient</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(cm$^{-1}$)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>450 nm</td>
<td>9.55</td>
<td>7.44</td>
</tr>
<tr>
<td>500 nm</td>
<td>9.12</td>
<td>6.92</td>
</tr>
<tr>
<td>550 nm</td>
<td>8.82</td>
<td>6.75</td>
</tr>
<tr>
<td>600 nm</td>
<td>8.47</td>
<td>6.45</td>
</tr>
</tbody>
</table>
2.8.2 Results

2.8.2.1 Monte Carlo Simulations of Sensing Depth

Figure 13 shows an example image of the weighted visiting frequency in each grid at 450 nm and 600 nm for positive tissue. To obtain a single value of weighted visiting frequency at each depth, the weighted visiting frequency was summed across the grids at that depth. This plot also shows how the sensing depth of the probe can change depending on the definition and the percentage used.

![Figure 13: Example images of the weighted visiting frequency in each grid separated by depth versus radial distance from the center of the illumination fiber. White lines represent the depth where a percentage (50% or 90%) of the detected photon weight distribution is contained within.](image)

Figure 14 shows normalized weighted visiting frequency versus penetration depth for malignant, adipose, and fibro-glandular tissue at the wavelengths of 450 nm and 600 nm to show the full range of the sensing depths across the wavelengths of interest. From the plot it is apparent that sensing depth increases with wavelength, as expected. Although sensing depth was only calculated to be about 1.5-2.2 mm at 600 nm, there are actually photons capable of reaching greater depths, since sensing depth
was defined as the depth at which 90% of photons reach before being collected. Therefore, we may be capable of probing tissue slightly deeper than what this figure depicts. Malignant tissue has the shallowest sensing depth while adipose has the greatest. This could be due to the increased number of blood vessels (and hence hemoglobin absorption) present in malignant tissues and the combination of relatively high scattering values.

Close margins do not have disease at the surface of the specimen but rather below a layer of benign tissue. To calculate the sensing depth of these close sites adipose or fibro-glandular tissues were considered to be the first layer of the two-layer tissue model, while the malignant tissue was designated as the second layer. Figure 15 shows normalized weighted visiting frequency versus penetration depth for a tissue with an adipose/malignant combination along with a fibro-glandular/malignant combination. The top layer thickness is set at 1 mm. Wavelengths of 450 nm and 600 nm are shown. At 450 nm there is very little difference in the sensing depth for the two different tissue models. However, at 600 nm, there is a greater difference in sensing depth; specifically the adipose/malignant combination has a much larger sensing depth than the fibro-glandular/malignant combination.
Figure 14: Simulated weighted visiting frequency as a function of depth for various tissue types (P=positive, A=adipose, FG=fibro-glandular) in a single layer model. Black circles correspond to the depth at which 90% of the collected photons reach.

Figure 15: Simulated weighted visiting frequency as a function of depth for a two-layer tissue model. The first layer is either adipose (A) or fibro-glandular (FG) tissue with a 1 mm thickness and the second layer is positive (P) with a 29 mm thickness. Black circles correspond to the depth at which 90% of the collected photons reach.

Figure 15 shows the range of 90% sensing depths from 450-600 nm for the single layer model and the two-layer model. Sensing depth increases from 450nm to 600nm for each tissue type and layered tissues.
Table 11: The simulated 90% sensing depth of the 8-channel probe for various tissues in a single layer model and two-layer model. In the two-layer model, the first layer was simulated with a thickness of 1 mm and the second layer with a 29 mm thickness. FG = fibro-glandular.

<table>
<thead>
<tr>
<th></th>
<th>90% Sensing Depth (mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>450 nm</td>
</tr>
<tr>
<td><strong>Single Layer</strong></td>
<td></td>
</tr>
<tr>
<td>Positive</td>
<td>0.50</td>
</tr>
<tr>
<td>Adipose</td>
<td>0.70</td>
</tr>
<tr>
<td>FG</td>
<td>0.60</td>
</tr>
<tr>
<td><strong>Two-layer</strong></td>
<td></td>
</tr>
<tr>
<td>Adipose-Positive</td>
<td>0.70</td>
</tr>
<tr>
<td>FG-Positive</td>
<td>0.60</td>
</tr>
</tbody>
</table>

2.8.2.2 Sensing Depth of Tissue Data

Figure 16 shows the distribution of optical properties stratified by distance of cancerous cells from the inked margin. The extracted $\mu_a$ (Figure 16A,B) and the $\mu_s'$ (Figure 16C,D) from the site-level tissue data are plotted. For sites where the disease depth was reported, the sites were grouped into different depths: 0 mm (n=10), 0-1 mm (n=17), 1-2 mm (n=10), and greater than 2 mm (n=406) which are considered negative. A depth of 0 mm is considered positive while depths of 0-1 mm and 1-2 mm are considered close. For each range of depths, $\mu_a$ and $\mu_s'$ for all sites were plotted at 450 and 600 nm. Absorption is highest for the positive sites and decreases with increasing disease depth. At both 450 nm and 600 nm there is a significant difference in $\mu_a$ between disease at 0 mm and disease 1-2 mm away, as well as between 0 mm and negative sites. At 450 nm there is a drop in the median value of $\mu_a$ from the positive sites (0 mm) to the other distances. However, the median value is roughly the same for the close (0-1 mm...
and 1-2 mm) and negative sites, 10.69, 13.78, and 11.09 respectively. At 600 nm, the median value of $\mu_a$ also decreases from positive to the other distances. The median value of $\mu_a$ also slightly decreases from the close sites (0-1 mm) to the negative sites, 0.68 and 0.54 respectively; and from the close sites (0-1 mm) to the close sites (1-2 mm), 0.68 and 0.51 respectively. At both 450 and 600 nm scattering is highest in the close sites between 0-1 mm. Scattering decreases in the close sites 1-2 mm and is lowest in the negative sites. The lower scattering in the positive sites (0 mm) is most likely due to the various tissue types that make up the close sites; fibrous tissue shows higher scattering values than malignant tissue as seen in Table 10 which may be increasing the scattering in the close sites 0-1 mm.
Figure 16: Box and whisker plots of site-level data. $\mu$ (A,B) and $\mu'$ (C,D) coefficients at 450 nm and 600 nm as a function of depth. Positive sites correspond to a depth of 0 mm. Close sites are 0-1 mm and 1-2 mm. Negative sites are greater than 2 mm. P-values are only shown for categories with significant differences. The box represents the median, 25th percentile, and 75th percentile; the whisker represents values within 1.5 times the interquartile range.

2.9 Evaluation of Cross-talk at the Tissue Surface

2.9.1 Methods

A potential source of error arises with the simultaneous use of 8 channels and the possibility of cross-talk from adjacent channels. The current multi-channel probe was designed to have minimal cross-talk. Cross-talk can be defined in terms of a signal-to-noise ratio (SNR) and is depicted in Figure 17. The signal is defined as the sum of the
diffuse reflectance collected at C1, C2, C3, and C4 for photons launched from I6. The noise is the sum of the diffuse reflectance collected at C1, C2, C3, and C4 launched from the illumination fibers (I1-I5, I7-I8) of the 7 adjacent channels. An SNR > 100 is equivalent to less than 1% cross-talk from the adjacent channels, and is considered to be comparable to the inherent SNR of the system. Again, the site-level data from Table 10 were used to simulate cross-talk at the tissue surface with a Monte Carlo model [107, 108]. Cross-talk is going to be worst when the tissue has a low $\mu_a$, for any given scattering coefficient. Channels must be spaced far enough apart at the tissue surface in order to minimize cross-talk for the worst case scenario. The optical properties used in the simulation represented the lowest $\mu_a$ (found at 600 nm), along with $\mu_s'$ at the same wavelength for positive, adipose, and fibro-glandular tissue. An illumination source (r=0.515 mm) was modeled with 4 collection fibers (r=0.100 mm) and separation distances (S.D.) between channels was varied from 3 to 10 mm. SNR was calculated for each separation distance for the 3 different tissue types.
2.9.2 Results

The amount of cross-talk between individual channels was calculated for the worst case scenario, a low absorbing tissue for a channel in the center of the multichannel probe array. Table 12 represents the SNR for 8 different separation distances between channels for the three different tissue types. The optical properties at 600 nm (the wavelength with lowest absorption) from Table 10 were used for the cross-talk simulations. In order to have less than 1% cross-talk between channels an SNR greater than 100 is necessary. The current multi-channel probe (with channel-to-channel spacings of 10mm) has little to no cross-talk and each channel is capturing information about a specific site. With these optical properties and fiber geometry it is possible to reduce the channel spacing and still minimize cross-talk from adjacent channels.
Table 12: SNR due to cross-talk calculated using MC simulation. Simulations are based on radius=0.515 mm for illumination core and 200 µm collection fibers.

<table>
<thead>
<tr>
<th>Tissue Type</th>
<th>Positive</th>
<th>Adipose</th>
<th>Fibro-glandular</th>
</tr>
</thead>
<tbody>
<tr>
<td>( \mu_a \text{ (cm}^{-1})</td>
<td>1.42</td>
<td>0.55</td>
<td>0.92</td>
</tr>
<tr>
<td>( \mu_s' \text{ (cm}^{-1})</td>
<td>8.47</td>
<td>6.45</td>
<td>11.28</td>
</tr>
<tr>
<td>Separation Distance (S.D.)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3 mm</td>
<td>5.48</td>
<td>2.38</td>
<td>5.98</td>
</tr>
<tr>
<td>4 mm</td>
<td>16.58</td>
<td>5.24</td>
<td>18.07</td>
</tr>
<tr>
<td>5 mm</td>
<td>43.80</td>
<td>10.57</td>
<td>49.16</td>
</tr>
<tr>
<td>6 mm</td>
<td>107.81</td>
<td>19.87</td>
<td>125.43</td>
</tr>
<tr>
<td>7 mm</td>
<td>277.27</td>
<td>37.10</td>
<td>287.63</td>
</tr>
<tr>
<td>8 mm</td>
<td>700.97</td>
<td>68.78</td>
<td>635.52</td>
</tr>
<tr>
<td>9 mm</td>
<td>1545.29</td>
<td>114.04</td>
<td>1751.43</td>
</tr>
<tr>
<td>10 mm</td>
<td>3945.91</td>
<td>203.93</td>
<td>3276.21</td>
</tr>
</tbody>
</table>

2.10 Reproducibility of the Imaging Platform

2.10.1 Methods

To test the reproducibility of the probe-tissue interface, the multi-channel probe was secured in the 4x2 array with channels spaced 10 mm apart and a partial mastectomy specimen was placed in the plexi-glass box (Figure 5). The multi-channel probe was then interfaced with the specimen via the holes of the plexi-glass box in three different orientations: from the top of the box, the side, and bottom (shown in Figure 18). Diffuse reflectance spectra were collected for each of the 8 channels. The probe was removed and placed back into the same location as the first measurement and another diffuse reflectance measurement was made. This was repeated 10 times over a period of less than 5 minutes. [THb], \(<\mu_s'>\), [\(\beta\)-carotene], [THb]/\(<\mu_s'>\), [\(\beta\)-carotene]/\(<\mu_s'>\), and [\(\beta\)-
The reproducibility of the clinical data can be seen in Figure 19. The coefficient of variation (standard deviation / mean) was calculated from 10 serial measurements of the extracted parameters from each site. The median coefficient of variation was calculated over all channels (a total of 32 sites from 4 specimens) for each of the three orientations (Figure 19). The median coefficient of variation is well below 1 for all extracted parameters ([β-carotene], [THb], [β-carotene]/<μ' >, [THb]/<μ' >, and [β-carotene]/[THb]) indicating that there is little deviation from the mean in all measurements. This data also shows that the side orientation is better than the top or
bottom orientations with the median coefficient of variation being less than 0.11 for all extracted parameters. This was the orientation used in all of the clinical studies on tumor margins to date.

![Figure 19](image.png)

Figure 19: This plot shows the median coefficient of variation for the 32 sites which was calculated from 10 measurements on 4 separate lumpectomy specimens. $\beta = \beta$-carotene.

### 2.11 Discussion and Conclusions

In this chapter we have looked at the performance metrics of our clinical instrument for application to intra-operative imaging of partial mastectomy margins. Our measured SNR results show that average SNR across all channels is $>100$. The channels with the lowest SNR are at the edge of the CCD. There is a possibility that some of the signal collected from these channels is being lost because they are on the edge. A CCD with a few more pixels in the vertical direction could fix this problem.

The phantom study results showed that the extracted data is fairly accurate over a wider range of optical properties than our group has previously explored. Although,
the errors are slightly higher than previously reported, we do not think it will have much impact on the optical contrast seen between malignant and non-malignant tissues. The percent difference was calculated between the medians of positive and adipose tissue as well as the medians of positive and fibro-glandular tissue. Table 13 compares these percent differences to the average percent errors from the phantom results for the extracted parameters. For all of the parameters, with the exception of $\beta$-carotene, the percent difference is much larger than the average percent error in extraction accuracy. The small percent difference between positive and fibro-glandular tissue for $\beta$-carotene is probably due to the fact that both malignant tissue and purely fibro-glandular tissue have small amounts of fat present in the measurement site; $\beta$-carotene is likely not a good variable to differentiate these two particular tissue types. However, the higher error is likely due to propagation of errors in the actual phantom studies. Previously, we have shown that crocin concentrations can be extracted with an accuracy of 4.4$\pm$4.0% in a turbid phantom containing hemoglobin, polystyrene spheres, and crocin [118]. In the previous study, the hemoglobin concentration ranged from 6.47-11.77 $\mu$M and crocin concentration ranged from 0-468.50 $\mu$M; the ranges for this phantom study were 41.13-96.88 $\mu$M for hemoglobin and 0-337.32 $\mu$M for crocin. The higher $\beta$-carotene errors seen in the phantom study reported here are likely due to the lower ratio of crocin to hemoglobin concentrations in this set of phantoms. This is supported
by the fact that in the current phantom study when the ratio of crocin to hemoglobin concentration increases, the percent error in crocin decreases.

Table 13: Comparison of the percent error in accuracy versus percent differences between tissue types for the extracted parameters.

<table>
<thead>
<tr>
<th>% Difference</th>
<th>% Error</th>
<th>Positive vs. Adipose</th>
<th>Positive vs. Fibroglundular</th>
</tr>
</thead>
<tbody>
<tr>
<td>[THb]</td>
<td>5.57 ± 3.89</td>
<td>101.18</td>
<td>92.26</td>
</tr>
<tr>
<td>[β-carotene]</td>
<td>14.99 ± 13.6</td>
<td>29.91</td>
<td>9.69</td>
</tr>
<tr>
<td>&lt;μs&gt;</td>
<td>9.81 ± 6.89</td>
<td>28.11</td>
<td>29.81</td>
</tr>
<tr>
<td>[THb]/&lt;μs&gt;</td>
<td>6.07 ± 4.02</td>
<td>92.09</td>
<td>108.27</td>
</tr>
<tr>
<td>[β-carotene]/&lt;μs&gt;</td>
<td>11.76 ± 12.3</td>
<td>14.14</td>
<td>88.30</td>
</tr>
<tr>
<td>[β-carotene]/[THb]</td>
<td>13.21 ± 12.6</td>
<td>107.67</td>
<td>80.68</td>
</tr>
</tbody>
</table>

Based on the simulations, we have found that the sensing depth of the multi-channel probe ranges from 0.5-2.2 mm over the wavelength range of 450-600 nm. There are variations in the literature of the definition of a clear margin. DUMC uses < 2 mm to define a close margin, however, other institutions range from < 1 mm to < 1 cm in their definitions [80], with the majority of pathologists using 2 mm as the pathologic criteria for clear margins [82, 103-106]. With the sensing depth of the probe being ~1-2 mm we hope to compromise between the number of false positives and false negatives. If the mean sensing depth of the probe were 2 mm we may end up with more false positives because the probe could potentially sense positive tissue beyond the depth that pathology samples. In our previous 48-patient clinical study the sensitivity of the device was 82.4% for positive margins and 76.5% for close margins. The comparable
sensitivities suggest that although the simulated sensing depth was < 2mm for fibro-
glandular and positive tissue, the clinical results suggest that the probe has a sufficient
sensing depth that is consistent with the pathologic criterion for clear margins.
Compared to touch-prep we are capable of probing tissue at greater depths since this
technique only looks at the cells directly at the surface of the margin. In the future, it is
possible to change the sensing depth of the probe by altering the probe geometry and/or
wavelength range if a greater sensing depth is desired.

With Monte Carlo simulations we showed that cross-talk is minimal (<1%) with
the current separation distance between adjacent channels. Therefore, we are confident
that the photons captured for a single channel pertain only to the tissue directly
underneath it and not from tissue underneath a neighboring channel. With the optical
properties found in the ex vivo breast tissue, it may be possible to decrease the separation
distance between channels even further and still maintain <1% cross-talk.

The reproducibility experiment from partial mastectomy specimens showed that
in all possible probe orientations the median coefficient of variation is less than 0.17,
meaning that all orientations are fairly reproducible. Of the three possible orientations,
the side orientation showed the lowest coefficient of variation for all extracted
parameters (<0.11). Therefore, all measurements of partial mastectomy specimens
utilized the side orientation. The side orientation may be the most reproducible because
it is least affected by pooling of blood. With the desired margin at the top of the box,
blood may drain to the bottom of the specimen, thereby decreasing the total hemoglobin concentration over time. With the desired margin on the bottom of the box the opposite effect would take place and total hemoglobin may increase with time. It is also inconvenient to interface the probe to the specimen via the bottom of the box.

The 8CH probe covers an area of ~2 x 4 cm with 5 mm resolution for 4 consecutive placements of the probe. It takes ~15 seconds to image and 10 seconds to process the data for a single placement of the probe. The majority of margins we have imaged range from 3.1 x 3.1 cm (10 cm²) to 4.5 x 4.5 cm (20 cm²). The speed of the system could be significantly improved if it could be designed to cover a margin as large as 20 cm² (the large end of margin sizes) with millimeter resolution (comparable to the thickness of each bread loafed pathology specimen) with just a single placement. The number of channels needed to image the largest sized specimen can be determined as follows. The diameter of the sampling area of each channel is typically comparable to the sensing depth and should be between 1-2 mm, which is the accepted criterion for clear margins. If there are multiple channels in the device, intuitively, one would minimize spacing between channels and determine the number needed to ensure the entire tissue area is covered. However, because tissue is highly scattering, there should be spacing between channels to minimize cross-talk between adjacent pixels, thus minimizing the sampling of redundant information. To achieve a cross-talk of 5% or less (using the cross-talk of the multi-channel optical spectral imaging system as a
benchmark), a minimum center to center channel spacing of 7 mm is desired, as shown in our cross-talk simulation results. Assuming each channel has an active area that is approximately 2 mm in diameter and the center to center channel spacing is 7 mm, a 49 channel device would cover a 4.5 x 4.5 cm (20 cm²) margin with a single placement of the imaging probe. The scaling of the imaging probe from 8 channels to 49 channels can be readily achieved by incorporating a larger CCD that can resolve more channels, reducing the number of collection fibers to a single fiber per channel to minimize the number of pixels on the CCD occupied by each channel, and by illuminating every other channel at any given time such that with sequential illumination, twice as many channels can be imaged without the issue of cross-talk between adjacent channels.
3 Effects of Inter-patient Variability on Optical Data and the Ability to Predict Surgical Margin Status

This chapter evaluates the effects of inter-patient variability on the optical data and uses a conditional inference tree (CIT) model to predict surgical margin status in 70 patients. Specifically, this chapter evaluates the effects of mammographic breast density on negative margins to determine how changes in benign tissue composition affect optical contrast between negative and close/positive tumor margins. In addition, the performance of the spectral imaging platform was evaluated taking breast density into account.

3.1 Introduction

Breast cancer is an enduring health problem with more than 200,000 patients diagnosed annually in the United States [119]. Most of these patients are eligible for breast conserving surgery (BCS) [120]. BCS, also known as a partial mastectomy or lumpectomy, is a recommended treatment for early stage breast cancer and for breast cancers that have been reduced in size by neoadjuvant therapy. The goal of BCS is to excise the tumor along with at least a 1-2 mm margin of surrounding normal tissue [9, 10, 79]. Post-operative histopathologic assessment of the resected specimen is the current gold standard by which completeness of excision is determined. Margin status is an important predictor of local recurrence of an invasive or in situ cancer after BCS [1, 2]. Unfortunately, as many as 17.7-72.0% of patients undergoing BCS require repeat surgeries due to a close or positive surgical margin [3, 6-11]. Younger women in
particular, typically have higher percentages of involved margins, requiring re-excision and higher local recurrence rates [1, 80, 82, 84, 121-123]. These results with age may be due to increased breast density; a study by Bani et al [124] found that higher mammographic breast density (MBD) was associated with higher re-excision rates, 18% (MBD-1), 18% (MBD-2), 22% (MBD-3), and 42% (MBD-4). One recent study observed that in over 2,000 women undergoing BCS, the variation in re-excision rate varied from 0-70% across surgeons, indicating that there is no reliable intra-operative standard for preventing re-excision [73]. Touch-prep cytology and frozen section analysis have been used to help address this need intra-operatively. However, these techniques require a trained pathologist to be present, prolong surgery time (20-40 minutes), and have technical challenges associated with processing fatty breast tissues. By 2015, it is expected that the number of patients undergoing BCS will rise from approximately 200,000 to more than 270,000 per year in the U.S., at an annual growth rate of 5.5% [120]. Thus, there is a significant unmet clinical need for effective intra-operative assessment of breast tumor margins.

The best available method for the detection of residual carcinoma on a surgical tumor resection specimen is post-operative histopathology, which is the gold standard. This approach uses light microscopy to detect the presence of disease in 5 micrometer-thick tissue sections (at micrometer image resolution), taken from 3 mm equidistant slices of the tumor margin (millimeter sampling frequency). An ideal intra-operative
tool would sample tumor margins at comparable or better sampling frequency and image resolution. However, the need for microscopic resolution results in a practical sampling limitation, since there is inherent difficulty in sampling, imaging and analyzing large tissue areas (ca. 10-100 cm\(^2\)) with microscopic resolution in intraoperative time-frames [125]. This is a particular problem in heterogeneous organs such as the breast, in which samples are routinely large, and it is not possible to grossly observe and preferentially sample small areas of residual disease, due to the surrounding mix of normal tissue types including fat, glands, and fibrous tissues. Achieving microscopic resolution of the tumor margin in an intra-operative tool also comes at the expense of sensing depth (2 mm clear margin criteria established by breast cancer pathologists). Thus it is not currently practical to achieve micrometer image resolution, centimeter-square coverage area, and millimeter sensing depth with the same technology. In an effort to address this important problem, our group has developed a strategy to “sense” the micro-morphology of the breast tumor margin over a wide field of view by creating quantitative hyperspectral maps of the tissue optical properties (absorption and scattering) [12, 111] where each voxel can be deconstructed to provide information on the underlying histology. This strategy provides a means to quickly survey centimeter-square tissue areas with quantitative analysis of spectral information serving to provide a surrogate for microscopic imaging resolution. Further, the visible spectral range (450-600 nm) of the electromagnetic spectrum provides the requisite
contrast while maintaining a sensing depth of 2 mm, the criterion for clear margins used by pathologists [12, 25, 115]. The primary absorbers in the breast over this wavelength range include β-carotene stored in adipocytes (reflective of fatty tissues) and hemoglobin found in blood cells in the vasculature (reflective of tissue vascularity). Likewise, the scattering properties are directly related to the collagen and cell density within the breast (reflective of fibroglandular content).

The study presented here demonstrates the capability to survey histology landscapes with quantitative spectral imaging, in the context of breast tumor margin assessment. First we established that the information inherent in spectral data was specifically related to salient tissue composition and micro-morphologic features in the breast, such that millimeter-resolution sampling using spectral information could provide a surrogate for micrometer-resolution imaging. Specifically, we quantified the relationship between the quantitative metrics obtained from the absorption and scattering endpoints, and the proportion of fat and collagen/glands-quantified from histopathology of specific sites from negative and positive margins. We then determined the ability of this spectral mapping technique to survey shifts in the landscape of normal breast histology, by analyzing the spectral information arising from inter-patient variations in mammographic breast density (MBD), which further established the micro-morphological features to which the hyperspectral maps are sensitive. Finally, we determined the utility of this surveillance approach to detect shifts
in the histologic landscapes caused by the presence of residual carcinoma, by imaging breast tumor resection margins intra-operatively in 70 patients undergoing BCS, and predicting the presence of residual disease through a statistical predictive modeling approach for automated, unbiased selection of predictor variables.

3.2 Methods

3.2.1 Patient Population

Patients over age 18 undergoing BCS were consented under a Duke University Institutional Review Board approved clinical protocol. The following characteristics were recorded for each patient (if available): radiographic breast density, menopausal status, neoadjuvant treatment status (chemotherapy or endocrine therapy), age, body mass index (BMI), race, parity, lactation history, and surgical re-excision status. For mammographic breast density (MBD), each patient was assigned a value based on their pre-surgery mammogram: 1 (fatty), 2 (scattered fibrous), 3 (heterogeneously dense), or 4 (extremely dense). For the analyses in this paper an MBD score of 1 or 2 was considered to be low density, while a score of 3 or 4 was considered to be high density; the data was binned this way since the majority of the patients had 2’s or 3’s.

3.2.2 Instrumentation

The 8CH-1 instrument (described in detail in Section 2.2) was utilized to measure diffuse reflectance spectra from 8 discrete sites in a single acquisition. For ease of use and to avoid crosstalk between adjacent probes at the tissue surface, the 8 channels of
the probe were secured in an aluminum adaptor in a 4×2 array with a center-to-center separation of 10 mm between each channel; a 5 mm sampling resolution was achieved by translating the probe over the tissue in 5 mm increments. Note that this channel spacing does not define the spatial sampling resolution of the probe, since the probe may be sequentially stepped in smaller increments over the tissue surface to provide a finer sampling resolution. A custom software application was written in-house and described in more detail in Section 2.3).

### 3.2.3 Measurement Procedure

At the time of surgery, partial mastectomy specimens were excised and oriented by the surgeon using surgical clips and sutures to mark the center of 4 of the 6 total margins. Specimens then underwent routine specimen mammography. QDRI imaging of the excised lumpectomy specimen was performed intra-operatively, either in the operating room or in an adjacent room. Approximately 16±5 minutes post-excision, the specimen was placed in a rectangular plexi-glass box for imaging, oriented such that the clip/suture was at the center of the box face. The imaging probe was interfaced to the lumpectomy specimen via holes in the plexi-glass box (Figure 8A). Diffuse reflectance measurements (450-600 nm) were collected from the tissue surface with 5 mm sampling resolution until the entire margin had been measured by simply translating the imaging probe to sample the interleaving pixels [12, 25, 115, 116]. One to five margins were imaged per specimen based on the surgeon’s recommendation and available time. Four
surgeons participated in this study. To increase the yield of positive margins measured by QDRI, the surgeon indicated if any margin on the primary specimen was likely to be positive, based on palpation of the specimen and specimen radiography. The surgeon was blinded to the imaging output and performed selective intra-operative re-excision based on only on the review of the specimen mammograms and gross examination, as is standard of care.

3.2.4 Margin-level Histology

Following imaging of the full margin surface, the four corners of the measured margin were marked with histological ink for later pathologic correlation with the imaged area (referred to as margin-level analysis). The orientation of the imaged margin was recorded (i.e., superior, inferior, medial, lateral, anterior, or posterior) and sent to surgical pathology to ensure orientation concordance. The specimen was further inked by surgical pathology per standard protocol. Post-operative pathology served as the standard to classify each margin as negative (malignant cells > 2 mm from tissue surface), close (malignant cells ≤ 2 mm from tissue surface), or positive (malignant cells at surface). At the Duke University Medical Center, the standard of care is to grossly section the specimen into 3 mm slices perpendicular to the long axis of the specimen. The tissue slices are then further sectioned to fit into histological cassettes for automated processing and paraffin-embedding. From each of the paraffin blocks, a single 5 µm thick section is taken for staining and histological review. If any of those sections
contained cancer within 2 mm of the specimen surface, then the entire margin surface was deemed “close” or “positive” for residual cancer, regardless of the location of the residual cancer on the specimen surface. Therefore, for our purposes, an entire parameter map of the specimen surface was only paired with the overall pathologic diagnosis of that surface (i.e., negative, close, or positive), as no spatial information about the specific location of the malignancy on the tissue surface was available from pathology. For the purposes of this study, the close and positive margins are lumped together as “positive” since, clinically, both require re-excision.

3.2.5 Site-level H&E Image Analysis

In addition to the margin-level analysis, up to 10 sites per margin were inked with a unique color histological ink at the time of optical assessment and denoted as “research sites,” which were analyzed in detail to provide a histologic assessment of the underlying tissue composition; this site-level analysis is described in greater detail by Kennedy et al [25]. In the cases where individual imaged pixels were marked with ink, the resulting pathologic tissue sections could be paired with quantitative optical parameters from those pixels. Hematoxylin and eosin (H&E) stained sections of histologically-confirmed adipose-A (n=84), malignant-M (n=5), fibroglandular-FG (n=12), and fibroadipose-FA (n=40) tissues were imaged. The images were acquired with a Zeiss Axio Imager upright microscope with a 10% neutral density filter, 2.5X (for FG and FA tissues) and 10X (for adipose tissue) objectives, a halogen light source, and a
QImaging MicroPublisher 5.0MP color camera. MetaMorph 7.6.5 was used to adjust the acquisition time and RGB gain. The field of view (FOV) in the resulting FG and FA images was 5 mm x 4 mm with a resolution of 4.4 µm. The FOV for the adipose images was 1 mm x 1.3 mm with a resolution of 1.1 µm.

H&E-stained adipose tissue sections were analyzed with an automated image processing algorithm to extract average cell area and cell density. The green channel of the RGB images obtained from the color camera was used in the algorithm as it provided a convenient method of separating the primarily pink and blue stained tissue from white fat. All images were preprocessed with a 2-D implementation of an edge-preserving bilateral filter. Subsequently, the MATLAB implementation of the Canny edge detector was used to extract the outlines of the adipocytes. The interior of each outlined shape was measured and the number of shapes was counted to provide an estimate of cell density. Empirically-determined cell-area thresholds of 129.3µm² and 22,569µm² were used to limit the counted results to those with a high probability of being an adipocyte.

H&E images of histologically-confirmed fibroglandular, fibroadipose, and malignant images were analyzed manually by overlaying a grid pattern of 200µm x 200µm boxes and manually counting the number of boxes with specific tissue types (adipose, collagen, glands, vessels). From this, the percentage of fat, collagen, glands,
and vessels were calculated for each site and compared to the \([\beta\text{-carotene}]\) and \(<\mu_s'>\) values. Correlations were computed with a Pearson’s correlation.

### 3.2.6 Spectral Data Analysis

Details of the analysis of the diffuse reflectance data from the partial mastectomy specimens can be found in prior publications [12, 25, 115, 116] and in Section 2.5.

### 3.2.7 Empirical Cumulative Distribution Functions

Using all pixels from all parameter maps acquired from lumpectomy specimens, four empirical cumulative distribution functions (eCDFs) for each parameter were created for positive and negative margins from high density and low density breasts (negative, high density; negative, low density; positive, low density; positive, high density). To calculate statistical differences between the eCDFs, empirical p-values for a Kolmogorov-Smirnov statistic were computed using blocked permutation to maintain the correlation structure of multiple site level measurements within each margin.

### 3.2.8 Conditional Inference Tree Models

An advantage of the QDRI system is that it provides full parameter maps of the tissue surface with 5 mm lateral sampling resolution and 1-2 mm axial sensing depth. However, as mentioned in the previous section, detailed information about the exact spatial location and extent of residual disease was not available for the imaged specimen surfaces. Since there was no way to exploit the spatial information inherent in the images in a specific way, we adopted three approaches for reducing the 2-D parameter
maps to image descriptive scalar variables, which are more easily paired with the overall
binary margin diagnosis. The first approach was to simply take the median value of the
image. The second approach was to quantify differences in the distribution of values
within an image by computing the percentage of image pixels which lie below a
particular value threshold for each image. For each of the 5 quantitative parameter
maps (β-carotene, total hemoglobin, <µ′>, β-carotene/<µ′>, and total hemoglobin/<µ′>),
19 variables were computed which represented 19 thresholds. These thresholds were
pre-selected for each quantitative parameter by placing all image pixels from all margins
into a single vector, and then selecting the 0.05 – 0.95 data quantiles in 0.05 quantile
increments as the threshold values. This method ensured that the threshold values were
selected to evenly split the data on the actual data distribution, as opposed to the data
range (which is sensitive to outliers and extremes in the data). Then, for each individual
margin sample and quantitative parameter combination, these 19 thresholds were used
to compute the percentage of image pixels below each threshold. The third and final
approach was a statistical approach which compared the distribution of a given image’s
pixels to the distribution of all pixels from all (positive + close) margins. Specifically, the
two-sided Kolmogorov-Smirnov test was used to compute the likelihood that the
distribution of pixels in the image of interest came from a “positive” pixel distribution,
and the resulting Kolmogorov-Smirnov statistic was directly used as an image-
descriptive variable.
Based on the image-reduction schemes outlined above, for each margin, a set of 105 image descriptive values were computed (5 quantitative parameters × (19 thresholds + 1 image median + 1 Kolmogorov-Smirnov statistic) = (5 × 21) = 105 total variables). We hypothesized that, for each quantitative parameter, one threshold variable could be selected which best separated positive from negative margin samples based on the image distributions. Therefore, for each quantitative parameter, we selected the single threshold value which best separated positive/close margin samples from negative margin samples by calculating Wilcoxon rank-sums and selecting the threshold value with the lowest $p$-value. We thus arrived at a final set of $(5 \times 3) = 15$ image descriptive variables per imaged margin which were used in construction of a predictive model.

We employed a conditional inference tree (CIT) model for automated selection of predictor variables and estimation of prediction accuracy [126]. This was accomplished using the ‘ctree’ function of the library(party) in the R programming environment. All 15 variables for all imaged margins were fed into the CIT model, which selected the predictor variables, and optimum cut-points on those variables giving the best classification accuracy for the entire patient cohort. Separate MBD-specific CIT models were created by restricting the imaged margins to those from breasts with low or high breast density. This, in effect, created a second decision tree model in which the first decision node is MBD1/MBD2 or MBD3/MBD4. The statistical significance of the models was computed using Fisher’s exact test (1000 permutations), by randomly
sampling the margin diagnosis vector without replacement to shuffle the margin classifications, before building the CIT models. For each of the permutations, the performance of the permuted model was compared against the performance of the observed model using the quantity \( F = (1 - \text{sensitivity})^2 + (1 - \text{specificity})^2 \), which is minimized in well-performing models. The \( P \)-value (probability of a random model performing better than the observed model) was computed as the fraction of \( F_{\text{permuted}} < F_{\text{observed}} \).

### 3.3 Results and Discussion

Table 14 contains a breakdown of statistics for patients enrolled in the study, stratified by mammographic breast density. Note that in the subsequent results, all margin statistics are related to the margin status of the initial excised surgical specimen only, and do not include information about intra-operative re-excision specimens; therefore, the positive margin rates reported here are not the same as the final margin status for these patients (since the surgeons in some cases performed an intra-operative re-excision which “corrected” a positive margin on the primary specimen). Margins were imaged in 99 neoadjuvant-therapy naïve patients; 88 margins imaged from 70 patients were retained for analysis in this paper. Patients were excluded due to instrumentation failure (n=3), discrepant pathological orientation (n=20), atypical ductal hyperplasia (n=1), re-excision lumpectomy (n=2), and incomplete images (n=3). The low density group consisted of n=11 MBD-1 patients and n=27 MBD-2 patients; the high density group consisted of n=25 MBD-3 patients and n=7 MBD-4 patients.

<table>
<thead>
<tr>
<th></th>
<th>Low Density Patients</th>
<th>High Density Patients</th>
</tr>
</thead>
<tbody>
<tr>
<td># of Patients</td>
<td>38</td>
<td>32</td>
</tr>
<tr>
<td>Avg. Age (range)</td>
<td>61.9 (43-87)</td>
<td>56.8 (36-83)</td>
</tr>
<tr>
<td>Avg. BMI (range)</td>
<td>31.7 (18.3-49.2)</td>
<td>28.1 (18.4-43.7)</td>
</tr>
<tr>
<td>Tumor Receptor Status</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ER +,-</td>
<td>33 (86.8%), 5 (13.2%)</td>
<td>27 (84.4%), 4 (12.5%)</td>
</tr>
<tr>
<td>PR +,-</td>
<td>29 (76.3%), 9 (23.7%)</td>
<td>25 (78.1%), 6 (18.8%)</td>
</tr>
<tr>
<td>HER-2/neu +,-</td>
<td>0 (0%), 33 (86.8%)</td>
<td>5 (15.6%), 20 (62.5%)</td>
</tr>
<tr>
<td>Triple-negative</td>
<td>4 (10.5%)</td>
<td>0 (0%)</td>
</tr>
<tr>
<td>Avg. Lumpectomy Volume (range)</td>
<td>63.7 cm$^3$ (10.2-192.0 cm$^3$)</td>
<td>49.5 cm$^3$ (9.5-175.9 cm$^3$)</td>
</tr>
<tr>
<td>Primary Tumor Histology</td>
<td></td>
<td></td>
</tr>
<tr>
<td>IDC</td>
<td>4 (10.5%)</td>
<td>3 (9.4%)</td>
</tr>
<tr>
<td>DCIS</td>
<td>4 (10.5%)</td>
<td>5 (15.6%)</td>
</tr>
<tr>
<td>IDC/DCIS</td>
<td>16 (42.1%)</td>
<td>17 (53.1%)</td>
</tr>
<tr>
<td>Other</td>
<td>4 (10.5%)</td>
<td>3 (9.4%)</td>
</tr>
<tr>
<td>No Tumor</td>
<td>0 (0%)</td>
<td>1 (3.1%)</td>
</tr>
<tr>
<td>Measured Margin Histology</td>
<td></td>
<td></td>
</tr>
<tr>
<td>IDC</td>
<td>8 (16.7%)</td>
<td>6 (15.0%)</td>
</tr>
<tr>
<td>DCIS</td>
<td>8 (16.7%)</td>
<td>9 (22.5%)</td>
</tr>
<tr>
<td>IDC/DCIS</td>
<td>1 (2.1%)</td>
<td>2 (2.5%)</td>
</tr>
<tr>
<td>Other</td>
<td>1 (2.1%)</td>
<td>5 (12.5%)</td>
</tr>
<tr>
<td>No Tumor</td>
<td>25 (52.1%)</td>
<td>17 (42.5%)</td>
</tr>
<tr>
<td>Surgical Margin Status</td>
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<td></td>
</tr>
<tr>
<td>Negative (&gt;2mm)</td>
<td>25 (52.1%)</td>
<td>17 (42.5%)</td>
</tr>
<tr>
<td>Close (&lt;2mm)</td>
<td>15 (31.3%)</td>
<td>14 (35.0%)</td>
</tr>
<tr>
<td>Positive</td>
<td>8 (16.7%)</td>
<td>9 (22.5%)</td>
</tr>
<tr>
<td>Measured Margin</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Anterior</td>
<td>10 (20.8%)</td>
<td>7 (17.5%)</td>
</tr>
<tr>
<td>Posterior</td>
<td>11 (22.9%)</td>
<td>12 (30.0%)</td>
</tr>
<tr>
<td>Superior</td>
<td>7 (14.6%)</td>
<td>8 (20.0%)</td>
</tr>
<tr>
<td>Inferior</td>
<td>8 (16.7%)</td>
<td>4 (10.0%)</td>
</tr>
<tr>
<td>Medial</td>
<td>6 (12.5%)</td>
<td>6 (15.0%)</td>
</tr>
<tr>
<td>Lateral</td>
<td>6 (12.5%)</td>
<td>3 (7.5%)</td>
</tr>
<tr>
<td>Re-excision Rate</td>
<td>8 (21.1%)</td>
<td>11 (34.4%)</td>
</tr>
</tbody>
</table>
3.3.1 Spectral imaging surveys tissue composition and micro-morphology in the normal breast

We hypothesized that by obtaining spectral information (Figure 8A) and using quantitative spectral analysis methods to quantify the absorption and scattering (Figure 8B) properties of the tissue, we would be able to leverage low-spatial-resolution spectral imaging as a surrogate for high-spatial resolution methods, but with the added advantage of an adequate sensing depth of 2 mm and the ability to more practically survey large tissue areas (e.g., tumor margins). The success of this approach hinges on the relationship between the optical sources of contrast and the histologic parameters of interest (primarily, the relative contributions of various histologic tissue types including fat, collagen and glandular tissue). Specifically, with quantitative spectral imaging, the concentrations of β-carotene and hemoglobin can be quantified, which should be directly related to the amount of fatty tissue and vasculature, respectively. Likewise, the reduced scattering coefficient has been shown to be sensitive to changes in cellular density and collagen content [127]. In the present study, although the tissue samples were measured within 20 minutes of excision from the patient, there existed the possibility that the blood found in the vasculature could be lost or re-distributed due to removal from the blood supply. Therefore, for the purposes of this study, the hemoglobin content was not considered further.

We previously hypothesized that [β-carotene]/<μs'> is sensitive to the relative amount of fat (from the [β-carotene] parameter) to fibro glandular tissue (from the <μs'>
parameter) [115]. Figure 20A is an image of \( [\beta\text{-carotene}]/<\mu.'> \) from a negative margin with histologically-confirmed adipose and fibroglandular plus adipose sites highlighted, which demonstrates a decrease in this parameter as the fat content decreases, as expected. This is also reflected in the absorption and scattering spectra shown in Figure 20B, in which the absorption of \( \beta\text{-carotene} \) at ~480 nm is highest in the adipose tissue (solid line), and the scattering coefficient of the fibroglandular/fibroadipose tissue is higher than the pure adipose tissue at all wavelengths. To confirm the relationship between the quantitative parameters obtained from spectral data and the composition of the breast tissue at the micron scale (in particular the \( [\beta\text{-carotene}]/<\mu.'> \) parameter), a subset of data from these images for which site-level histopathology were obtained was used to compare the optical data to tissue histology. In Figure 20C, two representative H&E images are shown corresponding to one site with a high fat to collagen ratio and another with a low fat to collagen ratio, with the corresponding \( [\beta\text{-carotene}] \) and \( <\mu.'> \) values shown below each image. As expected, the \( [\beta\text{-carotene}] \) concentration is increased in the tissue with higher fat content and \( <\mu.'> \) is increased in tissue with higher fibroglandular content. These trends were confirmed in a larger cohort of pathologically-confirmed sites from the margins imaged in this study; H&E images of the sites were broken down into specific categories: fibroglandular (collagen and normal glands), fibroadipose (collagen and adipose), and adipose. The empirical cumulative distribution functions (eCDFs) in Figure 20D show that \( [\beta\text{-carotene}]/<\mu.'> \) decreases as
the tissue changes from predominantly adipose tissue to predominantly fibroglandular tissue components (i.e. decreasing fat and increasing collagen/glands).

Figure 20: A) 50x bicubic interpolated image of [β-carotene]/<μs'> from a negative margin. Sites with corresponding histopathology are highlighted with diagnoses of adipose (A) or fibroglandular plus adipose (FG+A). B) Absorption (μ_a) and scattering (μ_s') spectra for representative adipose (A), fibroadipose (FA), and fibroglandular (FG) sites from a negative margin. C) Representative H&E images of a predominately collagen site and another predominately fat. D) Empirical cumulative distribution functions (eCDFs) of the site-level data for fibroglandular (FG), fibroadipose (FA), and adipose (A) sites.

3.3.2 Spectral imaging tracks shifts in histological landscapes associated with breast density

To further elucidate the sensitivity of this technique to the histological landscape in the breast when it is used to “map” large areas, we used mammographic breast density (MBD) in cancer-free margins as a model system. We hypothesized that as breast density increases, the percentage of collagen/glands in the overall breast would
increase and the percentage of fat would decrease. Based on the findings in Figure 20 this would suggest a decrease in [β-carotene] and an increase in scattering. To test this hypothesis, we used spectral information from negative (cancer-free), neoadjuvant-naïve margin images to investigate whether the β-carotene and $<\mu'>$ parameters would reflect this shift in the histologic landscape due to differences in breast density between patients. Figure 21 shows eCDFs of all pixels from negative margins, separated by low (MBD = 1 or 2) and high (MBD = 3 or 4) density breasts. Interestingly, [β-carotene] and [β-carotene]/$<\mu'>$ were significantly higher in the negative margins of high density compared to low density breasts, which is counter intuitive (since low density breasts are associated with higher proportions of fatty tissues). Also, $<\mu'>$ was non-significantly increased in high density breasts compared to that in low density breasts. The increased $<\mu'>$ was likely associated with an increase in glandular tissue and collagen in the breasts of these patients, as expected. However, the significant increase in [β-carotene] with breast density, and thus the ratios of [β-carotene] to $<\mu>$, could not be attributed to differences in the relative percentage of adipose tissue, since low density breasts should by definition have a higher percentage of this tissue type.
Figure 21: A) Parameter maps from a low density margin; B) Parameter maps from a high density margin; blue indicates higher values of the corresponding variable. C) eCDFs of all measured sites from negative, neoadjuvant naïve margins, separated by mammographic breast density. *P*-values were calculated with modified Kolmogorov-Smirnov statistics.

### 3.3.3 Adipocytes in high density breasts are smaller and have a higher baseline β-carotene concentration

Since β-carotene is stored in adipocytes, we hypothesized that this unexpected finding was related to adipocyte morphology. Under normal conditions β-carotene is absorbed through the intestines and circulates in the blood before being transported to the liver; excess β-carotene is stored in adipose tissue [35-37]. Inside the adipocyte, β-carotene is converted into retinaldehyde via central cleavage by the enzyme β-carotene
monoxygenase type I (Bcmo1) [38]. Retinol, a byproduct of β-carotene is also converted to retinaldehyde with aldehyde dehydrogenase [38]. Retinaldehyde can then be converted into retinoic acid [38]. This β-carotene/retinoic acid pathway is important in adipocyte differentiation and has also been shown to play an important role in modulating adipocyte size [38, 39]. This suggests that β-carotene concentration is related to adipocyte size which prompted an investigation into whether any differences in adipocyte size existed between high and low density breasts.

H&E images of optically-sampled adipose tissues from breasts covering the full range of density classes are shown in Figure 22A. Qualitatively, the average adipocyte size appears to decrease as breast density increases. A more quantitative analysis of the adipose tissue images and optical data (n=84) showed increased [β-carotene] (p=0.0038), increased adipocyte density (p=0.093), and smaller adipocyte areas (p=0.17) in the adipose tissues of high density breasts as compared to low density breasts (Figure 22B). After restricting the body mass index (BMI) range of the patients in this analysis to BMI = 25-30 (n=25) to correct for the influence of BMI on adipocyte size, the same trends were noted: increased [β-carotene], increased adipocyte density, and smaller adipocyte areas in the adipose tissues of high density compared to low density breasts (Figure 22C). This analysis suggests that increased [β-carotene] is associated with smaller adipocytes, and that high density breasts overall have smaller adipocytes, thus resulting in an increased baseline level of [β-carotene] in the fatty tissues of high density patients.
Figure 22: Analysis of adipose tissue between low and high density breast tissue. A) Representative H&E micrographs (100x) from all 4 MBDs. Cell area and cell density were calculated from an automated image analysis algorithm. [β-carotene] and $<\mu_s>$ were measured via quantitative spectral imaging. The adipose sites are from B) the negative margins of neoadjuvant-naïve patients and C) the negative margins of neoadjuvant-naïve patients with a BMI restricted to 25-30. P-values were calculated with a Wilcoxon rank-sum.
3.3.4 Spectral surveying of histologic landscapes can be leveraged in detection of residual cancer on a tumor margin

We hypothesized that when areas of malignancy are present, the morphological and compositional landscape shifts to one with less fat and more fibroglandular components. This would result in a shift of the histological landscape to one with lower [β-carotene] and higher $\mu_s'$. Figure 23A is a depiction of this shifting landscape of the ratio of [β-carotene] to $\mu_s'$ in a positive and negative margin from patients matched for breast density (MBD-3). In the positive margin there are lower values of [β-carotene]/$\mu_s'$ compared to the negative margin; in this particular image, these lower values were histologically-confirmed to correspond to sites with ductal carcinoma in situ (DCIS) that was less than 0.5 mm below the margin surface. The benign sites, which had fattier compositions, had higher [β-carotene]/$\mu_s'$ values. Figure 23B displays representative absorption and scattering spectra of three benign sites and a malignant site. The malignant site shows similar absorption trends as the fibroglandular site but has much higher scattering. Figure 23C contains example H&E images of a fibroadipose and DCIS site; where the DCIS site has higher scattering. [β-carotene]/$\mu_s'$ eCDFs of the site-level data are shown in Figure 23D. [β-carotene]/$\mu_s'$ is very similar between the malignant sites and fibroglandular sites likely due to decreased fat content and increased collagen/cellularity in these tissues. Figure 23E further demonstrates the positive correlation between the scattering coefficient $\mu_s'$ and the percentage of glandular tissue vs. the percentage of collagen present in the sensing volume indicating
that $\mu_\text{s}'$ is more sensitive to the presence of glands which is the predominant constituent in malignant tissues.

Figure 23: A) 50x bicubic interpolated images of $[\beta\text{-carotene}]/\mu_\text{s}'$ from a negative and positive margin in 2 different patients with MBD-3. Sites with corresponding histopathology are highlighted with diagnoses of adipose (A), fibroglandular (FG), ductal carcinoma in situ (DCIS). B) Absorption ($\mu_a$) and scattering ($\mu_\text{s}'$) spectra for representative adipose (A), fibroadipose (FA), fibroglandular (FG), and malignant (M) sites. C) Representative H&E images of a fibroadipose and DCIS site. D) Empirical cumulative distribution functions (eCDFs) of the site-level data for A, FA, FG, and M sites. E) Scatterplots of the wavelength-averaged reduced scattering coefficient ($\mu_\text{s}'$) versus the percent of glands to the percent of collagen in benign sites containing glands.

Figure 24 shows eCDFs of all measured image pixels from negative and positive margins, stratified by breast density. These eCDFs show the trends that we expected for this shifting biological landscape: decreased $[\beta\text{-carotene}]$, increased $\mu_\text{s}'$, and decreased $[\beta\text{-carotene}]/\mu_\text{s}'$ in the positive margins compared to the negative margins. The significant increase in baseline $[\beta\text{-carotene}]$ levels in the negative margins of high density patients, although originally unexpected, actually served to markedly improve
contrast between positive and negative margins in this cohort of patients. The ratio of $[\beta$-carotene] to $<\mu_s>$ also benefited from the differences in the negative margins due to breast density, and was found to be the most useful parameter in discriminating positive and negative margins.

Figure 24: A-B) eCDFs of all measured sites from negative and positive, neoadjuvant naïve margins, separated by radiographic breast density. The p-values indicated correspond to modified Kolmogorov-Smirnov tests between: 1) negative versus positive margins in low density patients and 2) negative versus positive margins in high density patients. Cartoon showing β-carotene contrast between tumor and the surrounding adipocytes; little contrast is observed when adipocytes are large, more contrast is observed when adipocytes are smaller. C) Delta eCDFs between the positive and negative margins for both low and high density patients. Negative values indicate the negative distribution has higher values.
Figure 25 shows the CIT model fitted to the dataset using the 15 image descriptive variables described in the Methods. For this model, the margin samples were first classified into low and high breast density subgroups (MBD ≤ 2 and MBD ≥ 3, respectively) prior to construction of the predictive model – this was in an effort to account for the differences in baseline optical variables between MBD subgroups previously observed. This in effect creates a single decision tree model, in which the first decision node is MBD subgroup, as shown in the figure. The primary quantitative parameter chosen in both the low and high MBD subgroups was the ratio of \( \beta\)-carotene/\(\mu_s'\); however, the statistical parameter computed on that variable was different between the MBD subgroups. The other quantitative parameter selected by the CIT model was \(\mu_s'\), and in particular, the Kolmogorov-Smirnov statistic, which compared a given margin sample \(\mu_s'\) distribution to the distribution of \(\mu_s'\) from all pixels from all positive margins. For each terminal node, a barchart is shown which indicates the relative fraction of negative and positive margins classified into that node; the number of margins classified into each node is also indicated above the barcharts. These barcharts are significant in that they indicate the likelihood that future samples classified into each node will be classified accurately.
Figure 25: Conditional inference tree predictive model for the 88 margin (70 patient) dataset, with mammographic breast density as the first decision node. The bar plots at each terminal node indicate the relative fraction of n margins classified into that node that are negative or positive.

The eCDFs shown in Figure 26 are helpful in interpreting the CIT model. For each decision node (shown by the ovals in Figure 25), the empirical eCDFs for positive and negative margins classified into the resulting terminal nodes (shown by the bar charts in Figure 25) are plotted in Figure 26. The CIT model can be thought of as a set of “rules” which are applied to any candidate margin image to determine whether it will be classified as positive or negative. For the low breast density subgroup (MBD ≤ 2), the first decision node is the percentage of [β-carotene]/µ pixels in the image that are less
than 1.2388. In this case, margin images with less than 4.8% of image pixels below this value (i.e., characterized by higher values of \([\beta\text{-carotene}]<\mu>'\)) are classified into Node 2 as negative; all 12 of these samples were grouped together and their cumulative distribution is shown by the blue curve in Figure 26A. As seen in the plot, samples that are ultimately classified into Node 2 as negative are characterized by higher \([\beta\text{-carotene}]<\mu>'\) values, which are indicative of histological landscapes with higher adipose and lower fibrous/glandular content. The red curve in Figure 26A represents samples ultimately classified into terminal Nodes 5-7, which are composed of 21 positive margins and 15 negative margins. Without additional discrimination, samples classified to the right of decision Node 1 would be classified as positive, but with a \(21/(21+15) = 58\%\) probability of actually being positive. Fortunately, additional variables were identified by the model to further improve the classification of those samples. In this case, the Kolmogorov-Smirnov statistic for \(<\mu>'\), which compares the eCDF of any given margin image to the eCDF of all pixels from all positive margins (the reference distribution), is helpful in further classifying these margin samples. Samples with a Kolmogorov-Smirnov statistic of 0 indicate that their pixel distribution was drawn from the reference distribution; therefore, samples with lower Kolmogorov-Smirnov statistics have distributions which more closely mimic the reference distribution of all positive margins, and are therefore more likely to be positive. Figure 26B shows the samples classified by decision Node 3, which is the Kolmogorov-Smirnov statistic with a cut-off
value of 0.339. Margin samples with a Kolmogorov-Smirnov statistic > 0.339 (i.e., less similar to the positive reference distribution) were classified into terminal Node 7 (and are shown by the blue curve in Figure 26B). The eCDF of the combined samples in Node 7 are clearly less similar to the positive reference distribution, shown by the black curve in Figure 26B. Conversely, margin samples with a Kolmogorov-Smirnov statistic <0.339 (i.e., more similar to the positive reference distribution) were classified into terminal Nodes 5 and 6. Again, these margin samples classified to the left of decision Node 3 were composed of 18 positive margins and 6 negative margins, for a conditional class probability of 69%. However, these margin samples were further classified by decision Node 4, which is the Kolmogorov-Smirnov statistic with a cut-off value of 0.16. Interestingly, although the eCDFs of Figure 26C show that the samples classified into Node 6 (red curve in Figure 26C) more closely resemble the reference positive distribution (black curve in Figure 26C) and were also classified as positive by the CIT model, they were characterized by a higher Kolmogorov-Smirnov statistic than the samples classified in Node 5. The samples classified into Node 5 (blue curve in Figure 26C) visually appear to deviate more from the positive reference distribution and are characterized by a slightly greater proportion of negative margins, but are characterized by a lower Kolmogorov-Smirnov statistic (remember that a low Kolmogorov-Smirnov statistic indicates similarity to the positive reference distribution). This discrepancy is likely due to the fact that the Kolmogorov-Smirnov statistic is known to have poor
sensitivity to deviations from the reference distribution at the tails of the distribution, as is observed in the samples classified into Node 5 (blue curve) [128].

For the high breast density subgroup (MBD ≥ 3), a single decision node was selected by the CIT model for classification of margin samples in that cohort. In this case, it is the median value of $[\beta$-carotene]/$<\mu'>$ in the margin image that was chosen as the predictive variable, where images with a median value of less than 3.401 were classified into Node 2 (83% positive conditional class probability) and images with a median value greater than 3.401 were classified into Node 3 (76% negative conditional class probability). Figure 26D contains the eCDFs for these two terminal nodes – the median value is defined as the value at the 0.5 quantile, therefore, the positive margins (red curve) are characterized by a curve shift to the left and a lower median value than the negative margins (blue curve). Taken together, these results indicate that the presence of residual carcinoma on the cancer margin indeed results in a shift of the margin histological landscape to one with less fat and more fibrous and glandular components, which is reflected as a shift to lower values of $[\beta$-carotene]/$<\mu'>$. In addition, the presence of residual carcinoma results in a $<\mu'>$ image distribution which is similar to the $<\mu'>$ distribution for all pixels from all positive margin samples.
Figure 26: eCDFs for samples classified into each terminal node of the MBD-specific CIT model, separated by the corresponding decision node listed in each figure panel.

In B and C, the $\langle\mu_s^\prime\rangle$CDFs for the margin samples classified into those nodes are shown along with the eCDF for all pixels from all positive margin samples in the study, which served as a reference distribution for two-sided Kolmogorov-Smirnov testing. Red curves indicate distributions composed of majority positive margin samples, and blue curves indicate distributions of majority negative margin samples.

Table 15 contains a summary of the performance of the technology for intra-operative detection of close or positive margins. Margin assessment based on quantitative spectral imaging would have detected close/positive margins with 74% sensitivity (65% and 83% in low and high density breasts, respectively) and 86% specificity (92% and 76% in low and high density breasts, respectively). Overall, the surgeon sensitivity was 65% (69.6% and 60.9% in low and high density breasts, respectively), which was calculated based on the number of positive primary margins.
for which an additional intra-operative shaving was not obtained. However, determination of the true surgeon specificity is not straightforward, since at our institution some surgeons obtain additional shavings of multiple margins as a matter of course, whether or not they believed residual disease to be present – this had the effect of artificially increasing the false-positive rate if we calculate it as the number of times the surgeon took a shaving of a negative primary margin. Therefore, although we compute the surgeon specificity (26%, Table 15) for comparison to the device, the preceding caveat applies. The surgeon sensitivity is determined and is reflected in the eventual repeat surgery rates (27.1% of patients overall, or 21.1% (8/38) in low MBD patients and 34.4% (11/32) in high MBD patients). The performance of the model in this work represents a substantial improvement over the unaided surgeon, 74% of the margins imaged in this study were in the MBD2 and MBD3 subgroups which indicates that the lowest and highest density subgroups (MBD1 and MBD4) were unequally represented in this dataset. The fact that we observed differences in adipocyte density and [β-carotene], and the fact that breast density is associated with fibroglandular content which is associated with the $\mu_r$ parameter, suggests that ultimately the use of MBD a priori should provide the most robust method for accurate detection of residual cancer in BCS. Although hemoglobin content was excluded from the results presented here, we did examine the effects of adding total hemoglobin ([THb]) and [THb]/$\mu_r$ to the CIT model. Adding these additional parameters resulted in exactly the same
performance, suggesting that [THb] is not a unique predictor of surgical margin status, and justifying its exclusion due to concerns about stability over time after excision.

Table 15: Sensitivity (Se), specificity (Sp), positive predictive value (PPV), negative predictive value (NPV), and classification accuracy (A) of the device and the surgeon. Performance within each MBD subgroup is given. The surgeon’s performance is based on the primary specimen (and no additional shavings) taken during the first operation. Fisher’s empirical P value is provided for each CIT model.

<table>
<thead>
<tr>
<th>Samples (n)</th>
<th>Se (%)</th>
<th>Sp (%)</th>
<th>PPV (%)</th>
<th>NPV (%)</th>
<th>A (%)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Device Performance</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>All (88)</td>
<td>74</td>
<td>86</td>
<td>85</td>
<td>75</td>
<td>80</td>
<td>&lt;0.096</td>
</tr>
<tr>
<td>MBD 1-2 (48)</td>
<td>65</td>
<td>92</td>
<td>88</td>
<td>74</td>
<td>79</td>
<td>&lt;0.096</td>
</tr>
<tr>
<td>MBD 3-4 (40)</td>
<td>83</td>
<td>76</td>
<td>83</td>
<td>76</td>
<td>80</td>
<td>&lt;0.096</td>
</tr>
<tr>
<td><strong>Surgeon Performance</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>All (88)</td>
<td>65</td>
<td>21</td>
<td>48</td>
<td>36</td>
<td>44</td>
<td>NA</td>
</tr>
<tr>
<td>MBD 1-2 (48)</td>
<td>70</td>
<td>28</td>
<td>47</td>
<td>50</td>
<td>48</td>
<td>NA</td>
</tr>
<tr>
<td>MBD 3-4 (40)</td>
<td>61</td>
<td>12</td>
<td>48</td>
<td>18</td>
<td>40</td>
<td>NA</td>
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</table>

Table 16 provides a breakdown of the false negatives and false positives for the CIT model and the surgeons’ performance. These numbers are broken down by the type of cancer found at the margin, the surgical margin status, and MBD. Of the 12 false-negatives in the CIT model, close margins and margins containing DCIS had higher percentages (75% and 33%, respectively) of being missed than positive margins or margins that contained IDC (25% and 33%, respectively). This was also true of the surgeons’ performance where 38% DCIS margins were missed and 63% close margins were missed. Four out of the 6 false positives in the model were associated with histologic features that may explain why they were incorrectly diagnosed. One of the measured margins consisted of fibrocystic change and fibroadenoma; two of the
measured margins had adjacent margins that contained atypical ductal hyperplasia and fibrocystic change; and another measured margin had cancer ~4mm from the surface.

Table 16: Number of false negative (FN) and false positive (FP) margins (margin histology and surgical margin status) and patients (MBD) calculated from the surgeon performance, as well as, the performance of the device.

<table>
<thead>
<tr>
<th>Margin Histology</th>
<th># of FN (%)</th>
<th># of FP (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Surgeon</td>
<td>Device</td>
</tr>
<tr>
<td>IDC</td>
<td>3 (19%)</td>
<td>3 (25%)</td>
</tr>
<tr>
<td>DCIS</td>
<td>6 (38%)</td>
<td>4 (33%)</td>
</tr>
<tr>
<td>IDC/DCIS</td>
<td>1 (6%)</td>
<td>1 (8%)</td>
</tr>
<tr>
<td>other</td>
<td>4 (25%)</td>
<td>2 (17%)</td>
</tr>
<tr>
<td>no tumor</td>
<td>0 (0%)</td>
<td>0 (0%)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Surgical Margin Status</th>
<th># of FN (%)</th>
<th># of FP (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Surgeon</td>
<td>Device</td>
</tr>
<tr>
<td>Negative</td>
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<td>0 (0%)</td>
</tr>
<tr>
<td>Close</td>
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<td>9 (75%)</td>
</tr>
<tr>
<td>Positive</td>
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<td>3 (25%)</td>
</tr>
<tr>
<td>MBD</td>
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<td>2 (13%)</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>5 (31%)</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>8 (50%)</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>1 (6%)</td>
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</table>

3.4 Conclusions

The technology presented here seeks to bridge the gap between the requirement for microscopic resolution and cm² imaging areas and millimeter sensing depth for tumor margin assessment, by leveraging quantitative optical spectral imaging to report on the histological make-up of the interrogated tissue. An important advantage of this technique is the ability to be sensitive to the tissue down to 2 mm below the tissue surface, which is important for detection of residual cancer lying surreptitiously below
the surface. Emerging technologies meant to address this problem include traditional light microscopies and optical coherence tomography [31, 33, 116]. Although light microscopy (such as confocal microscopy) can achieve the required resolution for tumor margin assessment, the ability to see beneath the tissue surface is limited to less than 1 mm, which limits the utility of such devices for assessment of the excised specimen, which is the current clinical paradigm. Optical coherence tomography, on the other hand, is a depth-resolved technique which observes information related to light scattering of tissue from suitable depths, but at this time has not been scaled for observation of cm² fields of view [31, 33]. In addition, any microscopic imaging approach will result in a preponderance of image data which must be analyzed with an appropriate algorithm which can detect spatial signatures associated with residual cancer (i.e., more data than a human can reasonably assess in an intraoperative timeframe). The technique presented here, although not capable of microscopic image resolution, does offer a pragmatic solution that has potential as a clinically translatable tool for tumor margin assessment. Not only might this technology aid the surgeon in identifying margins suspicious for residual carcinoma, it can potentially guide tissue sectioning for frozen-section analysis or for routine processing of breast specimens by the pathologist. Currently, pathologists cannot physically section and review an entire specimen, especially large volume specimens. A device that could quickly assess the
margin and highlight specific areas to sample would help reduce the amount of tissue to be sectioned, and perhaps increase the sensitivity of permanent histopathology.

The differences in adipocyte size and [β-carotene] with breast density were an unexpected finding, but actually served to increase optical contrast in high density breasts. This was a fortuitous finding given that surgeons have an easier time excising a tumor with clear margins within a fattier background (low density patients) than excising a tumor from a very dense background (high density patients). From this cohort of 70 patients the percentage of patients who returned for a second surgery was 21.1% (8/38) of low density patients and 34.4% (11/32) of high density patients. This higher re-excision rate is consistent with others reported in the literature where Bani et al [124] found that higher MBD was associated with higher re-excision rates; specifically their re-excision rates were: 18% (MBD-1), 18% (MBD-2), 22% (MBD-3), and 42% (MBD-4). These studies speak to the fact that surgeons face greater difficulties in excising tumors in a denser breast and that an optical device would benefit this patient population.

In addition to being a tool for intra-operative margin assessment, this technology could examine micro-morphological changes in the tumor micro-environment through measures of collagen content, glandular content and β-carotene. Recent studies have focused on the tumor micro-environment and an understanding of how this impacts the growth and aggressiveness of a tumor. Some studies [57, 58] have focused on the
orientation of collagen to the tumor boundary, and others [53, 54] have focused on cancer-associated adipocytes (adipocytes near a cancer that are in constant cross-talk with the tumor cells). The research on cancer-associated adipocytes (CAAs) suggests that crosstalk between tumor cells and nearby adipocytes leads to a reduction in adipocyte size, decreased levels of PPAR-γ, aP2, C/EBPα, resistin, and hormone-sensitive lipase, and increased levels of inflammatory markers, such as IL-6. In our study we found that increased levels of β-carotene were associated with smaller adipocytes and high density patients. Interestingly, high density patients are typically at increased risk for developing breast cancer which leads us to question whether β-carotene may be associated with breast cancer risk. There have been a number of epidemiological studies [62, 70, 129] that have looked at [β-carotene] in serum with breast cancer risk but we offer a technique that can measure [β-carotene] in tissue. A better understanding of the tumor micro-environment, facilitated by large-scale sampling of surgical breast specimens with optical spectral imaging to report on collagen (<μs>) and adipocytes (β-carotene), may offer insight into the relationships between tissue composition at tumor boundaries, risk, and eventual clinical outcomes.
4 Effects of Surgical and Excisional Factors on Optical Data

Surgical procedure varies from patient to patient and from surgeon to surgeon. This chapter quantifies the effects of three different surgical and excisional factors that may impact quantitative optical parameters. Specifically this chapter addresses the presence of Lymphazurin™, a blue-dye used for sentinel lymph node mapping, on the optical images, the use of a cautery tool to excise the tissue, and how post-excision tissue kinetics affect optical data.

4.1 Introduction

Breast conserving surgery (BCS) is a recommended treatment for early-stage breast cancer and for breast cancers that have been reduced in size by neoadjuvant therapy. The goal of BCS is to excise the tumor along with a margin of normal tissue, while preserving as much of the normal breast tissue as possible. Unfortunately, as many as 18-72% of patients undergoing BCS require repeat surgeries due to a close or positive surgical margin diagnosed post-operatively and thus, require a re-excision surgery to achieve cancer free margins [3-11]. These re-excision surgeries are not only a burden to patients financially but also physically and psychologically and can delay recommended adjuvant therapies. Additionally, 10-36% of women requiring re-excision will undergo mastectomy which significantly alters a patient’s initial treatment decision [73]. The large variation in re-excisions is thought to be due to differences in surgeon’s training, in
the definition of a close margin, and in the perceived risk of focally positive margins versus extensive involvement [73].

Histopathology is the current gold standard for determining surgical margin status. At many hospitals, including Duke University Medical Center (DUMC), the standard of care is to grossly section the specimen into 3 mm slices perpendicular to the long axis of the specimen. The tissue slices are then further sectioned and from each of the resulting paraffin blocks, a 5 µm thick section is taken for staining and histological review. The pathologic margin status is an important predictor of local recurrence of an invasive or in situ cancer after BCS [74, 75]. Thus, re-excision of the tumor margin is essential to reduce the risk of local recurrence [76]. In post-operative pathology, it is not feasible to section and analyze the entire specimen, especially when the specimens are large. This issue was evaluated by Guidi et al. [130] where they looked at the presence of tumor in perpendicularly sliced sections of the inked margin (the type of analysis done here at DUMC) versus evaluating tissue from an en face cut of the margin (i.e. a shaved margin). They found that of 69 positive shaved margins, only 42 inked margins were found to be positive, indicating that residual carcinomas may be missed with the current approach of sampling tissue every couple of millimeters.

A small number (less than 5%) of hospitals which perform BCS currently utilize intra-operative cytologic or pathologic analysis of tumor margins. Touch-preparation (touch-prep) cytology is a technique in which cells on the surface of the tissue are
transferred to glass slides by touching the specimen to the glass, and are then stained for pathologic observation. For frozen section analysis, the tissue is frozen and select microscopically thin sections are cut from the specimen for pathologic observation. Typically a much smaller fraction of the tumor margin is sampled in frozen section than in post-operative pathology. Touch-prep cytology and frozen-section analysis can reduce surgical re-excision rates; reported sensitivities and specificities for touch-prep are 38-100% and 83-100%, respectively [85-92]. Sensitivity of frozen section ranges from 59-91% and specificity ranges from 86-100% [88, 93-99]. Although these two approaches have been shown to be beneficial to the surgeon, there are a number of limitations with each. Both procedures are time consuming and require special expertise by a pathologist at the time of surgery. Additionally, touch-prep cytology allows for the evaluation of the whole lumpectomy surface but is not capable of detecting close margins since only cells at the specimen surface are sampled. Frozen section analysis may not be utilized on every patient but may be determined in collaboration with the surgeon, pathologist, and radiologist after a laborious process of gross examination and specimen mammography [99]. Sampling issues are also a problem since the entire specimen cannot be evaluated.

The above discussion points to the fact that surgery to remove the cancer and obtain clear margins is a collaborative effort between the surgeon and the pathologist (and in some institutions, the radiologist). In spite of this, there can be substantial
variability in the prediction of positive margins in the intra-operative and post-operative settings. Surgeons do not have adequate intra-operative assessment tools to ensure that the cancer has been completely removed at the time of first surgery. Pathologists do not have adequate tools for sampling from areas on large tumor margins. The lack of these capabilities represents a significant unmet clinical need for margin assessment for both the surgeon and pathologist.

Optical imaging of tissue is an attractive solution to this problem because it is relatively fast and non-destructive. Optical techniques can also measure features related to the histological landscape without the need for labels. Table 1 provides a breakdown of the different optical tools that have been leveraged to measure breast tissue constituents for different applications in breast cancer ranging from diagnostic biopsy to margin assessment to monitoring of response to neoadjuvant therapy. This table shows that no matter what tool is used, the primary sources of contrast in breast tissue are scattering (which primarily reflects the fibroglandular content of breast tissue), lipid and carotenoid concentration (which reflects the fatty content of the breast tissue content), hemoglobin (which reflects tissue vascularity), and in the case of fluorescence, metabolism of the tumor cells.

Pioneering optical studies to characterize breast tumor margins was carried out by Bigio et al [131] where they used reflectance spectroscopy in the UV-Visible range to look at sites within the tumor bed in 24 patients (13 cancer and 59 normal sites). This
work was important in that it represented initial evidence of absorption and/or scattering contrast in residual breast cancer. Keller et al published on diffuse reflectance and fluorescence spectroscopy to detect cancerous sites on excised breast tumor margins in 32 patients (145 normal and 34 individual tumor sites), and reported a sensitivity and specificity of 85% and 96%, respectively, for classifying individual sites (not margins) [132]. Haka et al published on Raman spectroscopy of tumor sites on freshly sliced lumpectomy specimens in 21 patients (123 benign and 6 malignant tissue sites) and exploited fat and collagen contrast to achieve sensitivity and specificity of 83% and 93%, respectively for classifying individual sites [28]. Nguyen et al [31] demonstrated that optical coherence tomography detects ex vivo margin positivity in 20 patients (11 positive/close margins and 9 negative margins), with sensitivity and specificity of 100% and 82%, respectively by exploiting scattering associated with increased cell density. Nachabe et al [110] used diffuse reflectance spectroscopy to acquire spectra from 102 ex vivo samples that consisted of adipose, glandular, fibroadenoma, invasive carcinoma, and DCIS. Using a K-nearest neighbor algorithm, malignant and non-malignant samples were separated with a sensitivity of 94±4% and a specificity of 98±2%.

We published recently on using a quantitative diffuse reflectance spectral imaging technique to non-destructively image lumpectomy margins surrounding a mass in 48 patients [115, 133]. What is unique about our published work on breast tumor margin assessment is that we demonstrated the capability to image an entire tumor margin,
which has yet to be demonstrated by previously published optical techniques. The engine of this bench-top spectral imaging system is a broadband source that emits at visible wavelengths, an imaging spectrograph, and a CCD camera [20, 134]. Light is relayed between the instrument and each discrete site on the margin within a specimen box via an imaging probe [133]. The diffuse reflectance spectra per site were analyzed with a feature extraction algorithm based on a fast, scalable Monte Carlo model developed by our group [111, 112] to quantitatively determine absorption (β-carotene and hemoglobin) and scattering contrast in the breast. These sources of contrast were used to create tissue morphology maps which were used in a decision-tree model to differentiate positive from negative margins. We reported sensitivity and specificity of 79% and 67% respectively on 55 margins from 48 patients [115, 133] imaged 16±5 minutes post-excision. We have since accrued images from 88 margins in 70 patients and the results are consistent with those reported previously [135]. In summary, optical imaging technologies can aid the surgeon in finding positive margins and they can also be used to guide pathological assessment of tissue and provide insight into where to sample the tissue, thereby improving sampling yield, particularly in larger tumor specimens in both the intra-operative and post-operative setting.

Before this technology can be used in an intra-operative setting or in a post-operative setting, systematic studies have to be performed to determine which surgical and post-surgical factors affect the precision and accuracy with which this technology
maps optical contrast. This is true not only for our technology but other technologies, both optical and non-optical that are intended for this application. Specifically, if the technology is to be used on the excised margin (which is the way in which intra-operative pathology is performed), then there must be an understanding of how the presence of the blue sentinel lymph node mapping dye, Lymphazurin™, and cautery could influence the primary sources of contrast in the breast. Another important variable to characterize is the impact of the time delay after excision on the primary sources of optical contrast in the breast. Given that all of the recent studies reporting on optical technologies have been carried out on resected tumor margins [24, 28, 31, 115, 136] and the fact that frozen section and post-operative pathology are necessarily carried out on resected specimens, characterizing the effects of these potential sources of error will be important in the context of developing optically based margin imaging tools for use by surgeons and pathologists.

In this study, we examine the effects of time after excision on the following quantitative optical parameters in breast tumor margins which include: [β-carotene], oxygenated and deoxygenated hemoglobin, total hemoglobin concentration ([THb]), the wavelength-averaged reduced scattering coefficient from 450-600nm (<μ'>), [β-carotene]/<μ'>, [THb]/<μ'>, hemoglobin saturation (HbSat), and [Lymphazurin™]. In addition, we evaluate the effects of varying Lymphazurin™ concentration and cautery on the optical absorbers and scatterers. Finally, we evaluated how all of these results
impact optical contrast between negative and close/positive margins for the purposes of breast tumor margin assessment.

4.2 Methods

4.2.1 Clinical Protocol

Patients undergoing partial mastectomy were consented under a Duke University Institutional Review Board approved protocol. Patient enrollment was limited to patients who had not received a prior surgical excisional biopsy for cancer diagnosis or prior chemotherapy or endocrine therapy. Standard surgical protocol was followed. Following excision, orientation by the surgeon, and specimen mammography, the specimen was placed in a plexi-glass box. Diffuse reflectance spectra were obtained from 8 discrete sites on the tumor margins and all of these sites were inked for histopathology.

Patients undergoing mastectomy were also consented. Patient enrollment was limited to patients with palpable tumors (approximately >1 cm) who had not received a prior surgical excisional biopsy for cancer diagnosis or prior chemotherapy or endocrine therapy. Standard surgical protocol was followed. Immediately following excision, the breast was inked and the dimensions (anterior-posterior, inferior-superior, and medial-lateral) were measured for standard surgical pathology. A single incision was then made through the posterior or anterior aspect of the mastectomy specimen into the center of the tumor by the surgeon or a board-certified pathologist (JG) present in the
operating room. Diffuse reflectance spectra were measured from two locations from each mastectomy specimen; one corresponding to grossly benign tissue and the other grossly malignant tissue as identified by the pathologist. Once measurements were completed, the two sites were inked for histopathology.

The lumpectomies were used to quantify the degree to which each optical endpoint changed over time. These specimens were also used to determine the effects of Lymphazurin™ on the primary sources of optical contrast in the breast. Benign sites from both lumpectomies and mastectomies were used to evaluate the effect of cautery (incised mastectomies did not undergo cautery within the measurement area). Finally, because the yield of positive sites was low in the lumpectomy specimens, only mastectomies were used to compare the kinetics in the primary sources of optical contrast between benign and malignant breast tissue.

4.2.2 Instrumentation and Measurements

The 8-CH optical imaging device (described in detail in Section 2.2) was used in both the mastectomy and lumpectomies. In the lumpectomy study, the specimen was placed in a plexi-glass box and interfaced with the 8 channels of the probe via the holes in the box (Figure 27B). For this study, a diffuse reflectance spectrum (450-600 nm) was collected periodically, with intervals of ≤ 1 minute (average of 0.93 minutes apart) between each successive spectrum. The first measurement was taken between 2 and 12
(7±3) minutes post-excision. Spectra were measured for as long as possible without interfering with the surgical team (in practice, this was a range of 10-21 minutes).

After the mastectomy specimen was sliced to expose the tumor, one channel of the 8-channel fiber optic probe was placed on the tumor while another was placed on grossly benign tissue within the excision (Figure 27A); the probes were held securely in place with adjustable laboratory clamps. Note that the measurements were made on non-cauterized freshly cut tissue surfaces. For this study, a diffuse reflectance spectrum (450-600 nm) was collected periodically, with intervals of ≤ 1 minute (average of 0.42 minutes apart) between each successive spectrum. The first measurement was taken between 10 and 27 (17±4) minutes post-excision. Spectra were measured for as long as possible without interfering with the surgical team (in practice, this was a range of 10-32 minutes).

The measurement times were consistent with our previous lumpectomy study [115, 116] where measurements commenced 16±5 minutes after excision. In that study, the total time from excision to the end of margin imaging was 29±15 minutes (average and standard deviation). Each diffuse reflectance spectrum was divided by the CCD integration time, and corrected for daily variations in optical throughput by dividing the tissue spectrum by a spectrum collected from a 99% Spectralon reflectance standard (LabSphere) at each wavelength.
4.2.3 Histopathology

Upon completion of the measurements, the measured sites were inked for histological correlation. The specimens were then transferred to the surgical pathology laboratory for routine pathologic processing, and following routine diagnostic workup the inked sites were evaluated microscopically by the study pathologist (JG). The benign sites were classified as fat, fibro-adipose, fibro-glandular, or mixed/other; mixed/other refers to any site with some combination of fat, collagen, glands, or vessels. The malignant sites were classified as invasive ductal carcinoma (IDC), ductal carcinoma in situ (DCIS), or mixed/other; for these, mixed/other refers to sites with some combination of IDC, DCIS, or lobular carcinoma. If tumor cells extended to the inked surface, the margin was considered positive. If they were within 2 mm of the inked surface, the margin was considered close.
4.2.4 Data Analysis of Mastectomies and Lumpectomies

All data were analyzed over a wavelength range of 450-600 nm. Our inverse Monte Carlo model [12, 111, 118] was used to extract the optical property spectra ($\mu_a$ and $\mu'_s$) of each tissue site from the calibrated diffuse reflectance spectrum. Using the extracted optical properties, tissue parameters including [oxy-hemoglobin], [deoxy-hemoglobin], hemoglobin saturation (HbSat), total hemoglobin ([THb]), [$\beta$-carotene], [Lymphazurin™], and the wavelength-averaged reduced scattering coefficient from 450-600 nm ($<\mu'_s>$), were calculated for every site at each time point. Ratios of these tissue parameters were also calculated as additional endpoints.

The extracted tissue parameters were fit to a longitudinal mixed-effects model, which is an appropriate method for evaluating the trends over time in optical measurements across different tissue types. Longitudinal models were performed in R version 2.7.2 (www.r-project.org) using the lme4 package. The fixed-effect terms in the models were the time from surgical excision of the specimen and the histological subtype of the measured site. This model resulted in a fitted slope for every measured site. In all tests of main effects and interactions, statistical significance was considered to be $p < 0.05$.

4.2.4.1 Lumpectomy Kinetics

Our first step was to characterize how the optical properties changed over time in lumpectomies. To calculate the rate of change, sample-specific slopes were estimated
from the mixed model. To determine the most robust optical parameters for imaging lumpectomy margins, the percent change was calculated for all lumpectomy sites over a 30 minute time window (to be consistent with the average time it took to fully image the lumpectomy margins in our previous study). The percent change was used for this comparison since rates of change cannot be compared across tissue parameters since they have different units and magnitudes. The percent change was calculated by dividing the rate of change by the absolute value of the intercept from the fitted data. The association between the first measurement and time from excision were also evaluated using Spearman correlations.

4.2.4.2 Cautery

To determine the effect of cautery on the optical parameters, the initial values and the rates of change of the benign sites were compared between lumpectomies and mastectomies using Wilcoxon rank-sum tests. Lumpectomies and mastectomies were compared since measurements were made from the cauterized lumpectomy surfaces versus the non-cauterized incised mastectomy tissue.

4.2.4.3 Effects of Tissue Histology

Likelihood ratio tests were used to determine whether the rate of change was dependent on tissue type; specifically whether there were differences in the rates of change in the optical parameters between benign and malignant tissue. Spearman
correlations were also computed for both benign and malignant sites between the first measurement and time from excision.

### 4.2.5 Lymphazurin™ Simulations and Phantom Studies

Lymphazurin™ is a blue dye used for sentinel lymph node mapping with an extinction coefficient that partially overlaps with the alpha and beta bands of oxygenated and deoxygenated hemoglobin (Figure 28C). Although the inverse Monte Carlo model [111, 112] accounts for Lymphazurin™ by assuming this to be a primary absorber in breast tissue, it is unknown how accurately the model can extract [THb], [β-carotene], and scattering in the presence of varying concentrations of Lymphazurin™. A forward Monte Carlo model was used to obtain simulated diffuse reflectance spectra with known absorption and scattering levels. From our previous site-level study [25] with 854 sites from lumpectomy specimens, the median, 25th and 75th quantiles were computed for $\langle \mu_s' \rangle$, [THb], and [β-carotene]. These 3 levels can be seen in Table 17. The full range of Lymphazurin™ concentrations were also determined from the site-level data and are shown in Figure 28A. The majority of the measured sites have less than 5µM of Lymphazurin™ but go as high as 72.7µM. The effects of these different concentrations are reflected in the absorption spectra of Figure 28B where $\mu_a$ at 600 nm increases with increasing [Lymphazurin™]. Therefore, for the simulations, a diffuse reflectance spectrum was created for every combination of absorption and scattering
levels, and [Lymphazurin™] was input in 10µM increments from 0 to 70µM. This resulted in 216 simulated spectra (3x3x3x8).

Table 17: Planned and actual absorber and scatter levels for the tissue mimicking phantom. Scattering levels were calculated for a wavelength range of 450-600nm.

<table>
<thead>
<tr>
<th></th>
<th>&lt;\mu_s'&gt; (cm(^{-1}))</th>
<th>THb (µM)</th>
<th>β-carotene (µM)</th>
<th>Lymph (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Planned</td>
<td>6.68</td>
<td>16.97</td>
<td>10.29</td>
<td>0-70</td>
</tr>
<tr>
<td>Actual</td>
<td>5.81</td>
<td>16.69</td>
<td>11.23</td>
<td>0-79</td>
</tr>
</tbody>
</table>

Figure 28: A) Histogram of the extracted [Lymphazurin™] for the site-level data (854 sites). Maximum extracted [Lymphazurin™] was 72.7 µM. B) Extracted absorption coefficient spectra for 3 different fat sites with varying [Lymphazurin™]. C) Extinction coefficients for oxy-hemoglobin (HbO2), deoxy-hemoglobin (HbH), and β-carotene measured by Prahl [117]; and Lymphazurin™ measured by our group.

The simulated spectra were then inverted in the same manner as the clinical data to extract the concentrations of THb, β-carotene, Lymphazurin™, and <\mu_s'>. This inversion process used known extinction coefficients for β-carotene, oxy-hemoglobin, and deoxy-hemoglobin. The extinction coefficient for Lymphazurin™ was measured by our group previously. To determine the accuracy of the model at extracting the
absorbers and scattering information with varying degrees of [Lymphazurin™], the percent error was calculated between the expected data and extracted simulated data.

We have previously tested the accuracy of our system using phantoms containing hemoglobin, crocin (a substitute for β-carotene), and polystyrene spheres (a scatterer), and showed that all parameters could be extracted with <15% error [12]. Similarly, tissue mimicking phantoms consisting of 1.025µm diameter polystyrene spheres (Polysciences), hemoglobin (H0267 – Sigma-Aldrich), crocin (17304 Standard Fluka, Sigma-Aldrich), and Lymphazurin™ (TycoHealthcare) were created to access the effect of [Lymphazurin™] on the accuracy of the Monte Carlo model [111, 112] to extract [THb], [β-carotene], and <μs>. The absorber concentrations and scattering for the phantoms were also based on the ranges seen in the site-level clinical study [12, 25]. However, for the phantom study the median scattering level, and minimum [THb] and [β-carotene] levels were chosen. The lowest levels were selected because, theoretically, these would be most affected by high concentrations of Lymphazurin™. The actual absorber and scattering levels for the phantom were as follows: 5.81 cm⁻¹ (<μs>), 16.69 µM ([THb]), 11.23 µM ([β-carotene]), and 0-79 µM ([Lymphazurin™]). One phantom containing the spheres, hemoglobin, and crocin was made; and 12 titrations with increasing [Lymphazurin™] were added. The inverse Monte Carlo model was used to extract the concentrations of the absorbers and <μs>, and the percent error was
calculated between the expected and extracted values to determine the effect of varying concentrations of Lymphazurin™.

The effects of Lymphazurin™ in tissue were also evaluated by computing correlations between the extracted [Lymphazurin™] and each of the extracted optical endpoints ([THb], [β-carotene], and \(<\mu'>\)). These correlations were computed for the initial measurement from the lumpectomy sites since these specimens typically had Lymphazurin™ for sentinel lymph node mapping.

4.3 Results
4.3.1 Sample Sizes

From March 2011 to September 2011, lumpectomies were analyzed from 10 patients resulting in 80 sites. A total of 7 sites were excluded due to poor probe-tissue contact. The tissue was submitted for histopathology on the remaining 73 sites. However, histopathology could only be obtained for 61 of the sites. From May 2009 to October 2010, mastectomies from 19 patients were analyzed, resulting in 38 individually-measured tissue sites. The optical parameters were plotted versus time for every site and inspected for trends; 4 sites were removed due to poor probe-tissue contact and/or motion artifacts observed in the data, 2 additional sites (1 patient) were removed because the optical measurements were made 85 minutes after excision which was much longer than the other sites. Of the remaining 32 sites, 20 had microscopic histological confirmation. Samples with histology confirmation were given an overall
diagnosis of benign or malignant, and were then given a further classification by specific histological subtype. The breakdown of sample sizes and tissue subtypes is shown in Table 18.

**Table 18: Sample sizes of histologically-confirmed sites.**

<table>
<thead>
<tr>
<th></th>
<th>Lumpectomies</th>
<th>Mastectomies</th>
</tr>
</thead>
<tbody>
<tr>
<td>FG</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>FA</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>Adipose</td>
<td>29</td>
<td>4</td>
</tr>
<tr>
<td>Mixed/Other</td>
<td>27</td>
<td>6</td>
</tr>
<tr>
<td><strong>Total Benign</strong></td>
<td><strong>59</strong></td>
<td><strong>13</strong></td>
</tr>
<tr>
<td>IDC</td>
<td>1</td>
<td>5</td>
</tr>
<tr>
<td>DCIS</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>Mixed/Other</td>
<td>0</td>
<td>2</td>
</tr>
<tr>
<td><strong>Total Malignant</strong></td>
<td><strong>2</strong></td>
<td><strong>7</strong></td>
</tr>
<tr>
<td><strong>Total # of Sites</strong></td>
<td><strong>61</strong></td>
<td><strong>20</strong></td>
</tr>
<tr>
<td><strong>Total # of Patients</strong></td>
<td><strong>10</strong></td>
<td><strong>12</strong></td>
</tr>
</tbody>
</table>

**4.3.2 Which parameters are least affected by excision in lumpectomies?**

The purpose of doing these studies was to identify the optical parameters that are least affected by kinetics, the presence of Lymphazurin™, and cautery for optical margin assessment of lumpectomy specimens. Figure 29A-B show representative optical images of the ratios of [β-carotene] to $\mu_s'$ and THb to $\mu_s'$ for a negative (no residual carcinoma within 2mm of the surface) margin and a positive margin.
Histologically confirmed sites are highlighted corresponding to adipose, fibroadipose, or ductal carcinoma \textit{in situ}. Empirical cumulative distribution functions are shown in Figure 29C representing the distribution of the data in the negative and positive representative images. Both the margin-level data and site-level data show that [$\beta$-carotene]/$<\mu>$ and THb/$<\mu>$ decrease with malignancy and were important parameters in differentiating margins in our initial 48-patient study [115].
Figure 29: Example data acquired from 2 lumpectomy margins in our previous study [115]. A) 50x bicubic interpolated images of $\beta$-carotene/$\mu s^{-1}$ from a negative margin and a positive margin. Benign (fat and fibro-adipose tissue) and malignant (ductal carcinoma in situ – DCIS) sites are highlighted. B) Images of THb/$\mu s^{-1}$ with the same sites highlighted. C) Cumulative distribution functions of the pixels in the negative (N) and positive (P) margins.

Our first set of analyses examines the kinetics of lumpectomy specimens to determine how optical images of margins are impacted. Figure 30 shows three representative sites measured from the lumpectomy specimens diagnosed as adipose,
fibroadipose, and fibroglandular. The linear longitudinal model was used to fit lumpectomy data from all 10 patients (Table 20). In this example, β-carotene, <μs>, THb, and the ratios showed little change over time. There was no Lymphazurin™ in these sites. HbSat as well as, oxy-hemoglobin and deoxy-hemoglobin exhibited marked changes over time. Oxy-hemoglobin decreased and deoxy-hemoglobin increased due to the tissue being metabolically active and unable to replenish the oxygen supply. These three parameters all reach a fundamental limit that is dependent on the total amount of hemoglobin present in the vasculature. For this reason, oxy-hemoglobin, deoxy-hemoglobin, and HbSat were not linear throughout the entire measurement window. Therefore, oxy and deoxy-hemoglobin, and HbSat are not shown for the remainder of this manuscript for any analyses.
Figure 30: Example plots of the tissue parameters versus time for three histologically known sites (adipose-A, fibroadipose-FA, fibroglandular-FG) from three different lumpectomy specimens. Symbols are the measured data and lines are the fitted data.

The rate of change could not be compared across tissue parameters because the units and magnitudes of the variables were not the same. Therefore, the percent change at various time points post-excision was calculated to identify the optical parameters with the smallest percent change. Figure 31 shows the percent change in the optical parameters for various time points post-excision. A maximum of 30 minutes is shown here to correspond with our previous lumpectomy study [12, 25, 115, 116] where the
average amount of time elapsed between excision and the end of imaging was 29 minutes. These results show that \([\beta\text{-carotene}], <\mu',>\text{, and } [\beta\text{-carotene}]/<\mu'>\) had the lowest percent changes over a 30 minute time window (median percent changes of -8.2, -13.8, and -8.0%). \([\text{THb}]\) and \([\text{THb}]/<\mu'>\) had larger percent changes of -44.2% and -40.8% respectively; and \([\text{Lymphazurin}^{\text{TM}}]\) had the largest percent change of -228.7%.

This data was also evaluated for correlations between the first optical measurement and the time from excision. Spearman correlations were computed for all measured sites and no significant correlation was found between any of the optical parameters and time from excision to measurement, except for \([\text{Lymphazurin}^{\text{TM}}]\) with \(p=0.0007\). This was likely due to Lymphazurin\(^{\text{TM}}\) draining from the tissue after excision.
Figure 31: Percent change in each tissue parameter versus time from excision. Percent change is calculated as the fitted rate of change divided by the absolute value of the fitted intercept, multiplied by time from excision. Data is from histologically-confirmed lumpectomy sites (not all outliers are shown). The median percent change at 30 minutes is shown for each parameter.

4.3.3 Does the addition of Lymphazurin™ affect the optical absorbers and scatterers in the breast?

In Figure 31 we showed that [Lymphazurin™] had the highest percent change over a 30 minute period and that it was correlated with time from excision. This led us to question whether the amount of Lymphazurin™ would impact the other optical absorbers and scattering. Therefore, Monte Carlo simulations and a tissue mimicking phantom study were carried out to address this question. The simulated data covered the full range of absorption and scattering levels seen in our previous breast studies [12, 25], while the phantom data was for a subset of the breast optical properties that would
result in the worst case scenario, i.e. where Lymphazurin™ dominates the absorption spectrum. The percent error in [THb], [β-carotene] (crocin for the phantoms), and <µ'> as a function of [Lymphazurin™] is shown in Figure 32 for both simulated and phantom data. The simulated data had negligible error, while the phantoms had slightly higher error attributed to experimental measurements. The simulated and phantom data both showed <3.3% error in extracted [THb], [β-carotene], and <µ'> even in the presence of high concentrations of Lymphazurin™ (up to 80µM). With [THb] and [β-carotene] there did not appear to be any relationship of error with increasing [Lymphazurin™]. In the phantom data, when [Lymphazurin™] was approximately 10µM <µ'> was underestimated by the model and as [Lymphazurin™] was increased, <µ'> was overestimated. This should not be a concern though as the percent error was <1.5% and the simulated results showed no trend. In addition, no significant correlation was found between [THb], [β-carotene], or <µ'> and [Lymphazurin™] for the initial measurements in the lumpectomy sites (data shown in Table 19). Overall, these results indicate that Lymphazurin™ in concentrations up to 80µM do not impact the ability to quantify [THb] or [β-carotene], or <µ'> within the wavelength range of 450-600nm.
Figure 32: Average percent errors for Monte Carlo simulated data and phantom data. Data is shown for a single reference “phantom” (\(<\mu_a>=3.85\text{cm}^{-1}, \langle\mu_s'\rangle=6.79\text{cm}^{-1}\) for the simulated data; \(<\mu_a>=3.02\text{cm}^{-1}, \langle\mu_s'\rangle=5.81\text{cm}^{-1}\) for the phantom data). Simulated: 216 diffuse reflectance spectra were created consisting of 3 levels of scattering (4.85, 6.68, 9.15 cm\(^{-1}\)), THb (16.97, 31.03, 55.09 µM), and β-carotene (10.29, 16.29, 24.37 µM) and 8 levels of Lymphazurin\(^{TM}\) (0:10:70 µM). Phantom: Lymphazurin\(^{TM}\) was titrated 12 times (0-79µM) into a phantom consisting of 5.81cm\(^{-1}\) (<\mu_s'>), 16.69µM (THb), and 11.23µM (β-carotene).

Table 19: Correlations of [Lymphazurin\(^{TM}\)] with each optical variable for the initial measurements in the lumpectomy sites (n=61).

<table>
<thead>
<tr>
<th>Initial Measurement</th>
<th>ρ</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>β-carotene (µM)</td>
<td>0.039</td>
<td>0.765</td>
</tr>
<tr>
<td>(&lt;\mu_s'&gt;) (cm(^{-1}))</td>
<td>0.030</td>
<td>0.818</td>
</tr>
<tr>
<td>β-carotene/&lt;\mu_s'&gt; (µM-cm)</td>
<td>-0.014</td>
<td>0.917</td>
</tr>
<tr>
<td>THb (µM)</td>
<td>0.194</td>
<td>0.134</td>
</tr>
<tr>
<td>THb/&lt;\mu_s'&gt; (µM-cm)</td>
<td>0.058</td>
<td>0.658</td>
</tr>
</tbody>
</table>

4.3.4 Are there differences between cauterized and non-cauterized tissue?

Cautery artifacts on tissue in H&E stained histology slides have proved to be problematic for diagnosing surgical margin status [137]. Given that cauter is visible under H&E we questioned how this might impact optical imaging of tumor margins. To evaluate this effect we compared benign sites from cauterized lumpectomies and non-
cauterized, incised mastectomies. Figure 33 shows the initial measurement of each optical endpoint separated by specimen type. [THb] and [Lymphazurin™] were the only parameters that were significantly higher (p=0.013 and 0.0004, respectively) in the lumpectomies compared to mastectomies.

Figure 33: Optical parameters of the first time point from the histologically-confirmed benign sites of mastectomies (M) and lumpectomies (L). Statistical significance (*) indicates p<0.05) calculated with a Wilcoxon rank-sum test.

We also examined the differences in the rates of change (constrained to a 10 minute time window) between mastectomy and lumpectomy benign sites (Figure 34). [β-carotene] and [β-carotene]/<µs> were the only parameters that were not significantly (p=0.13 and 0.36 respectively) different between the two types of specimens.
Figure 34: Rate of change (fitted values from the model) in the tissue parameters from the histologically-confirmed benign sites of mastectomies (M) and lumpectomies (L) constrained to a time window of 10 min for all sites. Statistical significance (*) indicates p<0.05 calculated with a Wilcoxon rank-sum test.

4.3.5 Are kinetics different between benign and malignant tissue?

In section 3.2 we quantified the percent change in lumpectomy sites over different time points post-excision. In terms of margin assessment, it is important to ensure that contrast between benign and malignant regions is preserved over time and that the rate of change is not different between tissue types. Ideally this analysis would have been carried out with lumpectomy specimens, however, isolating malignant sites on a lumpectomy margin is difficult. Therefore, to compare the rates of change between benign and malignant tissue, we utilized mastectomy specimens that could be incised to reveal gross tumor.
Figure 35 shows representative plots of two sites from two different patients, one measured with the shortest time from excision and the other with the longest time from excision. For each patient the histologically-confirmed benign and malignant site are shown. From the measured data we see that [β-carotene], <µs>, and [β-carotene]/<µs> were relatively invariant with time as was observed above. [THb] and [THb]/<µs> were also relatively invariant, although the benign site of Patient 1 had a slightly higher slope. The fitted data shown in this figure were from the longitudinal model. The model provided excellent fits to [THb], [β-carotene], <µs>.

Figure 35: Example plots of the tissue parameters versus time for four histologically known sites from two mastectomy patients. Symbols indicate the measured data, lines are the model fits for the benign and malignant tissues.
To quantify how much the tissue parameters changed over time, the data from the mastectomy sites was fit with the longitudinal model. Table 20 shows the fitted rate of change in the tissue parameters; a negative value indicates a parameter that decreased over time and a positive value indicates a parameter that increased with time. Most of the tissue parameters decreased with time and the rate of change was similar between the benign and malignant mastectomy sites. An interaction test was used to determine if the histology of the measured sites affected the rate of change. The results (Table 20) indicate that the rate of change did not differ significantly between the benign and malignant sites for any of the tissue parameters. This data was also evaluated for correlations between the first optical measurement and the time from excision. Spearman correlations were computed for both benign and malignant sites and no significant correlation was found between any of the optical parameters and time from excision (lumpectomy or mastectomy).

Table 20: Rate of change per minute (fitted values from the model) for each tissue parameter of the benign (n=13) and malignant (n=7) sites measured in mastectomy specimens and benign (n=59) sites measured in the lumpectomy specimens. Reported values indicate the average ± standard deviation. P-values indicate the statistical differences in the rate of change between the benign and malignant mastectomy sites.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Lumpectomy Benign</th>
<th>Lumpectomy Malignant</th>
<th>Mastectomy Benign</th>
<th>Mastectomy Malignant</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>β-carotene (µM/min)</td>
<td>-0.027±0.143</td>
<td>0.015±0.062</td>
<td>0.002±0.045</td>
<td>0.627</td>
<td></td>
</tr>
<tr>
<td>&lt;μs'&gt; (cm⁻¹/min)</td>
<td>-0.034±0.040</td>
<td>-0.010±0.019</td>
<td>-0.008±0.023</td>
<td>0.829</td>
<td></td>
</tr>
<tr>
<td>β-carotene/&lt;μs'&gt; (µM-cm/min)</td>
<td>0.010±0.020</td>
<td>0.005±0.009</td>
<td>0.001±0.005</td>
<td>0.304</td>
<td></td>
</tr>
<tr>
<td>THb (µM/min)</td>
<td>-0.537±0.750</td>
<td>-0.036±0.191</td>
<td>-0.123±0.112</td>
<td>0.256</td>
<td></td>
</tr>
<tr>
<td>THb/&lt;μs'&gt; (µM-cm/min)</td>
<td>-0.062±0.098</td>
<td>-0.005±0.043</td>
<td>-0.011±0.012</td>
<td>0.698</td>
<td></td>
</tr>
</tbody>
</table>
4.3.6 What happens to optical contrast for margin assessment?

The results in this manuscript indicate that [β-carotene] and [β-carotene]/<μ'> are the most robust variables but still change ~8% in 30 minutes. The question that arises is: what happens to contrast between negative and positive margins if a negative margin is imaged immediately after excision and a positive margin is imaged 30 minutes after excision, or vice versa? Figure 36 helps to illustrate the extent to which kinetics affect optical contrast. Images of a positive and negative margin from two different lumpectomies imaged at approximately the same time points post-excision are shown. The “initial” image is the actual parameter map that was measured. The median percent change for each variable at 10, 20, and 30 minutes post-excision (data from Section 3.2) was applied to either the negative or positive image to artificially decrease contrast. In Figure 36A this was done by multiplying the negative image by the median percent change. For [β-carotene], [THb], and [THb]/<μ'>, the percent change was in the negative direction and positive margins have lower values for these variables. To show the worst case scenario, the percent change was applied to the negative margin to decrease contrast. In Figure 36B, [β-carotene]/<μ'> is lower in positive margins but increases over time; therefore, the percent change was applied to the positive margin to decrease contrast. In Figure 36C, <μ'> is higher in positive margins but decreases over time, so the percent change was applied to the positive margin. These images show that an 8% change in [β-carotene] and [β-carotene]/<μ'> does not alter the contrast between
the negative and positive margin. $<\mu_s/>$ contrast also does not change significantly; however the initial contrast is not as apparent. By 30 minutes, the $>40\%$ change in [THb] and [THb]/$<\mu_s/>$ greatly reduces the contrast between the positive and negative margin; however, contrast is preserved.
Figure 36: 50x bicubic interpolated images of a negative and positive margin from different patients, where the “initial” images of the margins were imaged at approximately the same time points post-excision. The “initial” images represent the actual data measured. The median percent change at 10, 20, and 30 minutes was applied to either the negative or positive image to show how an image would change if measured at various time points beyond the “initial” image. A) For β-carotene, THb, and THb/<μ‘>, the negative margins have higher values and the kinetics decrease over time. Therefore, the percent change is applied to the negative margin to show decreasing contrast (worst case scenario). B) For β-carotene/<μ‘> the negative margins have higher values and the kinetics increase over time. C) For <μ‘> the positive margins have higher values but the kinetics decrease over time.

Using our previous site-level data [12, 25], a percent difference was calculated between adipose and positive malignant sites; fibroadipose and positive sites; and
fibroglandular and positive sites. These values were compared to the percent change at 10, 20, and 30 minutes for the lumpectomy kinetics data. The percent change is smaller than the percent difference for all optical parameters, indicating that optical contrast should be preserved within a 20 minute time window.

Table 21: Comparison of the percent change in each optical parameter at 10, 20, and 30 minutes post-excision to the percent differences between 1) adipose (A) and positive (P) sites, 2) fibroadipose (FA) and positive sites, and 3) fibroglandular (FG) and positive sites from our initial site-level study [12, 25]. A negative value in the percent difference indicates that positive sites were greater; a positive value means the benign tissue was greater.

<table>
<thead>
<tr>
<th></th>
<th>% Change</th>
<th>% Difference</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>At 10 min.</td>
<td>At 20 min.</td>
</tr>
<tr>
<td>β-carotene (µM)</td>
<td>-2.7</td>
<td>-5.5</td>
</tr>
<tr>
<td>&lt;µ_s’&gt; (cm⁻¹)</td>
<td>-4.6</td>
<td>-9.2</td>
</tr>
<tr>
<td>β-carotene/&lt;µ_s’&gt; (µM-cm)</td>
<td>2.7</td>
<td>5.3</td>
</tr>
<tr>
<td>THb (µM)</td>
<td>-14.7</td>
<td>-29.4</td>
</tr>
<tr>
<td>THb/&lt;µ_s’&gt; (µM-cm)</td>
<td>-13.6</td>
<td>-27.2</td>
</tr>
</tbody>
</table>

4.4 Discussion and Conclusions

Quantitative spectral imaging can be used to accurately quantify optical parameters related to tissue morphology which can be used to identify residual disease in breast tissue margins. The context in which this technology is applied whether it is in the intra-operative setting or post-operative setting is influenced by time after excision and imaging of the resected specimen is influenced by surgical factors including cautery.
and the presence of Lymphazurin™. This work underscores the importance of understanding the effects of time after excision, Lymphazurin™ and cautery on the primary sources of optical contrast in the breast.

This technology is not only important for breast margin assessment but also for other applications where optical devices are used on excised tissues. A number of other groups have investigated the use of diffuse reflectance spectroscopy in the measurement of ex vivo breast tissue [15, 22, 23, 138-140]. However, there is a lot of variation in the types of breast specimens (biopsies, lumpectomies, mastectomies, reduction mammoplasties) that have been measured, whether they had been stored in solutions and/or partially processed, and the amount of time that lapsed post-excision before measurements were made. All of these reports involved spectroscopic measurement of breast tissues over a wide range of intervals (<30 minutes to 12 hours) after excision from the body. These changes may be reflected in the optical measurements of the tissue, and the lack of consistency in measurement time intervals post-excision in the reported studies could make it difficult to confidently compare results across studies. The work presented here provides a framework in which investigators using similar technologies can interpret data, design experiments and conduct their own quality control measurements.

In our previous margin-level study [12, 25, 115, 116], where we performed quantitative diffuse reflectance spectral imaging of ex vivo breast lumpectomy margins,
tissue kinetics and cautery were not accounted for explicitly in the analysis of those data. Sites where the diffuse reflectance at 600 nm was lower than at 450 nm were excluded to account for high concentrations of Lymphazurin™ but the actual impact had not been examined. However, it is not surprising that we did find that the ratios of [β-carotene]/<μ'> and [THb]/<μ'> were the best parameters to differentiate cancer-free margins from margins that contained residual cancer (sensitivity = 79.4% and specificity = 66.7%) [115]. From the current study we determined that HbSat cannot be fit with a linear model due to excessive changes in oxygenated and deoxygenated hemoglobin post-excision. This is likely due to oxygen being consumed by the metabolically active tissue immediately after excision. Although HbSat may be a useful in vivo parameter for determining tumor hypoxia, or for examining the local microenvironment, or even for margin assessment of the resected cavity, it is not reliable in ex vivo margin assessment of breast tissue specimens. In this study we show that [THb] and [THb]/<μ'> are less likely to be affected by post-excision kinetics than HbSat or [Lymphazurin™], though both variables had >40% change in a 30 minute time window. Interestingly, the percent differences between positive malignant sites versus adipose, fibroadipose, and fibroglandular sites were much larger than the percent change in 30 minutes. In fact, it would have taken 63 minutes for the percent change in [THb] to exceed the percent difference between positive malignant and fibroglandular sites. Therefore, parameters involving [THb] may have large percent changes over time but the contrast between
benign and malignant tissues appears to be greater within a reasonable time window. 

\[ [\beta\text{-carotene}], \langle \mu_s \rangle, \text{ and } [\beta\text{-carotene}]/\langle \mu_s \rangle \] were least affected by kinetics (<14% in 30 minutes).

The results from both the simulated and phantom data for [Lymphazurin™] indicate that [Lymphazurin™] does not impact the extractions of [THb], [\beta\text{-carotene}], or \langle \mu_s \rangle from the diffuse reflectance spectra. The simulations had <0.5% error with Lymphazurin™ up to 70\text{µM}. In the phantoms, [THb], [\beta\text{-carotene}], and \langle \mu_s \rangle were all extracted with <3.3% error with Lymphazurin™ up to 80\text{µM}. Although the errors were higher in the phantom data (as would be expected), there was no trend in the percent error with increasing [Lymphazurin™]. In addition, no significant correlation was found between the extracted [Lymphazurin™] and [THb], [\beta\text{-carotene}], or \langle \mu_s \rangle in the lumpectomy sites for the initial measurements. Significant correlations were seen between [Lymphazurin™] and [THb] (and [Lymphazurin™] and [THb]/\langle \mu_s \rangle) when all time points were included which was likely due to draining of both Lymphazurin™ and blood throughout the measurements. Therefore, we can conclude that Lymphazurin™ up to 80\text{µM} adds minimal error to the extraction of the relevant sources of optical contrast; again, the highest concentration of Lymphazurin™ seen in the previous lumpectomy study was 72.7\text{µM}.

To determine the effects of cautery on the optical endpoints, we examined the differences in initial values and rates of change between the cauterized lumpectomy
sites and the non-cauterized mastectomy sites. In the initial values, we found that [THb] was significantly higher in the benign sites of the cauterized lumpectomies compared to the mastectomies. This initial difference could be due either to varying excisional times for mastectomy and lumpectomy procedures or due to cauterization. Since we observed no significant correlation between the initial value and time from excision, we assume that this difference in [THb] is due to cauterization of the vasculature to prevent blood from draining out of the vessels as rapidly as it would in mastectomy specimens. As for the rates of change, [β-carotene] and [β-carotene]/<μ> were the only parameters that were not significantly different between the lumpectomy and mastectomy specimens.

For all tissue parameters, the rate of change was not significantly different between the benign and malignant sites. This is an important finding for margin assessment which indicates that optical contrast between benign and malignant regions of a margin will be preserved, regardless of the time when the margin is imaged over a 30 minute window. We also showed that there was no correlation between the time from excision and the initial value (or first measurement) of the optical data. This suggests minimal change in the data within the time window that we examined (17±4 minutes post-excision and measured for 10-32 minutes). Additionally since there was no significant difference between the lumpectomies and mastectomies for [β-carotene] and [β-carotene]/<μ>, we can extrapolate these findings to benign and malignant tissue in cauterized lumpectomies.
5 Conclusions and Future Directions

5.1 Conclusions

The work in this dissertation demonstrates that variability in instrumentation, surgical procedure, time of specimen excision from the body, and inter-patient differences in micro-morphology affect the extractions of optical parameters for breast margin assessment. Despite all of these factors, this work shows the feasibility of using an optical device to assess the presence of residual carcinoma on the margins of lumpectomy specimens in hopes of reducing surgical re-excision rates in patients undergoing breast conserving therapy. Currently, patients have to return for additional surgeries up to 72% [141] of the time which is a burden to patients financially and emotionally. Histopathology is the current gold standard for identifying residual cancer at the margin; however, this is typically performed post-operatively and patients do not receive the results until about a week later. Thus, a device that can provide feedback on surgical margin status while the patient is still on the operating table would help reduce re-excision rates. However, developing a robust device to image excised breast tissue can be a challenge due to differences in surgical techniques, post-excision imaging times, and inter-patient variability, especially for optical devices which measure hemoglobin content, \( \beta \)-carotene content, and other parameters related to tissue micro-morphology.

The optical imaging platform that our group has developed can measure parameters that are related to breast micro-morphology and can be used for either intra-
operative margin assessment of breast tissue or for pathological sampling of breast tissue. The device, based on diffuse reflectance spectral imaging, consisted of a Xenon light source, spectrometer, CCD, and a multi-channel fiber optic imaging probe. This device was used to obtain optical images of lumpectomy margins. The current device has a sensing depth of ~2mm which is ideal for margin assessment at DUMC, however, the sensing depth can be tailored to meet the requirements of the particular institution or application. Table 22 provides a breakdown of the characteristics of the spectral imaging device in terms of margin assessment device needs and compares our technology to other optical devices that have been reported. The speed requirement is that an entire margin must be imaged in less than 20 minutes. Compared to other optical devices, diffuse reflectance spectral imaging is ideal for breast tumor margin assessment.

Table 22: Comparison of diffuse reflectance spectral imaging to other optical techniques for breast margin assessment. DRSI – diffuse reflectance spectral imaging; FL – fluorescence; SPX – spectroscopy; OCT – optical coherence tomography; HRME – high resolution micro-endoscopy.

<table>
<thead>
<tr>
<th></th>
<th>DRSI</th>
<th>FL</th>
<th>Raman SPX</th>
<th>OCT</th>
<th>Confocal Imaging/HRME</th>
<th>Molecular Imaging</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fast (&lt;20 min.)</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
</tr>
<tr>
<td>Non-invasive Coverage Area (&gt;10cm²)</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
</tr>
<tr>
<td>Resolution (&lt;5mm)</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
</tr>
<tr>
<td>Sensing Depth (0-2mm)</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
</tr>
</tbody>
</table>
Variability in breast tissue composition was evaluated to determine the impact on optical margin images. Mammographic breast density affected $[\beta\text{-carotene}], <\mu'>$, and $[\beta\text{-carotene}]/<\mu'>$ to varying degrees in negative margins. Thus, morphological differences in benign tissue composition impacted the optical contrast between negative and positive margins and the ability to predict surgical margin status. Increased breast density was associated with increased $[\beta\text{-carotene}], <\mu'>$, and $[\beta\text{-carotene}]/<\mu'>$. This observation was counterintuitive to our assumption that patients with high breast density breasts would have more fibroglandular tissue and less fat, which would result in decreased $[\beta\text{-carotene}]$. This spurred an investigation of adipose tissue, since $\beta$-carotene is predominately stored in adipocytes. $[\beta\text{-carotene}]$ was found to be higher in adipose tissue with smaller adipocytes and decreased with increasing adipocyte size. High MBD patients had smaller adipocytes and greater adipocyte density. This analysis of the adipose tissue was consistent with our margin-level $[\beta\text{-carotene}]$ values and suggested a biological reason for the trend which helped to improve contrast between negative and positive margins in high density patients. Predictive modeling of surgical margin status in neoadjuvant naive patients resulted in accuracies of 77% and 80% for low and high density patients, respectively. The increased accuracy in high density patients is important, since surgeons have a more difficult time determining margin status in these patients, which ultimately leads to higher re-excision rates.
Incised mastectomies and lumpectomy margins were used to understand tissue kinetics and the effect of cauterization on the margins. These studies showed that there was no significant difference in the rates of change between benign and malignant tissue for any of the optical parameters ([THb], [β-carotene], <μ'>, or HbSat). From this we also found that HbSat changed drastically over time and could not be characterized with a linear model; and [Lymphazurin™] also changed drastically over time (>200% in 30 minutes). Therefore, for the purposes of ex vivo margin assessment, HbSat (along with oxy-hemoglobin and deoxy-hemoglobin) and [Lymphazurin™] should not be used, and were therefore excluded from any further analysis of surgical margin status. Variables involving [THb] were found to be significantly affected by cautery and changed by >40% in a 30 minute time window. For these reasons [THb] and [THb/<μ'>] were likely not necessary for predicting surgical margin status with the CIT model. [β-carotene], <μ'>, and [β-carotene/<μ'>] were least affected by cautery and kinetics and were the three parameters that we determined to be suitable for margin assessment. These three parameters have also consistently been the best parameters for differentiating margins.

Although HbSat was found to change too drastically over time for the purposes of ex vivo margin assessment, this might still be a useful parameter with which to measure tumor hypoxia in vivo. In a previous study by our group [24], we found that HbSat was an important variable for differentiating benign and malignant tissue in vivo.
Lymphazurin™, a blue dye used for sentinel lymph node mapping, can be present on the excised tissue, sometimes in excessive quantities. The work in Chapter 4 showed that up to 80µM Lymphazurin™ added <3.3% error to the extracted values of [THb], [β-carotene], and <µ'.> The highest [Lymphazurin™] observed in our previous site-level study [25] was 72.7µM. Therefore, we can conclude that the Lymphazurin™ concentrations present on our lumpectomy specimens added little error to the optical images of the margins, even though Lymphazurin™ decreased quite significantly over time. To ensure >80µM Lymphazurin™ did not impact the data, diffuse reflectance spectra with raw intensities at 450nm greater than intensities at 600nm were excluded from the margin images.

This work demonstrated that diffuse reflectance spectral imaging can be used to accurately quantify optical parameters related to tissue morphology of excised specimens. It also shows the feasibility of using an optical device for margin assessment when surgical factors and inter-patient variability are accounted for. Such a device could be helpful in reducing surgical re-excision rates, especially in hospitals without specimen mammography, frozen section histology, touch-prep cytology, and/or limited pathology resources. The studies in this dissertation were performed specifically for breast margin assessment but point to the fact that this sort of device could also be used in other ex vivo applications, such as guiding surgical pathology for sampling of excised specimens to reduce the amount of tissue that would need to be evaluated.
5.2 Future Work

The results of this dissertation lay the groundwork for a number of future studies that can be completed to further understand the biology of breast tissue at the microscopic and macroscopic scales, and for improvements that can be made to optical imaging platforms for the purposes of breast tumor margin assessment.

5.2.1 Microscopic Scale: Assessing the Tumor Micro-environment

5.2.1.1 $\beta$-carotene, Adipocyte Size, and Breast Density

**Hypothesis:** Women with dense breast tissue have higher levels of $\beta$-carotene in their adipose tissue and have smaller adipocytes in truly normal breast tissue.

**Rationale:** One of the most important findings from this work was the relationship between $[\beta$-carotene], mammographic breast density, and adipocyte size. Although we found that $[\beta$-carotene] was inversely related to adipocyte size and that high density patients had smaller adipocytes, it is currently unknown why adipocytes would be smaller in high density patients. Under normal conditions, $\beta$-carotene is absorbed through the intestines and circulates in the blood before being transported to the liver. If there is an adequate amount of $\beta$-carotene in the body, the excess $\beta$-carotene is stored in adipose tissue. Inside the adipose cell, $\beta$-carotene is converted into retinaldehyde via central cleavage by the enzyme $\beta$-carotene monoxygenase type I (Bcmo1) [38]. Retinol, a byproduct of $\beta$-carotene is also converted to retinaldehyde with aldehyde dehydrogenase (ADH1) [38]. Retinaldehyde can then be converted into retinoic
acid with retinaldehyde dehydrogenase (Raldh1) [38]. This β-carotene/retinoic acid pathway is important in adipocyte differentiation and has also been shown to play an important role in modulating adipocyte size. Notable studies have found the following: inhibiting Bcmo1 increases adiposity [38], retinaldehyde inhibits adipocyte size and decreases adiposity [38], and retinoic acid induces lipolysis (the breakdown of lipids) [38]. Another study showed associations between circulating carotenoids and mammographic breast density. Tamimi et al [71] showed that mammographic breast density was higher in patients with greater levels of circulating β-carotene (although this was non-significant). Taken together, these findings show that [β-carotene] and adipocyte size are related, and that there may be some relationship between carotenoids and breast density, but do not indicate why [β-carotene] and/or adipocyte size are modulated by breast density. Further investigation into the molecular biology of adipocytes would be important for understanding this observation.

**Approach:** To begin, it would be important to look at [β-carotene] per cell in truly normal breast tissue. One way of doing this would be a combined confocal/spectroscopic imaging technique to obtain images of both the tissue morphology at the cellular level, with a map of [β-carotene] overlaid on the image. Biopsies from reduction mammoplasties (normal breast tissue) could be obtained and sectioned for imaging. It would be important to first establish that smaller adipocytes do indeed have higher [β-carotene] in truly normal tissues within a specific breast
density category (likely MBD-2 or MBD-3 since the majority of patients fall within this range). If this holds up, it would then be important to look at images from all breast densities (MBD-1 through MBD-4).

5.2.1.2 Surveillance of the Tumor Micro-environment Using Quantitative Spectral Imaging

**Hypothesis:** Adipose $[\beta$-carotene] decreases with increasing distance from malignant cells due to increasing adipocyte sizes and scattering decreases due to less collagen in the micro-environment.

**Rationale:** It is obvious from the work in this dissertation that tissue architecture greatly affects optical measurements of the breast. Therefore, it is possible that an optical device could be used to study the tumor micro-environment through measures of collagen content and $[\beta$-carotene]. Recently, there have been a number of studies focused on the tumor micro-environment and understanding how this impacts the growth and aggressiveness of a tumor. Some studies [57, 58], which were discussed in Chapter 1, have focused on the orientation of collagen to the tumor boundary, and others [53, 54] have focused on cancer-associated adipocytes (adipocytes near a cancer that are in constant cross-talk with the tumor cells). The research on cancer-associated adipocytes (CAAs) suggests that crosstalk between tumor cells and nearby adipocytes leads to a reduction in adipocyte size via lipolysis and a high fibroblast-like cell to adipocyte ratio in the stroma surrounding cancer cells. In these studies, CAAs also showed decreased levels of PPAR-$\gamma$, aP2, C/EBP$\alpha$, resistin, and hormone-sensitive lipase.
all of which are important in the differentiation and regulation of adipocytes. Increased levels of inflammatory markers, such as IL-6, were also seen in CAAs co-cultured with cancer cells. Increased IL-6 has been correlated with poor disease outcome and supports tumor growth and metastasis. CAAs which were once normal adipocytes, essentially dedifferentiate and signal pathways that are crucial for cancer cell survival and growth.

In Chapter 3 we showed that [β-carotene] is modulated by adipocyte size.

**Approach:** An interesting study that could be completed to better understand CAAs would be to determine how adipocyte size and [β-carotene] change as a function of distance from the tumor (irrespective of breast density). This could be done by slicing directly into a tumor and taking optical measurements at specific intervals from the center of the tumor to an outside area of grossly benign tissue. The exact distance interval would have to be determined; the work by Dirat et al [54] suggests that the changes to the adipocytes occur directly at the invasive front of a tumor (i.e. within a few cells layers), but this exact distance was not quantified. Finally, en face and perpendicular histological sections of the tumor and surrounding area would be important for co-registering the optical data. This type of study could also be used to determine how collagen fluorescence or scattering changes as a function of distance from the tumor. As noted in Chapter 1, other research [56-58] has focused on the way in which collagen organizes itself around a tumor and how that organization can promote tumor invasion.
Important questions that could be asked with this study would be: 1) does [β-carotene] decrease with distance from the tumor?; 2) can collagen fluorescence be used to determine collagen alignment near a tumor?; 3) are scattering signatures different at the invasive front of a tumor versus collagenous tissue that is farther away?; and 4) is collagen fluorescence or scattering better at detecting collagen alignment at the invasive tumor front?

5.2.1.3 β-carotene and Tumor Aggressiveness

**Hypothesis:** Adipose tissue adjacent to a high grade, invasive tumor has higher levels of β-carotene compared to a low grade, *in situ* tumor.

**Rationale:** Given that collagen and adipocytes are different at the invasive front of a tumor, and given that dense breast tissue, which has relatively more collagen and smaller adipocytes, increases breast cancer risk, one must ask the question: does high density breast tissue have different collagen and/or [β-carotene] levels near a tumor that make the tumor more aggressive? Or is [β-carotene] a marker for cancer risk? This would be a difficult study to complete, but it may offer insight into why dense breast tissue increases breast cancer risk.

**Approach:** Mastectomy specimens could again be used for this study by slicing directing into the center of the tumor. Spectral images could then be acquired of the tumor and surrounding normal tissue. [β-carotene] and scattering/collagen could be quantified as a function of distance from the tumor. For this type of study it would be
important to look at both invasive and in situ carcinomas and ductal and lobular types. Presumably invasive tumors would have smaller surrounding adipocytes and higher [β-carotene]. In addition, higher grade tumors that are more proliferative may also show higher [β-carotene] and smaller adipocytes.

5.2.2 Macroscopic Scale: Additional Studies for Margin Assessment

5.2.2.1 Benign Abnormalities and the Less Common Breast Cancers

Hypothesis: Spectral images of benign breast abnormalities with increased fibrous tissue (such as fibrocystic change) are similar to positive margin images and are misdiagnosed.

Rationale & Approach: The data presented in this dissertation is just an initial understanding of how an optical device can be used for margin assessment. The majority of the data was sampled from patients who had tumors consisting of ductal carcinoma in situ (DCIS) or invasive ductal carcinoma (IDC). There were also a handful of lobular carcinomas. Moving forward, it will be important to establish what optical images look like for other types of breast cancers such as tubular and mucinous carcinomas, as well as more lobular carcinomas. Additionally, measurements from tissue with atypical ductal hyperplasia should be thoroughly compared to measurements of DCIS, as these are often difficult to differentiate with standard histopathology [142]. This is also true of atypical lobular hyperplasia and lobular carcinoma in situ. Other types of tissue that were not sampled in this imaging study
were benign lesions such as papillomas, fibroadenomas, and fibrocystic change. The increased fibrous tissue in these lesions may result in increased scattering signatures, resulting in false positives. For this optical imaging device to be an effective intra-operative tool, the full spectrum of normal, benign, and malignant tissue must be sampled.

5.2.2.2 Neoadjuvant Therapy

**Hypothesis:** Optical images of negative margins from neoadjuvant therapy patients have increased scattering and decreased $\beta$-carotene resulting in misclassification using the CIT model for neoadjuvant-naïve patients.

**Rationale:** Patients who had undergone neoadjuvant chemotherapy or endocrine therapy were excluded from the analyses in this work due to potential effects on the optical data due to alterations in the tissue’s structure and composition. This data was not presented due to small sample sizes, especially among the endocrine patients, and since patients received a variety of neoadjuvant treatments, and no standardization method was available. From the data that was analyzed, $\langle \mu_s \rangle$ was the parameter that was most affected by neoadjuvant chemotherapy, likely due to increased fibrosis in these patients.

**Approach:** Margin images from patients undergoing similar neoadjuvant therapy treatments should be compared in a similar fashion to how the breast density data was analyzed in Chapter 3. A CIT model was developed specifically for patients
with different breast densities and it may be important to develop a separate predictive model for neoadjuvant patients which leverages optical contrast of different variables. Finally, response to neoadjuvant therapy also differs with tumor type. Optically probing this information could provide insight into how the micro-morphologically of a tumor responds to neoadjuvant therapies.

5.2.3 Technological Improvements

5.2.3.1 Footprint Reduction

The most important modification that is necessary for this imaging platform to serve as an effective intra-operative device is to decrease the footprint of the instrument for better portability in the operating room. The 8CH system has a large footprint of approximately 4x2x3 ft. (L,W,H). Our group is currently working on designing a smaller system with light emitting diodes to replace the Xenon light sources, and photodiodes to replace the current detection components (spectrometers and CCDs) [143]. This would reduce the footprint to approximately 1.2x1x0.7 ft., a 28x reduction in size.

5.2.3.2 Microscopic Imaging

Tissue sampling is an issue with current histopathological processing – with a sampling resolution between 3-5mm, while cancer cells are on the order of μm in size. Currently, the sampling resolution of the 8CH system is comparable to that of standard histopathological processing at 5mm. Given the amount of time required to survey
tissue, it is not feasible to look at the entire tissue surface. Therefore, it is possible that residual carcinoma at the margin may be missed, since only a 5µm slice is inspected every 3mm. This issue was evaluated by Guidi et al [130] where they looked at the presence of tumor in perpendicularly-sliced sections of the inked margin (the type of analysis done here at DUMC) versus evaluating tissue from an en face cut of the margin (i.e. shaved margin). They found that of 69 positive shaved margins, only 42 inked margins were positive. These results suggest that residual carcinomas may be missed with the current sampling resolution. Therefore, µm resolution would be ideal for a margin assessment device to cover as much tissue as possible to identify small areas of residual carcinoma. In modifying the device to achieve this resolution, care must be taken to maintain a sensing depth of 1-2mm capable of identifying carcinomas that are close to the margin surface.

5.2.3.3 Modeling Collagen and Cells in Breast Tissue Using Mie Versus Rayleigh Light Scattering Models

One of the main optical parameters analyzed in this work was the reduced scattering coefficient. Light scattering occurs due to differences in the refractive indices at a boundary. Light, therefore, is scattered by a variety of biological structures with different shapes and sizes when there is an index of refraction mismatch. It is believed that $\mu_s$ is a measure of light scattering from cell membranes, nuclei, mitochondria and other cellular organelles, and collagen; however, the exact source is not known [127]. In our Monte Carlo model [144], we currently model light scattering based on Mie theory,
which assumes that particles are spherical in shape. This may not be the most accurate model to use, especially if collagen, a fibrous structure, is the main source of scattering in breast tissue. Using a model that accounts for scattering from non-spherical particles in a variety of sizes may be important for accurately quantifying scattering in breast tissue. A Rayleigh model may also be useful in quantifying scattering from particles that are much smaller than the wavelength of light. To better understand the scattering results in this work, it will be important to determine what structures dominate light scattering and if the current Mie model is the most appropriate method for modeling light scattering from those structures.

5.2.3.4 Fluorescence Imaging and Contrast Agents

Adding the capability of fluorescence imaging to the device would provide additional information about the tissue micro-morphology. Our group has previously measured collagen and NADH fluorescence in bread-loafed breast tissues [19] and found that benign/fibrous and malignant tissue had significant differences in their collagen contributions to the fluorescence spectra; no significant difference was found with NADH contribution. This indicates that fluorescence from collagen could provide additional structural information, while fluorescence from NADH and FAD could potentially add information about the metabolic activity of the tissue (although the kinetics of these variables would need to be evaluated for ex vivo use). Examining collagen fluorescence could help address this question of whether collagen is a
significant contributor to $\langle \mu \rangle$. If $\langle \mu \rangle$ is not a measure of collagen content, knowing the amount of collagen present in the tissue could add additional information that is important for predicting surgical margin status.

All of our work thus far has relied on endogenous contrast from the tissue architecture. Recent advances in optical imaging coupled with contrast agents (such as acriflavine or 2-NBDG) enable enhanced visualization of microscopic features. In particular, Richards-Kortum et al have developed a high resolution fluorescence microendoscope (HRME) and used it in conjunction with acriflavine, a fluorescent nuclear stain, to image dysplasia and early cancer in the oral cavity and esophagus [145, 146]. While this tool has been used to diagnose cancer in these sites, its potential has yet to be determined in breast tissue. Our group is currently using optical spectral imaging to identify suspicious regions over large areas of breast tumor margins. Acriflavine is then applied to the margin and the suspicious regions are interrogated with the HRME device. The goal of this study is to evaluate the potential of HRME and acriflavine in the context of surgical margins specifically, to see if this tool can detect the presence of microscopic disease at margins intra-operatively without the need for time-intensive tissue processing.

Another potential contrast agent for breast margin assessment is the metabolic fluorescent contrast agent 2-[N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino]-2-deoxy-D-glucose (2-NBDG). 2-NBDG follows a similar metabolic pathway as D-glucose by
entering the cell via the glucose transporters and is then phosphorylated by hexokinases I-II. This resulting fluorescent metabolite remains in the cell until further decomposition. Therefore, this contrast agent can be used to monitor cellular metabolism and has been used by our group to examine 2-NBDG uptake in a variety of breast cancer cell lines [147]. Langsner et al [148] recently evaluated the effects of 2-NBDG on 14 breast cancer specimens by acquiring multi-spectral images of the specimens before and after incubating them in 2-NBDG. They found increased fluorescent signal in the malignant tissues after application of 2-NBDG, likely due to increased consumption of the contrast agent. This work demonstrates that 2-NBDG has the potential to be used as a contrast agent for breast margin assessment.

Additionally, recent advances in nano-particles and activatable cell-penetrating peptides (ACPPs) have shown promise in pre-clinical studies for margin assessment. Nguyen et al [149] developed a technique to better visualize tumor margins during surgery using ACPPs in a mouse model and compared residual tumor cells with and without guidance of ACPPs. They found that ACPPD-based (ACPPs conjugated to dendrimers) fluorescent imaging guidance resulted in ~90% fewer residual cancer cells following surgery compared to standard surgery since ACPPs were able to better delineate the tumor border. Bickford et al [150] reported on using nanoshells to enhance contrast in HER2-overexpressing cells with near-infrared reflectance confocal microscopy. Gold nanoshells were incubated with three different cell lines and tissue
sections. In both the cell and tissue studies, nanoshells bound to HER2+ breast cancer cells showed dramatically enhanced scattering compared to normal or HER2- cancer cells. These two studies suggest that molecular imaging may also be useful for breast tumor margin assessment.
References


162


Biography

Torre Bydlon was born March 18, 1984 in Dallas, Texas. She received her BS degree magna cum laude in Electrical Engineering from Tufts University in May 2006. She then moved to Durham, North Carolina where she completed her PhD in Biomedical Engineering at Duke University in May 2012.

Publications


Scholarships, Fellowships, Memberships, and Honorary Societies

- Harry Poole Burden Prize in Electrical Engineering (2006)
- Eta Kappa Nu Honor Society (2006)
- Optical Society of America
- Institute of Electrical and Electronics Engineers