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Cancer detection and treatment: surgical margin status evaluation and monoclonal antibody determination

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Cancer Detection and Treatment: Surgical Margin Status Evaluation and Monoclonal Antibody Determination

A dissertation submitted in partial satisfaction of the requirements for the degree Doctor of Philosophy

in

Bioengineering

by

Manuel Esteban Ruidíaz Ramos

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2011
The dissertation of Manuel Esteban Ruidíaz Ramos is approved, and it is acceptable in quality and form for publication on microfilm and electronically:

Co-Chair

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University of California, San Diego

2011
DEDICATION

To my wife, for her encouragement, patience and dedication.

Meep!
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LIST OF SYMBOLS AND ABBREVIATIONS

BMI  Body Mass Index
BNP  Bacitracin-Neomycin-Polymyxin
RF   Radiofrequency
SC   Scalpel
ES   Conventional Electrosurgery
PB   PEAK PlasmaBlade®
Coag Coagulation
LSM  Least-squares mean
SD   Standard Deviation of the Mean
SOC  Standard of Care (scalpel and conventional electrosurgery)
H&E  Hematoxylin and eosin
FTI  Fibrocollagenous Thermal Injury
CTI  Cellular Thermal Injury
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ABSTRACT OF THE DISSERTATION

Cancer Detection and Treatment: Surgical Margin Status Evaluation and Monoclonal Antibody Determination

by

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Doctor of Philosophy in Bioengineering

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Interactions between surgical implements, tissue types, and thermal injury at the surgical margin interface has clinical impacts that predict healing and disease progression. A thorough analysis of this interplay on surgical margins from human specimens is under-reported. Herein, the physiological changes associated with the healing process at the surgical interface in a human cutaneous model is described. Subsequently, an analysis of the primary thermal injury interactions based on electrosurgical blade type is evaluated for its direct effect on cancer margin determination. This analysis is then further expanded to describe the interactions between differing tissue type content near the incision site on thermal injury effects. Next, a system is proposed and tested for the
intraoperative determination of cancer margins to reduce the number of second surgeries required for total tumor excision. Finally, a novel monoclonal drug assay has been developed to quantify the levels of serum monoclonal antibodies for dosage determination. Based on these findings a more comprehensive understanding of the surgical margin is provided. A further elucidation of determinants on surgical margins is necessary which should improve the diagnostic and clinical utility of the margin interface as predictors of healing and morbidity.
Chapter 1

Introduction

Breast cancer is the leading cancer diagnosis for women in the United States and is estimated to represent about 30% of new cancer cases in 2011 [1]. Yet, the 5-year survival rate from 1999 to 2006 is 90% [1]. The most effective treatment for breast cancer remains surgical removal and several studies have demonstrated equal survival rates for mastectomy and breast conserving surgery [2–8]. Therefore the optimal treatment for early stage breast cancer is breast conservation therapy (BCT) [9, 10]. In BCT, the tumor is located by mammographic techniques pre-operatively and during surgery an attempt is made to remove the entire tissue mass along with a buffer of normal tissue. Due to limitations during surgery, the partial removal of breast tissue introduces a finite risk of local recurrence due to cancer tissue that is not fully removed. This risk can be reduced by removing additional breast tissue thereby increasing cancer margins but must be weighed against the decreased cosmesis of the breast [11].

Several studies on BCT have determined that margin status is the most important factor in predicting local recurrence [12–14]. The gold standard for the determination of margin status is permanent section analysis. This is a slow and time consuming process which involves embedding the breast tissue margins in paraffin wax followed by serial sectioning. Due to the time consuming nature of the analysis, it is generally performed post-operatively and the results are not obtained until the patient has already started the recovery process.

During surgery the margins of the excised tissue are inked followed by a patho-
logic evaluation. The ink that is present during evaluation is used as the determinant for margin status by evaluation of cancerous cells in relation to the inked surface. Typically margin status is divided into three categories:

1. Positive margin status indicating that cancer cells are at or extremely close to the inked surface

2. Close margin status with cancer cells being within a predefined distance to the inked surface (usually about 1-2 mm, but depends on institution)

3. Negative margins with cancer cells farther than the close margin case (typically greater than 1-2 mm).

In the case of close or positive margins, the standard of care is to have additional surgeries as necessary until the margins are negative. The requirement of a multiple surgeries results in decreased cosmesis, increased the risk of complications, and is costly to the health care system. Several techniques have been used to mitigate this problem such as wire guides, dye injections, and ultrasound. Consequently about 32-63% of BCT patients result in a positive margin resulting in cancerous tissue remaining in the breast [15].

The surgical margin interface has clinical implications that affect downstream decisions regarding patient treatment and is affected by several characteristics which are secondary to the cancer that is being excised. In this dissertation, an attempt is made to illustrate the various effectors that play a role in the surgical margin microenvironment. In Chapter 2, a comparison of healing dynamics based on the type of surgical blade used is performed on human cutaneous tissue by evaluating cellular markers which are indicative of tissue remodeling and proliferation. Chapter 3 provides an analysis of how the type of surgical implement used can affect the readability of the surgical margins for cancer margin diagnostics, which has been demonstrated to be an important predictor of local recurrence. A novel thermal injury model was implemented to provide a platform for comparative thermal injury analysis. Ancillary to the blade used, the tissue types near the incision site can also affect the thermal injury, and consequently readability, at the surgical margin. Chapter 4 provides insight into the effects of adipose
tissue content on thermal injury metrics. An automated analysis platform for intraoperative surgical margin evaluation using imprint cytology is detailed in Chapter 5. The implementation of such a system is expected to reduce the number of second surgeries required for total cancer removal. Finally, Chapter 6 presents a monoclonal antibody detection strategy which shows promise as a quick way to determine serum antibody levels for dosage determination and administration.

1.1 References


Chapter 2

Comparative Healing of Human Cutaneous Surgical Incisions Created by the PEAK PlasmaBlade, Conventional Electrosurgery, and a Standard Scalpel

2.1 Abstract

**Background:** The authors investigated thermal injury depth, inflammation, and scarring in human abdominal skin by comparing the histology of incisions made with a standard “cold” scalpel blade, conventional electrosurgery, and the PEAK PlasmaBlade, a novel, low-thermal-injury electrosurgical instrument.

**Methods:** Approximately 6 and 3 weeks before abdominoplasty, full-thickness incisions were created in the abdominal pannus skin of 20 women, using a scalpel (scalpel), the PlasmaBlade, and a conventional electrosurgical instrument. Fresh (0-week) incisions were made immediately before surgery. After abdominoplasty, harvested incisions were analyzed for scar width, thermal injury depth, burst strength, and inflammatory response.
**Results:** Acute thermal injury depth was reduced 74% in PB incisions compared to ES \((p < 0.001)\). Significant differences in inflammatory response were observed at 3-weeks, with mean CD3+ response (T-lymphocytes) 40% \((p < 0.005)\) and 21% \((p \approx 0.12)\) higher for ES and PB, respectively, compared to scalpel (SC). CD68+ response (monocyte/macrophages) was 52% \((p < 0.005)\) and 16% \((p \approx 0.35)\) greater for ES and PB, respectively. PB incisions demonstrated 65% \((p < 0.001)\) and 42% \((p < 0.001)\) stronger burst strength than ES with equivalence to SC at the 3 and 6 week time points, respectively. Scar width was equivalent for PB and SC at both time points, and 21% \((p = 0.01)\) and 12% \((p = 0.15)\) less than ES, respectively.

**Conclusions:** PlasmaBlade incisions demonstrated reduced thermal injury depth, inflammatory response, and scar width in healing skin compared to conventional electrosurgery. These results suggest that the PlasmaBlade may provide clinically meaningful advantages over conventional electrosurgery during human cutaneous wound healing.

### 2.2 Introduction

The hemostatic control and dissection capability of conventional electrosurgical (ES) devices [1] is fundamental to the practice of surgery. However, their underlying mechanism of action – thermal ablation of tissue by delivery of continuous-waveform radio frequency (RF) energy to the tip of an electrode “blade” [2, 3] – is associated with thermal damage to tissues, reduced surgical precision compared to a scalpel, the potential for injury to adjacent structures (e.g., bowel, nerves, blood vessels), and delayed wound healing [4–12]. Incremental improvements in conventional electrosurgical device design have resulted in some reduction in thermal injury [5–8, 12], however these improvements have been modest.

The PEAK PlasmaBlade® (PB) is a novel electrosurgical device that uses brief \((\approx40 \mu\text{sec})\), high-frequency pulses of RF energy to induce the formation of electrical plasma along the edge of a thin \((\approx 12.5 \mu\text{m})\), flat, 99.5 percent-insulated electrode [13, 14]. With a burst rate less than 1 kHz, a typical duty cycle that does not exceed 5% and a very small exposed electrode surface area, the operating temperature of the PlasmaBlade remains between 40°C and 100°C [14]. This technology has been shown
to effectively dissect ophthalmological tissues as precisely as a scalpel (SC) with the hemostatic control of conventional electrosurgery, even when completely submerged in a liquid medium.

Prior work comparing the healing dynamics of incisions made in porcine skin using scalpel, PlasmaBlade and conventional electrosurgery demonstrated that the PlasmaBlade reduced acute thermal injury depth by 7- to 10-fold, decreased T-lymphocyte (CD3+) and macrophage/monocyte (CD68+) inflammatory cell response, and produced an approximately 2.6- to 2.78-fold increase in wound burst strength after 6 weeks of healing and, ultimately, resulted in superior scar formation compared to conventional electrosurgery [15]. Additional work in rats examining the healing dynamics of midline fascial incisions created by these three instruments demonstrated similar results [16]. While these results are scientifically interesting, the effect of different forms of electrosurgery upon human wound healing has not been investigated.

The present study used all three instruments (SC, PB, and ES) to test the hypothesis that decreased thermal injury would lead to improved cutaneous healing in humans following full-thickness skin incision with primary closure. In addition to objective endpoints, such as acute thermal injury depth, healed incision burst strength and surface scar width, wound inflammatory response by quantifying T-lymphocyte (CD3+) and macrophage (CD68+) cell density in excised samples from healing incisions was evaluated.

### 2.3 Methods

#### 2.3.1 Subjects and Study Design

The protocol for this clinical study was approved by the Institutional Review Board of El Camino Hospital (Mountain View, CA) and was conducted in accordance with all accepted standards for human clinical research. All patients gave written informed consent prior to study enrollment. One half of the cost of the abdominoplasty procedure was covered for patients participating in the study.

This study was conducted as part of a randomized, controlled trial of 20 adult
female subjects undergoing abdominoplasty with either the PlasmaBlade or the Standard of Care (SOC: scalpel and conventional electrosurgery) [17]. The study population had a mean age of 42.7 ± 10.1 years and a mean body mass index (BMI) of 24.6 ± 3.4 kg/m². Approximately 6 and 3 weeks prior to abdominoplasty, each subject underwent placement and primary closure of three full-thickness skin incisions, made with the three instruments of interest (SC, PB, and ES) and located within the area planned for eventual excision (Fig. 2.1). An additional set was made immediately prior to abdominoplasty with the patient under general anesthesia. Following harvest of the abdominoplasty tissue mass, healing incisions were submitted for burst strength testing and manual and computer-assisted histological analysis.

### 2.3.2 Incisional Wound Model Surgical Procedure

Three separate sets of comparison incisions were placed within the subject’s planned abdominoplasty area as shown in Fig. 2.1. Incisions created prior to abdominoplasty were designated as the 6-week and 3-week time points; the fresh incisions made immediately prior to abdominoplasty were designated as the 0-week (acute) time point. Each incision was considered to be an independent data point.

The incision area was shaved, prepped with ChloraPrep (2% chlorhexidine gluconate/70% isopropyl alcohol solution; CareFusion, Inc. San Diego, CA) and draped
in the usual sterile fashion. The location for each incision was measured and labeled, and local anesthesia was induced via subcutaneous injection of approximately 5 mL of 1% lidocaine without epinephrine (VWR, West Chester, PA). Each incision was 5 cm in length and made through the full thickness of the skin in a single stroke, with repetitive strokes made only to ensure a full-thickness wound. Incisions were made in parallel orientation and separated from each other and the abdominoplasty border by a minimum of 2.5 cm in all directions. Incisions were made with a #10 scalpel blade (Bard-Parker, Franklin Lakes, NJ), the PlasmaBlade 4.0 using the PULSAR™ Generator (PEAK Surgical, Palo Alto, CA) on Cut setting 3 (6W), and the Valleylab Electrosurgical Pencil (Model E2516) with standard stainless-steel blade electrode (Model E1551X) using a Force 2 Generator (Valleylab, Boulder, CO) on Cut mode (30W). Settings for the PlasmaBlade and conventional electrosurgical instruments were chosen based on widely-accepted settings for routine use. The approximate cost of the PlasmaBlade device was $300; the Valleylab pencil $43; the scalpel blade $0.28.

Each incision was closed with 5-0 nylon suture (Johnson & Johnson / Ethicon, Somerville, NJ) in a running fashion, and covered with Bacitracin-Neomycin-Polymyxin (BNP) ointment (Johnson & Johnson) and sterile gauze. Sutures were removed after approximately 7 days and incisions monitored for healing.

2.3.3 Histological Preparation and Thermal Injury Examination

Following harvest of the abdominoplasty sample, the excess underlying adipose tissue was dissected away and 8 cm x 2 cm samples containing the healing incision for each instrument (SC, PB, ES) and time point (0, 3, 6 weeks) were labeled and excised (Fig. 2.2). Each healing incision sample was subsequently sharply divided in half. One half was immediately placed in sterile 0.9% NaCl solution (VWR, West Chester, PA) and submitted for burst-strength testing in a fresh state (described below). The remaining half was immersed in 10% neutral buffered formalin (VWR, West Chester, PA) for a minimum of 24 hours and then embedded in paraffin for histological analysis. Representative 4-µm sections were stained with hematoxylin and eosin, Masson’s Trichrome stain, and human immunohistochemistry stain for T-lymphocytes (CD3 M7254, Dako, Carpinteria, CA), and macrophages (CD68 NCL-L-CD68, Novacostra, Newcastle, UK).
Figure 2.2: Tissue dissection and preparation for burst-strength testing and histological analysis. Each healing incision sample was sharply divided in half. One half was immediately placed in sterile 0.9% NaCl solution and submitted for burst-strength testing in a fresh state. The remaining half was immersed in 10% neutral buffered formalin for a minimum of 24 hours and embedded in paraffin for histological analysis.

All slides were coded and the 0-week time point slides were evaluated by light microscopy (Olympus BX 40 microscope, with DP70 CCD camera, Center Valley, PA) for acute thermal injury depth by a single pathologist (E.J.H.) blinded to the wounding modality. The zone of coagulation necrosis resulting from thermal injury was determined in µm by direct microscopic measurement using the hematoxylin and eosin sections. Slides from all time points were then scanned for digital immunohistochemical analysis (described below).

2.3.4 Digital Immunohistochemical Analysis

Computational analysis of CD3+ T-lymphocyte and CD68+ macrophage/monocyte density was performed using a previously described image-analysis software package [18] adapted by the authors to the current study. (see Supplemental Content (Section 2.7) and Figures for a complete description of this method) Briefly, 632 slides of stained tissue sections from all study subjects were scanned (Fig. 2.3) on a ScanScope XT slide scanner (Aperio Technologies, Inc., Vista, CA) at 20x and processed in 2 x 2 mm increments. Following segmentation and colorimetric thresholding, noise correction, filtering and manual verification, a total of 605 slides were included in the subsequent statistical
Figure 2.3: Left: Typical 2x2 mm processing tile, from 3-week scalpel incision, stained for CD3. Center CD3+ cluster is zoomed at right. Top right: 20x representation of CD3+ cluster. Bottom right: Automated segmentation outlines of positively stained tissue.

2.3.5 Wound Burst Strength

The burst strength of freshly excised healing wound samples immersed in normal saline was measured in pounds-force/inch (lb-f/in) according to previously reported methods [15], using a Chatillon TCD200 digital force tester (Ametek TCI Division, Largo, Florida). Briefly, each incision sample was divided into three equally sized test units, each with the healing incision centered in the sample. The maximum width of each test unit at the location of the healing incision was then measured three times using digital calipers (Absolute 500 Digimatic Calipers, Mitutoyo America Corporation) and averaged. Each test unit was then secured in the jaws of the clamp, with the healing incision centered between 1 cm of tissue above and below the clamp jaws. Bursting strength of each incision was then determined by slow, progressive stressing of the segment to disruption at a speed of 2 in/min. The peak force was then recorded and converted to
lb-f/in.

2.3.6 Surface Scar Width

The width of the surface scars for the 6-week time point incisions were recorded at 3 and 6 weeks following placement; the 3-week incisions were recorded on the day of abdominoplasty. Measurements were made, in mm, by one author (H.L.R.) using a calibrated ruler from one edge of the scar to the other at three distinct points: scar center, 2 cm above and 2 cm below the center point. Measurements for each incision were averaged and recorded by age (in days) since incision for comparison.

2.3.7 Statistics

Statistical analysis was performed using the R statistical environment software program, version 2.11.0 [19]. All endpoints are reported as mean (standard deviation), except where indicated. A general linear model was constructed with main effects of instrument and time point to evaluate differences between study endpoints. Summaries of the Least Squared Means (LS Mean) differences between overall healing scores were evaluated with the model. Linear regression was also performed to evaluate each endpoint over the 6-week assessment period. Statistical significance was defined as $p < 0.05$.

2.4 Results

Mean (SD) operative time was 1 hr, 38 min, 40 sec (13 min, 9 sec) for patients in the PlasmaBlade abdominoplasty group and 1 hr, 35 min, 48 sec (9 min, 9 sec) for patients in the SOC abdominoplasty group ($p = 0.47$). The clinical course of healing and time to drain removal was comparable between the two groups.

Skin scar width, acute thermal injury depth and wound burst strength are summarized in Table 2.1. When compared to conventional electrosurgery, incisions made with the PlasmaBlade reduced thermal injury depth by 74% ($p < 0.001$). At the 3 and 6 week time points, PlasmaBlade incisions demonstrated equivalent burst strength to scalpel and
an improvement of 65% ($p < 0.001$) and 42% ($p < 0.001$), respectively, over conventional electrosurgery incisions. PlasmaBlade skin incision scar width was equivalent to scalpel at the 3 and 6-week time points with a 21% ($p = 0.01$) and 12% ($p = 0.15$) reduction in scar width, respectively, compared to conventional electrosurgery.

CD3+ and CD68+ responses by time point and instrument are shown in Figs. 2.4 and 2.5, and mean responses are summarized in Table 2.2. The week 0 samples served as an internal control; because there were no significant differences between the measured densities of CD3+ and CD68+ cells in week 0 samples from the three instruments, the data were judged to be reliable. After 3 weeks of healing, CD3+ and CD68+ inflammatory response in PlasmaBlade incisions was 21% ($p \approx 0.12$) and 16% ($p \approx 0.35$) greater compared to SC, respectively, however these differences were not statistically significant; conventional electrosurgery incisions demonstrated a 40% ($p = 0.01$) and 52% ($p = 0.05$) increase, respectively. There were no significant differences between any values at the 6-week timepoint (Figs. 2.4 & 2.5).

2.5 Discussion

The healing dynamics of surgical incisions made with various electrosurgical instruments have been explored in many animal models. However, a comparison of the healing dynamics of human cutaneous incisions made with conventional electrosurgical instruments versus lower thermal injury instruments, such as the PlasmaBlade, have not yet been described in the literature. In order to investigate this issue, a novel, in vivo human cutaneous wound-healing model that takes advantage of the planned removal and disposal of a large area of human skin during abdominoplasty was developed. By recruiting patients who have elected to undergo this procedure, the healing of incisions over a time frame that closely matches the clinical course of healing following a surgical procedure could be monitored and quantified, while ensuring patients are not subject to long-term residual scarring left by the experimental procedure.

In agreement with previous work in porcine skin [15] and rat fascia [16] models, it was found that use of a conventional electrosurgical instrument produced a wider scar, a deeper zone of thermal injury and weaker healed incision strength when compared to
Table 2.1: Comparison of Scar Width, Thermal Injury Zone, and Burst Strength Measurements in Human Skin Incisions Made with the PlasmaBlade, Electrosurgery, and Scalpel

<table>
<thead>
<tr>
<th></th>
<th>PlasmaBlade Mean (SD)</th>
<th>Electrosurgery</th>
<th>Scalpel</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean (SD)</td>
<td>%*</td>
<td>p</td>
</tr>
<tr>
<td>Acute thermal injury depth, µm</td>
<td>195 (127)</td>
<td>763 (208)</td>
<td>74† &lt; 0.001</td>
</tr>
<tr>
<td>Overall scar width, mm</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3 wk</td>
<td>2.0 (0.6)</td>
<td>2.5 (0.8)</td>
<td>25 0.01</td>
</tr>
<tr>
<td>6 wk</td>
<td>2.5 (0.7)</td>
<td>2.8 (0.5)</td>
<td>12 0.15</td>
</tr>
<tr>
<td>Wound burst strength, lb-f/in</td>
<td>43.44 (26.65)</td>
<td>26.28 (14.42)</td>
<td>65† &lt;0.001</td>
</tr>
<tr>
<td>6 wk</td>
<td>59.32 (37.53)</td>
<td>41.78 (21.71)</td>
<td>42† &lt;0.001</td>
</tr>
</tbody>
</table>

*Percentage differences and corresponding p values are calculated relative to the PlasmaBlade unless otherwise noted.
†Percentage difference calculated relative to electrosurgery.
Table 2.2: Density of CD3$^+$ and CD68$^+$ Cells in Sectioned, Immunostained, Human Skin Incisions Made with the PlasmaBlade, Electrosurgery, and Scalpel*  

<table>
<thead>
<tr>
<th>Time</th>
<th>SC</th>
<th>PB</th>
<th>ES</th>
<th>p†</th>
<th>LSM</th>
<th>SC</th>
<th>PB</th>
<th>ES</th>
<th>p†</th>
<th>LSM</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 weeks</td>
<td>138</td>
<td>93</td>
<td>120</td>
<td>0.33</td>
<td>9265</td>
<td>87</td>
<td>71</td>
<td>75</td>
<td>0.67</td>
<td>1355.9</td>
</tr>
<tr>
<td>3 weeks</td>
<td>171</td>
<td>207</td>
<td>239</td>
<td>0.02</td>
<td>42,928</td>
<td>173</td>
<td>201</td>
<td>263</td>
<td>0.01</td>
<td>77,827</td>
</tr>
<tr>
<td>6 weeks</td>
<td>196</td>
<td>216</td>
<td>223</td>
<td>0.71</td>
<td>7702.3</td>
<td>202</td>
<td>232</td>
<td>185</td>
<td>0.48</td>
<td>21,385</td>
</tr>
</tbody>
</table>

SC, scalpel; PB, PlasmaBlade; ES, electrosurgery; LSM, least-squared mean (the best unbiased linear estimate of the mean value at this time point).

*Mean cell density is expressed in cells per square millimeter.

†One-way analysis of variance $p$ values calculated across different blades by time point.

Figure 2.4: Box plot of CD3+ cell density by instrument and time point. The black bars denote median response, whereas boxes indicate interquartile range. The CD3+ responses at week 0 are similar as expected, whereas at week 3, median CD3+ response was 40 percent higher in the electrosurgery incisions than in PlasmaBlade or scalpel incisions ($p = 0.02$).
Figure 2.5: Box plot of CD68+ cell density by instrument and time point. The largest differences were observed during week 3, with electrosurgery incisions exhibiting a 52 percent increase in CD68+ cell density response compared with PlasmaBlade and scalpel incisions ($p = 0.01$).
scalpel and the PlasmaBlade (Table 2.1).

The correlation between thermal injury depth, scarring, and the inflammatory process associated with healing was also investigated. While inflammation marks a well-documented phase of wound healing [20], recent studies have suggested that post-injury inflammation is not necessarily an essential component of tissue repair, and that it may delay wound healing and worsen scarring [21]. It has been suggested that faster resolution of the inflammatory phase might lead to more rapid healing and less scarring. To further investigate the correlation between healing wound strength, scarring and healing-associated inflammation, an in-depth quantification of CD3+ T-cells and CD68+ macrophages was performed with our histological samples. These two cell types are central to the inflammatory phase of healing and, in this study, serve as a surrogate measure of inflammation [22–24].

For the quantification of cell density, a previously developed [18], color-based (YUV thresholding) computational method was adapted for this application. This method was employed because YUV transformation provides a computationally simple method for separating color information (chrominance) from image intensity (luminance). This focus on color allows for increased specificity and improved identification of cells with positive staining.

Cell densities were similar for the 0-week time point; this was expected as the incisions had been freshly harvested and analyzed, with no time for cell recruitment or full-scale initiation of the inflammatory phase. This time point served as a convenient internal control to validate the slide analysis method. The largest and most significant differences in instrument-dependent response were observed at 3 weeks, with inflammatory cell counts much higher in the conventional electrosurgery samples than in those created with the PlasmaBlade or scalpel (Figs. 2.4 & 2.5 and Table 2.1). Given the deeper thermal injury zone observed in the conventional electrosurgery incisions, the increased inflammatory response is likely attributable to phagocytotic removal of debris and necrotic tissue.

In contrast, by 6 weeks, when wounds would be expected to have progressed to the remodeling/proliferative phase, inflammatory cell density had essentially equalized between the three instrument types. Taken together with the other data presented here, it
is likely that the lower degree of inflammation at 3 weeks in the scalpel and PlasmaBlade groups is directly related to the observed improvement in scarring and wound burst strength at the end of the study.

These findings are consistent with the PlasmaBlade’s favorable effects on human skin, namely improvement in healed scar width and strength, and reduction in thermal injury depth and are similar to those seen in animal models and human ocular applications. Furthermore, this study begins to clarify the correlation between these observations and the reduced inflammatory response induced by the PlasmaBlade during incision. The results suggest that the PlasmaBlade provides useful advantages over conventional electrosurgery during human wound healing.

2.6 Acknowledgements

The authors would like to thank Jeanne McAdara-Berkowitz, Ph.D., Dominique Y. Atmodjo, B.A., and James R. Johnson, Ph.D., for their assistance in the preparation of this manuscript. Additionally, the authors thank Robbin Newlin, Ph.D., and Guillermina Garcia for assistance with the digital imaging of histological samples.

2.7 Supplemental Content

2.7.1 Detailed Slide Scanning and Analysis Methods

Tissue sections from 632 slides (≈15 slides per stain per patient) were imaged on a ScanScope XT slide scanner (Aperio Technologies, Inc., Vista, CA) at 20x (0.5μm/pixel) using JPEG compression for storing image data. Subsequent image analysis was performed using an internally developed image analysis software package for large datasets based on ImageJ [25]. To manage the large size of the digital slide imagery, processing was performed in 2 x 2 mm tile increments over the entire slide image (Fig. 2.6-1c, Fig. 2.6-1d).

For each 2 x 2 mm tile (Fig. 2.6-1e), CD3 and CD68 positive staining cells were identified by pixel thresholding and segmentation using a colorimetric threshold technique in YUV color space (intensity and color) [26]. Optimal selection of positively stained cells consisted of an 8-bit YUV thresholding operator with $Y = 0 – 255$, $U = 0 – 255$, and $V = 138 – 255$. A watershed algorithm for separating falsely concatenated outlines was then applied increase the correspondence between outlines and actual positively stained cells. Small ($\leq 5\mu m^2$) elements of dust and sectioning artifacts within the image were filtered out and the position, size and area of the remaining outlines were saved for further review at the tissue region analysis stage.

For tissue region analysis, tiles from the entire scan area were processed to yield an outlined data set across the entire scan area. A grayscale (8 bit) low-magnification overview image was used to identify valid tissue regions by a previously described Iso-data thresholding algorithm[27]. Tissue regions were then subjected to a series of 1 pixel morphological operations (dilate, fill, erode, erode, dilate) to reduce background image noise in the resulting outlines. The resultant outlines were subsequently filtered to exclude sizes less than 0.1 mm$^2$ in order to remove erroneously outlined fibers and histological debris. The remaining tissue region outlines were used to measure the area of the tissue present on the slide and to define the regions where the positive staining outlines were valid. Manual verification of the correspondence between outlines and actual tissue regions was then performed. Twenty-seven (27) slides exhibited excessive staining, scanner banding artifacts, and/or focus issues and were discarded from further
Figure 2.6: Tissue Collection Methodology and Analysis Hierarchy

1a: Location and time points of pre-abdominoplasty comparison incisions with the scalpel (SC), conventional electrosurgery (ES), and PlasmaBlade (PB).

1b: Each incision-time point had up to 2 associated slides, denoted as levels containing 2-3 consecutive sections.

1c: The slides containing tissue were scanned and tissue regions automatically determined.

1d: Digital representation processed in 2x2mm tile increments over entire image.

1e: Representative sub-tile used for segmentation of CD3+ and CD68+ cells.
analysis. Other non- correspondence due to isolated fibers which traversed tissue regions were corrected manually by delineating the outline representing the tissue region of interest. After verification, a total of 605 slides remained for subsequent statistical analysis.

2.8 References


Chapter 3

Quantitative Comparison of Surgical Margin Histology Following Excision With Traditional Electrosurgery and a Low-Thermal-Injury Dissection Device

Synopsis

Traditional electrosurgery and a low-thermal-injury dissection instrument are evaluated by a novel, in vivo, oncologic model. Observed differences in thermal injury at the surgical margin may have a significant impact on histopathological analysis and downstream treatment decisions.

3.1 Abstract

Background: This study is the first to examine in vivo the effect of thermal injury in breast conservation pathology in a direct comparison of traditional electrosurgery and an alternative low-thermal-injury device.

Methods: A prospective study of 20 consecutive subjects with biopsy-proven invasive ductal carcinoma (IDC) tumors > 1cm was conducted. Following excision,
incisions were made into the tumor with the two devices. Thermal injury depth, margin
distance, tissue type, and histological effect were compared on the same breast tissue
cut with each excision instrument. A probability evaluation of close and positive margin
cases for the true tumor margins was conducted.

**Results:** Compared to traditional electrosurgery, the low-thermal-injury instru-
ment reduced collagen denaturation depth from 435 µm to 102 µm (77%), fused tissue
depth from 262 µm to 87 µm (67%), and distortion depth from 1132 µm to 774 µm
(30%).

**Conclusions:** Based on analysis of the close subset of the true margins, using
the traditional electrosurgical device in place of the low-thermal-injury device would
have resulted in 48% of the close margin samples being negatively converted to false-
positive, and in 11% converting from close to false-negative. The methodology of this
work may be readily applied to larger, more definitive studies.

### 3.2 Introduction

Breast conservation therapy (BCT) is the standard surgical treatment for breast
cancer [1, 2]. The goal of BCT is total excision of the malignant lesion while simultane-
ously preserving the cosmetic appearance and functionality of the breast. The complete-
ness of resection and the potential for residual disease are determined by postoperative
histopathologic analysis of permanent tumor sections. A second surgery is indicated
when at least one of the following criteria are met: (a) malignant cells are identified at
the tissue margin (ink on cancer cells) or (b) malignant cells are identified adjacent to
the boundary that separates readable tissue from tissue that is unreadable due to ther-
mal damage (e.g. fused cells, loss of specific staining within nuclei, etc.). Despite
advances in surgical technique and pathologic analysis, approximately 20-50% of ma-
lignant breast lump excision margins are diagnosed “positive,” and yet, on reoperation,
residual disease is found to be present in only 40-70% of these cases [1, 3]. False posi-
tive margins can be created by (a) postoperative shrinkage of tissue, (b) the inking pro-
cess, (c) electrosurgical damage to the margin during excision, and/or (d) appropriately
conservative practices with respect to assigning indeterminate specimens. Therefore,
reducing thermal injury may reduce a percentage of false positive margins.

False-negative margin calls also present a serious issue, as they may result in under-staging and under-treatment, especially if the patient does not receive post-surgical radiation. For example, invasive breast cancers may have small, malignant foci near the margin which, when rendered unreadable secondary to thermal damage, can create the illusion of total excision when, in fact, residual cancer is present. The use of a low-thermal-injury technology in this setting, as well, could potentially decrease the number of erroneous negative evaluations.

Attempts to avoid unnecessary reoperation by developing an effective, reliable intraoperative analysis of surgical margins have met with limited success. For example, imprint cytology (i.e. Touch Prep) [4] has been tried in the past, but is not used routinely because an experienced cytopathologist may not always be available to correctly interpret the slides and, as with permanent sections, the reliability of test results are strongly affected by electrosurgical artifact at the sample margin [5]. At the University of California, San Diego, (UCSD), margins of breast malignancies are sub-classified. All measured distances to cancer cells are referenced to the inked surface since that is the most definitive reference point. However, classification of margins as “close” or “positive” is based on the distance between the cancer cells closest to the line dividing the histologically readable tissue (low thermal damage) and the histologically unreadable tissue (high thermal damage). Therefore, the three categories of margins at UCSD are: (1) “negative” when margins have greater than 1 mm of normal tissue from cancerous cells to the boundary between readable/unreadable tissue due to thermal injury (other institutions may use 2mm), (2) “close” when there is less than 1 mm of normal tissue between cancerous cells and the readable/unreadable boundary, and (3) “positive” when cancerous cells are in contact with the readable/unreadable boundary or are at the inked surface. Traditional electrosurgical devices impart thermal injury artifact to a depth of 1-2 mm from the surface, potentially destroying nuclear grading information and degrading fine tissue architecture throughout the critical margin depth [6–12].

At UCSD, a novel, low-thermal-injury technology has recently been evaluated for surgical soft-tissue dissection. The PEAK PlasmaBlade® (PEAK Surgical, Palo Alto, CA), uses brief (≈40 µs), high-frequency pulses of RF energy to induce the for-
formation of electrical plasma along the edge of a very thin ($\approx 12.5 \mu m$ wide), flat, 99.5 percent-insulated electrode [13, 14]. With a burst rate less than 1 kHz, a typical duty cycle (percentage of time that energy is transferred to the patient when the device is activated) that does not exceed 5% and a very small exposed electrode surface area, the operating temperature of the PlasmaBlade remains between $40^\circ C$ and $100^\circ C$ [14]. This technology, which received FDA 501(k) clearance in 2008 for general surgery applications, has been shown to effectively dissect ophthalmological tissues [14–21] and skin [22] as precisely as a scalpel with the hemostatic control of traditional electrosurgery, even when completely submerged in a liquid medium. In this report, a model is developed to quantify the effect of thermal injury on different breast tissue types and to predict the effect on margin status and final pathology. This model was created to address the absence of a standardized method for quantifying thermal injury, and may be adapted to reflect variation in parameters among individual institutions.

3.3 Methods

3.3.1 Study Design

The protocol for this prospective, controlled clinical study was approved by the Institutional Review Board of UCSD and was conducted in accordance with all accepted standards for human clinical research. All patients gave written informed consent prior to study enrollment. The study tested the hypothesis that reduced thermal injury during resection would result in superior diagnostic quality of tumor specimens, including increased effective distance from the visualized malignant tissue to the excision margin, compared to traditional electrosurgery. The primary quantification was depth of thermal injury artifact, by instrument, as evaluated by permanent histologic analysis. Secondary quantifications were the false-positive and false-negative margin call rates.

3.3.2 Study Population

The study population consisted of 20 adult females with biopsy-proven diagnosis of invasive ductal carcinoma (IDC) undergoing breast-conserving surgery, with
a minimum tumor diameter of at least 1 cm (established by mammography), and who were otherwise in good health. Exclusion criteria included women with tumors < 1 cm, and those undergoing total mastectomy or who had been previously treated with radiation therapy. All procedures, including the lumpectomy and pre- and postoperative visits, were performed by surgeons at the UCSD Thornton Medical Center (La Jolla, CA, USA). All histopathologic sample preparation and analysis was conducted by pathologists at UCSD who were blinded to the treatment instrument. Supplementary measurements were performed by the first author (M.E.R.) under pathologist supervision. Tissue generated for this study had no effect on final histopathologic diagnosis or margin status interpretation.

### 3.3.3 Tumor Excision

Patients were prepared for surgery in the usual sterile fashion prior to excision under general anesthesia. For all patients skin incisions were made with the PlasmaBlade 4.0 using the PULSAR™ Generator (PEAK Surgical, Inc.) on Cut setting 6 (20 W). Subcutaneous dissection and tumor excision were performed with the PlasmaBlade using Cut setting 6 (20 W) and Coag setting 6 (30 W). These settings are typically used during breast tissue excision. Directly prior to complete excision, a suture was inserted into the superior face of the tissue mass along with a double suture on the lateral face to allow for orientation of the excised mass. The excised mass was removed with approximately 1 cm of margin tissue surrounding the tumor as estimated by gross inspection. This was followed by excision of the cavity tissue and inking of the true margins. Subsequently, the primary excision specimen was placed on a small conductive strip for further processing of “calibrated” margin specimens and thermal injury measurements (described below).

### 3.3.4 Generation of Calibrated Margins for Comparative Histological Analysis

In order to compare the effects of the two instruments on margin quality, a set of deliberately “calibrated” margins were generated for each lumpectomy specimen in
order to provide an artificial margin that would mimic general representation of the tissue quality at the true margin surface (Figure 3.1). Briefly, the tumor was first placed on a conductive copper tape (3M, St. Paul, MN, USA) that was attached to the PULSAR generator grounding port. The approximate center of the tumor was located by palpation. Using the PlasmaBlade with the same settings during tumor excision (Cut setting 6, and Coag setting 6), an incision was made through the tumor tissue to a depth of approximately 45% of the diameter of the tumor mass. Subsequently, a #10 scalpel was used to remove a small strip of the tissue cut by the PlasmaBlade from within the invasive tissue area. The scalpel-exposed surface was inked, and the entire tissue sample was placed into a cassette and submitted for histological preparation. These steps were repeated on the opposite side of the tumor mass in the same plane as the original incision using the Valleylab Electrosurgical Pencil (Model E2516) with standard stainless-steel blade electrode (Model E1551X) using a Force Triad Generator (Valleylab, Boulder, CO, USA) on Coag mode (30 Watts), a widely accepted model and power setting for traditional electrosurgical technology.

3.3.5 Histological Preparation of Excised Samples

Excised tissue samples were fixed in 10% neutral buffered formalin (VWR, West Chester, PA) for a minimum of 24 hours, followed by paraffin embedding. During the embedding process, the tissue was oriented such that the inked surface (scalpel side) was placed on one side and the electrosurgically incised surface on the other side, thereby allowing slices to show the relationship of the tissue transitioning from unaffected histology to tissue with thermal injury. Ten histological levels were sampled from each paraffin block; five levels (each 5 μm thick with 30 μm spacing between levels) from each end of the tissue block. Sectioned levels were stained with hematoxylin and eosin (H&E), coverslipped, and digitally scanned at 20x (0.50 μm per pixel) magnification on a ScanScope XT (Aperio Technologies, Inc., Vista, CA) slide scanner followed by manual histopathological analysis of thermal injury.
Figure 3.1: General protocol for obtaining calibrated margin sections. A: Representation of excised tumor specimen showing PlasmaBlade (green) and traditional electrosurgery (blue) incision sites in the opposite sides of the excision. The cross-sectional plane (red) bisects both incision sites. B: Cross-section showing both incision types, with incision spread apart. PlasmaBlade (green) and traditional electrosurgery (blue) cauterized surfaces are highlighted. C: Magnification of PlasmaBlade (green) incision cross-section showing cauterized surface. A small scalpel incision (black) is made to remove a strip of tissue with thermal injury artifact. D: The small tissue strip is embedded and representative sections (red) are taken at each end of the tissue block. E: Sections are stained with H&E followed by scanning and measurement.
3.3.6 Thermal injury model

There is currently no standardized technique for the evaluation or quantification of thermal injury on tissue sections. Therefore, several quantifiers for thermal injury were created in this study to account for cellular and fibrocollagenous tissues. In our de novo thermal injury model, 2 zones of fibrocollagenous thermal injury (FTI) and 4 zones of cellular thermal injury (CTI) were defined by successive levels of heat-exposure-induced stress, which provide discernable visual characteristics on tissue sections. The interplay between CTI and FTI is intermixed and variable across samples. The FTI zones (collagen-denaturized versus undisturbed tissue) were delineated by the observed thermally induced smoothing of the collagen fibers and darkening of the tissue (Figure 3.2, Top) [23]. The CTI zones were defined as: (I) complete charring with no cellular structure; (II) fused tissue with severe tissue denaturation, few identifiable structures, indistinguishable nuclei, and tissue breakdown; (III) distressed cellular architecture with a wispy appearance, irregular elongated and spindled nuclei, smudged chromatin, visible distorted fibroblast nuclei, no clear cellular outlines, and cells visible but not clear whether they are epithelial or stromal; and (IV) undisturbed tissue with no signs of thermal injury, fibroblasts identifiable, clear distinction between epithelial and stromal cells, nuclear ultrastructures and chromatin identifiable. The differences are apparent in changes in staining property and tissue structure at low magnification, and changes in cell structures at high magnification (Figure 3.2, Bottom).

Thermal injury evaluation and categorization

Evaluation of digitized tissue levels was performed using the ImageScope Viewer (Aperio Technologies, Inc., Vista, CA) software. Margins exhibiting signs of thermal injury were identified by locating staining artifact at the margin interface, which appears as a darkening of the stain along with distortion of the cellular architecture. Measurements to quantify FTI and CTI were manually performed using the ImageScope Viewer’s measurement tool: (1) distance from the true margin to the end of the collagen denaturation (FTI), (2) distance from the true margin to the fused/distressed boundary (between CTI zones II/III), and (3) distance from the true margin to the distressed/undisturbed boundary (between CTI zones III/IV) (Figure 3.2).
Figure 3.2: Histopathologic features of thermal injury. The effects of thermal injury are characterized differently in the two tissue types observed: Fibrocollagenous Tissue (FTI, above) and Cellular Tissue (CTI, below). **Top:** The Fibrocollagenous tissue is characterized by a zone of collagen denaturation as demarcated by a darkening and smoothing of the collagen staining and a zone of undisturbed tissue. **Bottom:** The Cellular Tissue is composed of four zones. Zone I is characterized by extensive charring that is often lost during the tissue embedding processing. Zone II has few identifiable cellular structures and mainly consists of fused tissue. Zone III has distressed tissue architecture with a wispy appearance. Zone IV is undisturbed, with cells displaying no evidence of thermal artifact. Dominant characteristics are exemplified in the conceptual drawings below each zone categorization. **Fused tissue:** increased staining uptake, cells not identifiable. **Distressed tissue:** smudge nuclei, unclear cell outlines, distorted cellular arrangement. **Undisturbed tissue:** rounded nuclei, visible nuclear substructures, cell types are identifiable. **Note:** The illustration of the dominant characteristics for each zone categorization are conceptual ink drawings of typical cell types; they are not microscopy images.
Close margin measurement

Based on routine pathology reports, the true close margins of each patient were identified and digitally scanned at 40x (0.23 µm per pixel) magnification on a iScan Coreo Au (BioImagene, Inc., Sunnyvale, CA) followed by exact measurement of margin distances on the BioImagene Image Viewer (BioImagene, Inc., Sunnyvale, CA). Two distances were measured: the distance from the true inked margin to the closest cancer cells of the closest cluster of malignant cells, and the distance from the true margin to the farthest cancer cell in the closest cluster of malignant cells. In addition, the slides were categorized as FTI or CTI.

Evaluation of possible conventional electrosurgery effects on the true margin

For a negative or close margin, there is no direct extension of the malignant tissue to the true margin or to the boundary line between the readable and unreadable tissue zones (i.e. the FTI collagen denaturation boundary and the CTI fused/distressed boundary (zone II/III boundary)) (Figure 3.3, Top). For a margin to be called positive, the malignant cells are either at the true margin surface (touching the ink) or in contact with the zone of unreadable tissue caused by thermal injury adjacent to the true margin surface (Figure 3.3, Bottom). In excision with traditional electrosurgery, a region of unreadable tissue secondary to thermal injury is imparted just beyond the true margin, which decreases the effective readable distance between the excised cancer cells and the true margin. Therefore, to improve the sensitivity of the interpretation and potentially eliminate false negative reporting, a cluster of cancer cells that is associated with the unreadable portion of the true margin are called positive by default (Figure 3.3, Bottom).

The estimated probability of a thermal damage region extending beyond a location near to the true margin, if a traditional electrosurgical device was used in place of the low thermal injury device, was calculated by mathematical integration of the traditional electrosurgery thermal injury probability distribution (calculated from the calibrated margins). Each “close” low thermal injury true margin was evaluated as three possible binomial event categories, in the case that traditional electrosurgery was used instead: (1) there would be no change in switching to traditional electrosurgery (ther-
**Figure 3.3:** Electrosurgical excision creates two surface boundaries, the true margin surface (inked) and the readable/unreadable surface due to thermal injury. The surface boundaries affect the determination of margin status. Negative, Close, Positive (On Unreadable) and Positive (On Ink) margin outcomes with associated measurements are detailed.
mal injury did not extend sufficiently to the closest malignancy), (2) there would be a switch to a positive margin with traditional electrosurgery (the closest malignancy is partially unreadable due to thermal injury) or (3) there would be an obscured false negative margin with traditional electrosurgery (unreadable thermal injury extending beyond the farthest cancer cell of the closest malignancy to the true margin). Each category has an independent probability dependent on the exact margin distances and each was modeled as a Poisson binomial distribution and calculated with a previously described recursive algorithm [24].

3.3.7 Statistics

The R statistical environment software, version 2.12.0 was used for statistical evaluation [25]. Quantifications were described by univariate summaries unless otherwise indicated. Statistical significance was defined as \( p < 0.05 \). Estimates of probability distributions were performed with the density function in the stats package.

3.4 Results

3.4.1 Demographics

Patients were enrolled in the study from July 9, 2009 through July 20, 2010. All patients had core-biopsy proven diagnosis of invasive ductal carcinoma. Preoperative patient characteristics are summarized in Table 3.1.

3.4.2 Calibrated-Margin Thermal Injury Depth Metrics

Average, instrument-dependent depth of thermal injury to the calibrated-margin, measured from the cauterized surface to the respective boundary, is summarized in Table 3.2. Compared to traditional electrosurgery, average depth of collagen denaturation (FTI) was reduced by 77% (ES: 435 \( \mu m \), PB: 102 \( \mu m \)) in PlasmaBlade samples; fused/distressed boundary (between CTI zones II/III) by 67% (ES: 262 \( \mu m \), PB: 87 \( \mu m \)); and distressed/undisturbed boundary (between CTI zones III/IV) by 30% (ES: 3.3.7 Statistics

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Table 3.1: Pre-Operative Characteristics of Invasive Breast Carcinoma Patients.

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Mean (SD)(^a)</th>
<th>Range</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>58 (9.9)</td>
<td>34–79</td>
</tr>
<tr>
<td>Body-mass index (BMI) (kg/m(^2))</td>
<td>31 (8.3)</td>
<td>17–49</td>
</tr>
<tr>
<td>Modified Bloom–Richardson score</td>
<td>7.2 (1.6)</td>
<td>5–9</td>
</tr>
<tr>
<td>Sample volume (cm(^3))</td>
<td>97 (89)</td>
<td>29–414</td>
</tr>
<tr>
<td>Widest sample diameter (cm)</td>
<td>8.3 (1.95)</td>
<td>6.2–14.4</td>
</tr>
<tr>
<td>Tumor volume (cm(^3))</td>
<td>10.4 (15.6)</td>
<td>0.09–66</td>
</tr>
<tr>
<td>Widest tumor diameter(^b) (cm)</td>
<td>3.4 (1.6)</td>
<td>0.82–7.2</td>
</tr>
</tbody>
</table>

\(^a\)Sample size (N) = 20.

\(^b\)One tumor sample was poorly demarcated and unable to be measured.

1132 \(\mu m\), PB: 774 \(\mu m\) (p < 0.001 for all 3 measures). The distributions for FTI denaturation, CTI fused/distressed boundary and CTI distressed/undisturbed boundary are displayed in Figure 3.4.

3.4.3 Estimation of Thermal Injury Reduction with the Low-Thermal-Injury Device over Traditional Electrosurgery

Based on the probability distributions of each measurement modality of the model, it is possible to determine the probability distribution of the expected change in thermal injury should traditional electrosurgery be replaced with the PlasmaBlade for the calibrated margins (Figure 3.5). The average change for FTI collagen denaturation, CTI fused/distressed boundary and CTI distressed/undisturbed boundary was -333 \(\mu m\), -175 \(\mu m\) and -358 \(\mu m\), respectively. Should traditional electrosurgery be replaced with the PlasmaBlade, it is expected that a reduction in thermal injury would be observed 89%, 74% and 66% of the time respectively.

3.4.4 Close Margin Evaluation on the True Surgical Margins

From routine pathologic evaluation of the true surgical margins, 12 permanent sections from 7 cases were assigned pathologic diagnosis of “close” margins (cancer cells within approximately 1 mm of the inked margin surface). Average (SD) distances
Table 3.2: Comparison of Thermal Injury Depth in Invasive Breast Tumors, by Instrument.

<table>
<thead>
<tr>
<th>Metric</th>
<th>Instrument</th>
<th>Mean (SD) (µm)</th>
<th>Range (µm)</th>
<th>% Diff(^a) (P-value)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Collagen denaturation</td>
<td>Electrosurgery (N(^b) = 958)</td>
<td>435 (308)</td>
<td>36–2,437</td>
<td>77% (&lt;0.001)</td>
</tr>
<tr>
<td></td>
<td>PlasmaBlade (N = 1,334)</td>
<td>102 (114)</td>
<td>8–863</td>
<td></td>
</tr>
<tr>
<td>Margin to end of fused boundary</td>
<td>Electrosurgery (N = 109)</td>
<td>262 (226)</td>
<td>0–1,258</td>
<td>67% (&lt;0.001)</td>
</tr>
<tr>
<td></td>
<td>PlasmaBlade (N = 147)</td>
<td>87 (75)</td>
<td>0–434</td>
<td></td>
</tr>
<tr>
<td>Margin to end of distressed cell boundary</td>
<td>Electrosurgery (N = 119)</td>
<td>1132 (708)</td>
<td>215–3,065</td>
<td>30% (&lt;0.001)</td>
</tr>
<tr>
<td></td>
<td>PlasmaBlade (N = 153)</td>
<td>774 (670)</td>
<td>89–4,288</td>
<td></td>
</tr>
</tbody>
</table>

\(^a\)Percentage difference is calculated with respect to traditional electrosurgery.

\(^b\)N is defined as the number of total measurements (sample size).
Figure 3.4: Thermal injury distribution profiles for traditional electrosurgery (blue) and the PlasmaBlade (green). Top: FTI Collagen denaturation distance distribution. Middle: CTI Fused/Distressed boundary distribution. Bottom: CTI Distressed/Undisturbed distribution.

for all cases was 461 (471) µm and 1494 (976) µm for the closest and farthest cells, respectively, of the closest cluster of malignant cells (Table 3.3).

3.4.5 Thermal Injury Depth and Effect on Margin Evaluation

Traditional electrosurgery probability distribution estimates for each measurement modality were applied to the respective distances that were observed for the 12 “close” margin sections from our dataset, and this method was also used to derive expected changes in margin status (Figure 3.6). FTI best described 10 margins while the CTI tissue type best described 2 margins (Table 3.3). For the FTI tissue type, the margin malignancy is possibly affected in 3 ways; (1) no denaturation effects, (2) partial denaturation effects, or (3) fully affected by denaturation effects. For the FTI tissue of close margins (10 out of the 12 close margins), it is expected the following changes would occur: 47% to have no denaturation effects, 41% to have partial denaturation, and 12 fully consumed by denaturation. For the CTI tissue type, the malignancy at the margin could have been affected in 4 ways; (1) no thermal injury effects so cancer only in CTI zone
Figure 3.5: Determined thermal injury reduction (red) probability distribution that would be expected when replacing traditional electrosurgery with the PlasmaBlade. **Top:** Collagen denaturation reduction distribution profile. **Middle:** Fused/Distressed boundary distance reduction distribution profile. **Bottom:** Distressed/Undisturbed distance reduction distribution profile.
Table 3.3: Distances From the Inked True Margin to the Closest and Farthest Cells of the Closest Malignancy, in the “Close” Margin Cohort

<table>
<thead>
<tr>
<th>Case</th>
<th>Tissue block</th>
<th>Distance from true margin to closest cells of closest malignancy (µm)</th>
<th>Distance from true margin to farthest cells of closest malignancy (µm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2a</td>
<td>B13</td>
<td>366</td>
<td>NA (farthest cells not visible)</td>
</tr>
<tr>
<td>3</td>
<td>C3</td>
<td>998</td>
<td>1,865</td>
</tr>
<tr>
<td>3</td>
<td>D4</td>
<td>980</td>
<td>1,429</td>
</tr>
<tr>
<td>7</td>
<td>D7</td>
<td>1,568</td>
<td>3,070</td>
</tr>
<tr>
<td>7</td>
<td>B2</td>
<td>167</td>
<td>1,072</td>
</tr>
<tr>
<td>7</td>
<td>B1</td>
<td>391</td>
<td>614</td>
</tr>
<tr>
<td>9</td>
<td>B6</td>
<td>148</td>
<td>318</td>
</tr>
<tr>
<td>9</td>
<td>B5</td>
<td>67</td>
<td>636</td>
</tr>
<tr>
<td>10</td>
<td>C5</td>
<td>416</td>
<td>878</td>
</tr>
<tr>
<td>10</td>
<td>C6</td>
<td>169</td>
<td>2,660</td>
</tr>
<tr>
<td>17a</td>
<td>C4</td>
<td>120</td>
<td>2,861</td>
</tr>
<tr>
<td>20</td>
<td>B2</td>
<td>138</td>
<td>1,029</td>
</tr>
</tbody>
</table>

*Twelve permanent sections from 7 cases, out of 20 total study patients.

*aThese cases were designated as CTI due to the high degree of cellular tissue.
IV, (2) cancer cells in the distressed CTI zone III, (3) cancer cells partially in the fused CTI zone II, or (4) cancer cells fully in the fused/charred tissue, CTI zones II and I. For the CTI tissue of close margins (2 out of the 12 close margins in CTI IV), if low thermal injury excision was replaced by traditional electrosurgery excision, it is expected the following changes would occur: 4% no change in classification, 46% of close margins reclassified as in CTI III, 50% close margins reclassified as partially in CTI II, and 0% of close margin cancer cells totally obscured in CTI I and/or II.

Using the following definitions in our analysis, (1) cells within the CTI charred zone I or fused zone II are unreadable by pathologist, (2) cells within the CTI distressed zone III are readable 50% of the time, and (3) cells within the FTI denatured zone are unreadable, it is expected that from our 12 margin dataset, 41% of the margins would have been judged negative, 48% of the margins would have been judged positive, and 11% of the margins would have been obscured and therefore erroneously judged negative.

### 3.4.6 Study Cohort Clinical Outcomes

Four patients underwent re-excision for multiple close margins and had whole-breast radiation. Of the re-excisions, one patient had residual classic lobular carcinoma in situ (LCIS), one patient had 1 mm residual invasive ductal carcinoma (IDC) and two patients had no residual tumor. One patient refused re-excision and radiation, and her cancer recurred after one year.

### 3.5 Discussion

Currently, it is estimated that approximately 20-50% of breast lumpectomies are classified as having positive or close excision margins [26, 27]. As excessive thermal injury may confound this critical classification by creating false-positive (changing a close margin to a positive) or false-negative (thermal injury obscuring cancer at or near the margin) evaluations, it also may critically affect downstream treatment decisions. Therefore, a reduced thermal-injury profile that improves the specificity of the margin determination may in turn improve post-surgical treatment outcomes. To our knowl-
Figure 3.6: Probabilities of each of “close” margin having unchanged margin status, change from close to positive margin status or change from close to obscured (false-negative) margin status if traditional electrosurgery were used for tumor excision. Each “close” margin from the dataset is associated with a pre-cancerous zone (green), cancerous zone (red) and post-cancerous zone (blue). The probabilities of thermal injury only affecting the pre-cancerous zone, partially affecting the positive margin or totally obscuring the cancer clusters are within the green, red and blue. Note: The model defines a 50% probability of readability for cancerous tissue within the CTI distressed zone, therefore some probabilities will not sum to 100%.
edge, there is currently no published research on the impact of electrosurgical thermal injury on oncologic margin status, or even a standard technique for the evaluation of thermal injury on tissue sections.

Furthermore, there currently exists no standardized method to accurately quantify the various degrees of thermal injury imparted by surgical excision tools. Therefore, a novel model (Figures 3.1 and 3.2) was developed to quantify the degree of thermal injury from electrosurgical instruments on true margins. This model exemplifies the traditional types of positive margins that are encountered during routine breast conservation therapy. FTI is representative of normal tissue with small cancer foci while CTI, being a mostly cellular tissue type, better represents the thermal injury encountered with invasive malignancies. The results of probability distribution estimation (Figure 3.4) of the thermal injury quantifications, as defined by our model, showed that, by 3 different metrics, calibrated margins created using traditional electrosurgery demonstrated a significantly greater depth of thermal injury (Table 3.2). These distributions were used to (1) estimate the reduction in thermal injury when using the low-thermal-injury device over traditional electrosurgery and (2) to estimate the fraction of close margins, created with the low-thermal-injury device which would have altered the margin status had a traditional electrosurgical instrument been used.

### 3.5.1 Re-excision Indications Based on Close Margin Evaluation

Two potential histopathological outcomes were evaluated. By creating more thermal damage the effective readable distance between the margin and the “close” malignancy can be decreased, thereby forming a smaller “close” margin or an apparent positive margin (a false-positive indication for re-excision). A second possible outcome is that the thermal injury can obscure a small malignancy near the margin (a false-negative indication for re-excision). To properly account for the large variability in thermal injury from both instruments, the full probability distributions (Figure 3.4) of thermal injury were employed to calculate probabilities of both outcomes for the true margins.
3.5.2 Clinical Impact

Downstream treatment decisions are principally determined by margin status. Based on the 12 margin dataset, the replacement of traditional electrosurgery with the low thermal injury device, on average, results in 7 more margins being judged close instead of positive or obscured (false-negative), a conversion of approximately 58%. Therefore, a reduced thermal-injury profile improves the specificity of the margin determination, which in turn may improve post-surgical treatment efficacy. It is expected that the analysis of fibrocollagenous and cellular tissue thermal injury along with its relationship to oncologic margin status has broad applicability to other tissue types.

3.5.3 Study Limitations

Although this study presents FTI and CTI as two uniform entities consisting of mainly fibrocollagenous and cellular tissues, it is often the case that a combination of FTI and CTI within the same tissue type is observed. Furthermore, due to the small dimensions of the calibrated tissue margins, it is possible that the distances for the CTI distressed/undisturbed boundary are under-reported and possibly hiding a larger difference for this category when comparing traditional electrosurgery to the low-thermal-injury device.

In the false-positive margin evaluation, the derived number of close margins affected by thermal injury is dependent on the exact margin distance from the clinical samples obtained. Although these results are encouraging, the present false-positive margin evaluation is a case study for our specific 20-patient clinical study and additional work in larger populations should be undertaken.

3.6 Conclusions

This study is the first to quantify the effects of different sources of radiofrequency energy on the analysis and interpretation of surgical margins in cancer resection. Thermal-injury artifact has been shown to negatively affect margin status, which consequently affects treatment after surgery and perhaps probability of recurrence. This
is a small pilot study of 20 patients, but the methodology has been demonstrated for quantifying the impact of thermal injury on final pathology for use in a larger, definitive study.

3.7 Acknowledgements

The authors would like to thank Robbin Newlin, PhD, and Guillermina Garcia for assistance with the digital imaging of histological samples. We also thank Jeanne McAdara-Berkowitz, PhD, Dominique Y. Atmodjo, and Larissa K. Low for assistance with data gathering and manuscript preparation.

Chapter 3 in full, is a reprint of the material as it appears in Quantitative Comparison of Surgical Margin Histology Following Excision With Traditional Electrosurgery and a Low-Thermal-Injury Dissection Device in The Journal of Surgical Oncology 2011. Ruidiaz, Manuel E.; Cortes-Mateos, Maria J.; Sandoval, Sergio; Martin, David T.; Wang-Rodriguez, Jessica; Hasteh, Farnaz; Wallace, Anne; Vose, Joshua G.; Kummel, Andrew C.; Blair, Sarah L.; Wiley-Liss, Inc., 2011. The dissertation author was the primary investigator and author of this paper.

3.8 References


Chapter 4

Thermal Injury Dependence on Adipose Tissue Content

4.1 Introduction

The thermal injury dynamics of the surgical margin interface and its relation to prognostic outcomes are poorly understood in human models. Recent work has attempted to elucidate the effect of electrosurgical tool choice on thermal injury [1] and healing [2] observables. Via the use of a novel thermal injury model, up to a 77% difference in thermal injury was observed [1]. The influences on thermal injury for observables other than blade type has not yet been studied on this newly developed thermal injury model. Previous studies have determined differences in thermal conductivities between different tissue types [3–5]. The use of electrosurgical tools for general surgery on a wide variety of tissue types is thus expected to have differing effects dependent on the tissue type through which the incision occurs. It is therefore hypothesized that tissue type, in addition to blade type, will have an effect on thermal injury profiles.

In this study, the affect of thermal injury on adipose tissue is quantified based on the previous dataset acquired by Ruidiaz et al. [1]. The original model and definitions of fibrocollagenous thermal injury (FTI) and cellular thermal injury (CTI) are maintained.
4.2 Methods

Calibrated margin samples were collected as described previously by Ruidiaz et al. [1]. Briefly, biopsy proven diagnosis of IDC with a minimum 1cm tumor diameter, without prior radiation treatment, and not undergoing mastectomy was the criteria for inclusion. Twenty (20) adult females satisfying these conditions underwent routine breast conservation therapy, performed with a PlasmaBlade 4.0 using a PULSAR® Generator (PEAK Surgical, Inc.) to remove the tumor mass. Using both the PlasmaBlade or a standard Electrosurgical tool (Valleylab Electrosurgical Pencil (Model E2516) with Model E1551X stainless steel electrode blade and Force Triad Generator (Valleylab, Boulder, CO)), incisions were made partially through the midsection of the excision mass. The incision was spread apart manually and a small sliver of tissue was removed using a standard scalpel. These calibrated margins were then embedded and approximately 10 representative sections per blade type per patient were stained with H&E.

Measurements were taken from the true margin to the collagen denaturation boundary, the true margin to the fused/distressed boundary and true margin to the distressed/undisturbed boundary as described previously [1]. To further measure the affect of adipose distance and margin measurements, an additional measurement was taken from the general location of the three margin measurements to the nearest adipose tissue on the slide. If adipose tissue was not visible, then a measurement value of NA was assigned and only included in analysis subsets without upper bound measurement limits.

Statistical analysis was performed using the R statistical environment version 2.13.0 [6]. Summaries were performed with univariate statistics and significance was defined as $P < 0.05$.

4.3 Results

The difference in distance to the nearest adipose tissue deposit between blade types was not statistically significant (Table 4.1). Analysis of the adipose tissue distance to the incision site when comparing near-adipose (distance $< 500\mu m$) to far-adipose (distance $\geq 500\mu m$) tissue for FTI collagen denaturation and CTI fused/dist-
Table 4.1: Dependence of nearest adipose tissue on electrosurgical instrument used.

<table>
<thead>
<tr>
<th>Metric</th>
<th>Instrument</th>
<th>Mean (SD) µm</th>
<th>Range µm</th>
<th>% Diff(^{a}) (p-value)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Distance to Nearest Adipose Tissue</td>
<td>Electrosurgery (N=128)</td>
<td>978 (1168)</td>
<td>0 – 6432</td>
<td>-8% (0.5798)</td>
</tr>
<tr>
<td></td>
<td>PlasmaBlade (N=150)</td>
<td>1055 (1144)</td>
<td>0 – 7035</td>
<td></td>
</tr>
</tbody>
</table>

\(^{a}\): Percentage difference is calculated with respect to traditional electrosurgery.
Table 4.2: Effect of fat distance on collagen denaturation measurements.

<table>
<thead>
<tr>
<th>Metric</th>
<th>Fat Distance</th>
<th>Electrosurgery µm (SD)</th>
<th>PlasmaBlade µm (SD)</th>
<th>% Diff(^a) (p-value)</th>
</tr>
</thead>
<tbody>
<tr>
<td>FTI Collagen Denaturation Boundary</td>
<td>&lt; 500µm</td>
<td>550 (456)</td>
<td>167 (184)</td>
<td>70% (&lt;0.001)</td>
</tr>
<tr>
<td></td>
<td>≥ 500µm</td>
<td>427 (295)</td>
<td>99 (109)</td>
<td>77% (&lt;0.001)</td>
</tr>
<tr>
<td>% Diff(^b) (p-value):</td>
<td></td>
<td></td>
<td></td>
<td>22% (0.047) 41% (0.010)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Metric</th>
<th>Fat Distance</th>
<th>Electrosurgery µm (SD)</th>
<th>PlasmaBlade µm (SD)</th>
<th>% Diff(^a) (p-value)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CTI fused/distressed Boundary</td>
<td>&lt; 500µm</td>
<td>379 (272)</td>
<td>82 (67)</td>
<td>78% (&lt;0.001)</td>
</tr>
<tr>
<td></td>
<td>≥ 500µm</td>
<td>199 (168)</td>
<td>91 (79)</td>
<td>54% (&lt;0.001)</td>
</tr>
<tr>
<td>% Diff(^b) (p-value):</td>
<td></td>
<td></td>
<td></td>
<td>47% (0.0005) -11% (0.4647)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Metric</th>
<th>Fat Distance</th>
<th>Electrosurgery µm (SD)</th>
<th>PlasmaBlade µm (SD)</th>
<th>% Diff(^a) (p-value)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CTI distressed/undisturbed Boundary</td>
<td>&lt; 500µm</td>
<td>800 (434)</td>
<td>523 (287)</td>
<td>35% (0.009)</td>
</tr>
<tr>
<td></td>
<td>≥ 500µm</td>
<td>1294 (760)</td>
<td>906 (772)</td>
<td>30% (0.009)</td>
</tr>
<tr>
<td>% Diff(^b) (p-value):</td>
<td></td>
<td></td>
<td></td>
<td>-62% (&lt;0.001) -73% (&lt;0.001)</td>
</tr>
</tbody>
</table>

\(^a\): Percentage difference is calculated with respect to traditional electrosurgery.

\(^b\): Percentage difference is calculated with respect to fat distance < 500 µm.
tressed boundaries demonstrated statistically significant increases in the amount of thermal injury as the distance to the nearest adipose tissue deposit decreased (Table 4.2). In “near-adipose” tissue, the PlasmaBlade and traditional electrosurgery device demonstrated a 41% \( (p = 0.01) \) and 22% \( (p = 0.047) \) increase in FTI collagen denaturation over “far-adipose” tissue, respectively. For the CTI fused/distressed boundary a -11% \( (p = 0.4647) \) and 47% \( (p = 0.0005) \) increase was observed for PlasmaBlade and traditional electrosurgery. In the CTI distressed/undisturbed boundary an decrease of 62% \( (p < 0.001) \) and 73% \( (p < 0.001) \) was observed, respectively.

### 4.4 Discussion

Margin distance plays a key role in the final margin status determination. Depending on institution, a positive or close margin generally requires a second surgical procedure to remove the remaining cancer tissue while a negative margin does not. A characterization of FTI and CTI tissue has been performed previously [1] and additional measurements have been taken to determine the effect of adipose content near the site of incision on FTI and CTI measurements by blade type.

An initial comparison was done to ensure that no bias was introduced in the adipose tissue measurements when compared to the type of blade used during the creation of calibrated margins (Table 4.1). The difference of means was not statistically significant indicating that no bias was introduced. Quantification for the “near-adipose” \(< 500\mu m\) and “far-adipose” \(\geq 500\mu m\) effect on thermal injury is summarized in Table 4.2. An increase in the amount of collagen denaturation when adipose tissue was within 500 \( \mu m\) (Figure 4.1). In the CTI fused/disturbed boundary measurement, an increase was observed for the case of electrosurgery while the change in the PlasmaBlade was not statistically significant \( (P = 0.4647) \) (Figure 4.2). In the CTI distressed/undisturbed case a decrease was observed which was statistically significant \( (P < 0.001) \) for both measurement modalities (Figure 4.3).

Graphically, the thermal injury profiles for the FTI collagen denaturation and CTI fused/distressed boundaries have the majority of variability in the thermal injury measurements when closest to adipose tissue with the PlasmaBlade having a smaller dis-
**Figure 4.1:** Effect of adipose tissue distance to the FTI collagen denaturation boundary.

**Figure 4.2:** Effect of adipose tissue distance to the CTI fused/distressed boundary.
Figure 4.3: Effect of adipose tissue distance to the CTI distressed/undisturbed boundary.

dispersion. In the CTI distressed/undisturbed boundary case, the variation is more widely dispersed with no clear distinction between blade type. The lack of a clear separation between blade types is most likely due to the tissue section sizes being similar in size to the measured CTI fused/distressed boundaries.

Taking into account the FTI collagen denaturation and CTI fused/distressed boundary distance profiles, it is evident that the location of adipose tissue to the incision site has a strong positive influence on local thermal injury. According to the majority of previous studies, adipose tissue has a lower heat capacity and lower thermal conductivity than epidermal, dermal and muscle tissue types [7, 8]. This has been correlated to the reduced water content of adipose tissue (on the order of 20%) compared to other tissue types. These experimentally obtained measurements point to adipose tissue acting as an insulator when exposed to a thermal heat source. The insulation activity causes the surrounding collagenous and cellular tissue to be exposed to higher thermal stress causing increased thermal injury.
4.4.1 Study Limitations

Measurements of adipose tissue was performed on representative sections perpendicular to the thermal injury plane for each blade type. Due to the limitations of performing such two-dimensional measurements, the influence of adipose tissue that may have been closer but in a different tissue section may not be adequately reported.

Additional uncertainty may have been introduced due to the incisions not being performed on perfused tissue. It has been previously reported that the thermal conductivity of in-vivo tissue is higher than for dead tissue [7, 9]. This was primarily attributed to the presence of vascular perfusion in-vivo. In our assessment of thermal injury, calibrated margins were created as a surrogate for the true margin. These calibrated margins were created on ex-vivo tissue that has an inherent lack of vascular perfusion.

4.5 Conclusions

The primary determinant for the determination of margin status in carcinomas is the presence of malignant cells in an FTI or CTI tissue type. Therefore, adipose tissue content impedes the determination of margin status by increasing the amount of thermal injury observed. An initial analysis is provided which indicates an inverse relationship between thermal conductivity and thermal injury with adipose tissue being a negative effector of margin status.

Further study is warranted on the affect of vascular perfusion on thermal injury. Vascular perfusion could serve as an inhibitor of thermal injury by effectively transporting heat away from the blade. Conversely, a perfused vasculature may impart additional thermal injury, which is created by the surgeon placing the blade on tissue for longer time periods, for the purposes of maintaining hemostatic control. This may counteract the effect of increased thermal conductivity. To determine the net effect, it is proposed that further measurements be undertaken on the relationship between the vasculature and thermal injury as applied to our model.
4.6 Acknowledgments

The contents of Chapter 4 are part of a manuscript in preparation, Ruidíaz, Manuel E.; Kummel, Andrew C.; Thermal Injury Dependence on Adipose Tissue. The dissertation author was the primary investigator and author of this paper.

4.7 References


Chapter 5

Decision Support System for Tumor Margin Determination during Breast Conservation Therapy

5.1 Introduction

Approximately 32-63% of breast conservation therapy patients result in having positive margin status [1]. If the cancer tissue that remains within the breast is not removed it may continue to grow and/or become metastatic leading to further possible complications including death. Several studies have determined that negative margin status is required for control of local recurrence of disease [2–5]. Therefore, a positive margin results in possible multiple re-excisions surgeries, increases the likelihood of complications, produces physical discomfort, increases emotional distress and decreases cosmetic outcomes [1, 6].

Intraoperative margin evaluation has been determined to reduce the re-excision rates in patients [7–9]. Several techniques have been tested for the evaluation of intraoperative margins including gross examination [6, 9, 10], intraoperative ultrasound [7, 10–13], frozen section analysis (FSA) [14–18] and imprint cytology (IC) [5, 19–22] and are described below. However, currently these techniques are not routinely used.
**Gross Examination**  Gross examination involves the inspection of the excision and cavity surfaces for signs of disease by visual or palpatory means during the surgery. If cancer is detected, additional tissue is further excised from the corresponding margin of the patient. The identification of margin status by gross examination has been demonstrated to be of limited utility [6]. Difficulties in this approach include that most women do not have palpable masses [23], tumor cells may exist beyond the confines of a palpable mass [24], gross visualization of microscopic tumor cells is technically difficult, and the use of cauterization tools (electrosurgery) distorts the margin surface [25].

**Intraoperative Ultrasound**  In intraoperative ultrasound evaluation, the transducer is used on the excised tumor mass and remaining breast tissue to ensure the entirety of the cancer is removed during surgery [10]. This technique has been shown to be effective for breast tumor localization during biopsy [26] and localization during surgery compared to wire-guided excision of non-palpable breast cancer [27]. However, for the technique to be effective, the surgeon must be experienced in the use of ultrasound. Furthermore, only about 50% of nonpalpable lesion can be visualized in this manner [28] and ductal carcinoma in-site (DCIS) infiltration is difficult to detect [26].

**Frozen Section Analysis**  In FSA, the excised tissue mass is placed in an embedding solution and frozen immediately after removal from the patient [29]. Sectioning is then performed to provide a representative view of the margins of the tissue mass. Representative sections are stained followed by evaluation by pathologist. If cancerous tissue is present in the representative sections, they are then further shaved from excision cavity by the surgeon. Further representative frozen sections may then be taken of the newly removed tissue to ensure negative margin status. Although FSA provides a complete analysis of the margin state, the technique is difficult to perform for tissue with high fat content, it has limited utility with small tumors, disrupts subsequent permanent section analysis, it is labor intensive process that requires coordination of the surgeons, histology technicians and pathologists, and therefore costly which limits it use in the clinic [19, 30].
Imprint Cytology  IC is an alternative intraoperative technique used to determine the presence of cancer cells at the margin surface. In the operating room, once the tissue is excised from the patient, the tumor surface is sampled for cancerous cells [22, 31]. Exposed surfaces are imprinted on glass slides while keeping track of the location on the tissue from which the imprint was taken from. During the imprint process, cells on the surface of the tumor are removed from the tissue surface by electrostatic and hydrophobic interactions with the glass slide which preferentially adsorbs epithelial and blood cells over adipose tissue [32]. The preferential sampling of epithelial cells allows the imprint cytology samples to provide a representative view of cancer cells on the margin surface. It is also noted that cancer cells tend to lose their cellular adhesion properties in the tumor microenvironment which may further improve the preferential attachment of those cells during the imprint process [33–35]. Any cells that adhere to the slide surface are then appropriately stained and then analyzed by cytopathologist to identify cancerous cells [3, 19]. IC is a much simpler and less labor intensive technique than FSA which enhances the utility in the operating room but its utility for detecting close margins is poor because of the inability to sample cells below the excision surface [32]. Also, the imprinting process tends to introduce staining and drying artifacts because the monolayer of cells on the surface of the imprinted slide can easily become desiccated [31, 36].

5.1.1 Margin Evaluation Performance

Inherent in the evaluation of margin status is the determination of the presence of cancer cells at the tumor margin surface. Each of the above techniques vary in the ability to sample tumor margin cells which may affect the corresponding utility of the technique for tumor margin analysis. Each of the above techniques has utility in evaluation of the tumor that can be categorized into three groups: linear, surface, and volumetric sampling of the tumor. In the linear sampling modality, margin status is evaluated in discrete linear segments. Surface sampling is able to evaluate the entire surface of the tumor, while volumetric techniques are able to determine the three dimensional extent of the tumor.

Linear sampling techniques, such as FSA, involves obtaining thin strips of tissue
that are perpendicular to the margin surface. The limitation of linear sampling is primarily imposed by the poor ability of each section to give a representation of the entire margin surface. In an intraoperative assessment, this evaluation is limited to 10-15% of the margin surface [31]. Surface and volumetric techniques are more adept to fully evaluate the entire margin surface, but are currently limited by technical restrictions of the corresponding technology used.

Surface sampling techniques, such as visual gross inspection and CI, must get past the cautery artifact imposed by the use of haemostatic surgical instruments, such as electrosurgery, laser ablation and ultrasonic scalpel, to reduce bleeding during the surgery. Furthermore, a high spatial sampling resolution is required to adequately sample the entire surface. For visual gross inspection, the spatial resolution is low and consequently a poor technique for margin determination. On the contrary, imprint cytology has a much higher spatial sampling resolution, which enhances its margin determination ability.

Volumetric sampling techniques can theoretically provide the best assessment of margin status because of the ability to determine the extent of tumor infiltration at the surface and within the excised tumor mass. But currently used techniques, such as ultrasound and tactile gross inspection, are primarily limited by tumor type and accuracy.

Of the discussed techniques, imprint cytology has the ability to accurately sample the margin surface without affecting downstream permanent section analysis, which is the current “gold” standard for margin evaluation. Furthermore, the low cost, fast analysis and ease of use makes IC a practical intraoperative assessment technique.

5.2 Evaluation of Imprint Cytology Slides

The imprinting process creates a specimen that is sparsely covered by epithelial cells (including cancer cells if present), erythrocytes, leukocytes, cellular debris (organelles and cellular substructures), scrape debris, cauterized debris and staining and drying artifacts such as bubbles, stained non-cellular tissue, etc. For a given excision tissue, the imprinted specimen size is comparable in size to the 25 mm by 50 mm dimensions of the slide surface. Therefore, to adequately determine the status of the margin,
an experienced cytopathologist is required. Given that a reasonably sized excision tissue will be sampled on its 6 separate margin surfaces, this requires the search of 7500 mm$^2$ of slide area per tissue excision and is immensely time consuming.

The sparse nature of the imprint destroys any of the original architectural information from which the cells originated from, therefore it is difficult for a cytopathologist to use a low magnification (1x, 5x) to search across the entire slide to find areas of interest. To enable the cytopathologist to find cancerous cells on the slide and to differentiate from other types of debris and artifacts, a reasonable magnification level must be used (10x, 20x). It may also be necessary to increase the magnification to 40x to be able to differentiate between cancerous epithelial cells, normal epithelial cells and leukocytes. For a standard field of view of 1.4 mm by 1.0 mm (10x magnification), it is expected that 5357 fields of view must be thoroughly inspected to accurately determine margin status.

### 5.2.1 Automated Imprint Cytology Slide Evaluation

Identification and analysis of IC slides presents challenges which must be overcome for IC to gain widespread utility as an intraoperative margin analysis. 1. IC presents a highly heterogeneous slide surface consisting of several cellular types, no architectural features, and several types of debris and artifacts. For routine analysis, cytopathologists with experience in analyzing cellular features are required. 2. The large area of sparse cellular structure is difficult to thoroughly analyze intraoperatively due to the time restrictions inherent during surgery.

To overcome these limitations it is proposed that an automated analysis is better suited to the detection and identification of positive margins via IC. Current slide scanning technology has progressed to the point of being able to quickly scan through slides at various resolutions on the fly. Additionally, the sparse nature of the IC surface implies that a parallel processing architecture would be exploitable to analyze the entire slide simultaneously. This enables the use of modern multiprocessor computing architectures to process the slides enabling reduced processing time to enable intraoperative margin assessment.
Table 5.1: Positive Margin Training Subset Characteristics.

<table>
<thead>
<tr>
<th>Sample Number</th>
<th>Diagnosis</th>
<th>Positive Margin</th>
</tr>
</thead>
<tbody>
<tr>
<td>87</td>
<td>IDC</td>
<td>Deep</td>
</tr>
<tr>
<td>89</td>
<td>IDC/ILC</td>
<td>Deep</td>
</tr>
<tr>
<td>100</td>
<td>IDC/ILC</td>
<td>Lateral</td>
</tr>
<tr>
<td>111</td>
<td>IDC</td>
<td>Deep</td>
</tr>
<tr>
<td>113</td>
<td>DCIS/LCIS</td>
<td>Deep + Inferior</td>
</tr>
</tbody>
</table>

Table 5.2: Close Margin Training Subset Characteristics.

<table>
<thead>
<tr>
<th>Sample Number</th>
<th>Diagnosis</th>
<th>Close Margin</th>
</tr>
</thead>
<tbody>
<tr>
<td>91</td>
<td>DCIS</td>
<td>Lateral</td>
</tr>
<tr>
<td>95</td>
<td>IDC</td>
<td>Deep</td>
</tr>
<tr>
<td>104</td>
<td>IDC/DCIS</td>
<td>Deep</td>
</tr>
</tbody>
</table>

5.3 Methods

5.3.1 Study Population

Imprint cytology specimen collection was approved by the Institutional Review Board of the University of California, San Diego and was performed via accepted standards for clinical research. A retrospective analysis of 127 patients was performed to determine specimens with positive or close permanent section diagnosis. 18 positive/close margin cases were selected and an 8 slide training subset was culled. This subset consisted of 5 slides with positive margin determination (Table 5.1) and 3 slides with close margin determination (Table 5.2). An additional testing set was created consisting of 8 cases (exclusive of the training set) from the 18 positive/close margin selection (Table 5.3).

5.3.2 Slide Collection

Multiple 1-inch x 3-inch glass microscope slides coated with poly-L-lysine (PLL) (Newcomer Supply, Middleton, WI) were used for imprint cytology. Six (6) margin faces (Superficial, Deep, Superior, Inferior, Lateral, Medial) oriented by suture place-
Table 5.3: Testing Subset Characteristics.

<table>
<thead>
<tr>
<th>Sample Number</th>
<th>Diagnosis</th>
<th>Margin Status</th>
</tr>
</thead>
<tbody>
<tr>
<td>17</td>
<td>IDC</td>
<td>Positive</td>
</tr>
<tr>
<td>18</td>
<td>IDC</td>
<td>Negative</td>
</tr>
<tr>
<td>24</td>
<td>IDC</td>
<td>Negative</td>
</tr>
<tr>
<td>26</td>
<td>IDC</td>
<td>Positive</td>
</tr>
<tr>
<td>32</td>
<td>IDC</td>
<td>Positive</td>
</tr>
<tr>
<td>41</td>
<td>Lobular</td>
<td>Positive</td>
</tr>
<tr>
<td>44</td>
<td>IDC</td>
<td>Positive</td>
</tr>
<tr>
<td>58</td>
<td>Lobular</td>
<td>Positive</td>
</tr>
</tbody>
</table>

ment during initial excision were used for imprint cytology. Each imprint consisted of one PLL-coated slide being pressed against each margin surface for 10 seconds followed by immediate fixation in pure ethanol for 24 hours, after which the slide was stained with Hæmatoxylin and Eosin (H&E). Directly following each imprint, a #10 scalpel blade (VWR International, LLC., West Chester, PA) was placed perpendicular to the tumor surface and dragged across the tissue in a scraping fashion to remove cautery artifact. Immediately following scraping, an additional imprint cytology procedure was performed on the surface of the corresponding margin face, as described above. This resulted in 18 total slides per lumpectomy sample (6 pre-scrape slides, 6 debris slides, and 6 post-scrape slides). For the subsequent analysis, post-scrape slide specimens are used.

5.3.3 Slide Scanning and Image Processing

An IC image processing system was developed based on ImageJ [37] and adapted to function on images of H&E stained whole slides. Whole imprint cytology slides where digitized on an ScanScope XT slide scanner (Aperio Technologies, Inc., Vista, CA) at 40x (0.25 µm/pixel) and saved as SVS files with JPEG 2000 compression. Image analysis was done in two phases: 1. An outlining phase where raw pixel data was converted to objects representing cells, debris and artifacts; 2. An object analysis phase were individual objects were identified and quantified across the entire slide.
Phase I: Image Outlining

Each scanned IC slide was saved to an individual image file. Each image file was then broken down into 2000 x 2000 pixel subtiles with a 100 pixel overlap between tiles to ensure objects of interest were fully outlined. A series of image filters (Figure 5.1) were then applied in sequence to each subtile:

**Red Channel Filter**  In H&E staining, the Hæmatoxylin stain is selective and primarily stains the nucleus and related substructures a dark purple hue [38]. Eosin is more of a general stain which gives a light pink appearance to the entire tissue with erythrocytes having a strong red component. In IC specimens, the goal is to isolate cancer cells which tend to have a large nucleus due high DNA activity implicit in a cell that divides incessantly. These cell types would then be expected to have a strong Hæmatoxylin staining component.

The bright field nature slide images implies a white background which is composed of a combination of red, green, and blue primary colors in an additive color scheme. A red stained structure has a red component, but reduced green and blue components. Therefore the application of a red channel filter, which only allows the red component to be seen, will cause the red component to be isolated. The resultant image will then hide the strong red components because a strongly stained red structure and the white background have similar red component intensity (Figure 5.2).

**Laplacian of Gaussian Thresholding**  The filtered red channel image is then processed with a Laplacian of Gaussian (LoG) Filter. A Gaussian smoothing filter is first applied to reduce the noise in the underlying starting image. Subsequently, the Laplacian operation is performed. The zero crossings of the image are detected and used to define the boundaries of detected blobs. The resultant image at this point is a binary image (Figure 5.3).

**Binary Operations**  The LoG operation produces blobs of various sizes across the subtiles. Because some of these blobs were incorrectly identified as separated blobs, a dilation operation is performed on the binary image to concatenate closely associated
Figure 5.1: Image processing steps.
Figure 5.2: Isolation of red channel for minimization of blood and eosin staining artifacts.
Figure 5.3: Blob selection using Laplacian of Gaussian Thresholding.
blobs. A hole filling operation is then performed to include any structures with a dimly stained interior as a single object. Finally, a binary erode operation is performed to recover the original dimensions of the LoG selected blob (Figure 5.4).

**Watershed Operation** For darkly stained objects that are extremely close together, it is often the case that the LoG filter creates a concatenated blob composed of two smaller blobs connected by a small bridge. To correctly subdivided these types of concatenations, a watershed operation is performed. During this operation, a series of erode operations is performed to determine the ultimate points (Figure 5.5(b)). The valleys between the ultimate points correspond to the watershed lines (Figure 5.5(c)).
Figure 5.5: Watershed based division of binary blobs.
Object Selection and Outlining  Blobs are finally scanned and pixel area is measured. Detected blobs with sizes greater than 360 $\mu m^2$ were most associated with large debris and cauterization artifacts. Blobs that were smaller than 10 $\mu m^2$ corresponded to blobs that were associated with dust, staining artifacts, and small specks. During the selection range only sizes between 10 $\mu m^2$ and 360 $\mu m^2$ were chosen because they were indicative of biologically relevant blobs such as cancer, epithelial, and white blood cells. This size range also has coincidentally sized debris and artifacts. A sample IC specimen with locations of 118,897 size-selected blobs (Figure 5.6).
Object Measurements  Selected regions of interest (ROI) were then measured for 179 characteristics. These characteristics can be divided into five classes: Grey Descriptors, Shape Descriptors, Local Area Descriptors, Inner Color Descriptors, Outer Color Descriptors.

Grey Descriptors  The class of grey descriptors consists of raw pixel RGB features mapped to 8-bit (0-255) grey scale using a 1/3 intensity for each RGB channel. In the grey color space, the raw pixel distribution of the selected ROI are used to calculate the following features:

- Minimum grey value
- Maximum grey value
- Mean grey value
- Median grey value
- Standard Deviation of the selected 8-bit pixel values
- Integrated density: Area $\times$ Mean Grey Value
- Grey skewness value: Measurement of grey symmetry within the ROI
- Grey kurtosis value: Measurement of grey flatness within the ROI
- The dimensionless standard deviation of intensity:
  
  Grey Standard Deviation/Mean grey value

Shape Descriptors  The class of shape descriptors are based on characteristics of the ROI boundary values.

- Area
- Perimeter
- Circularity: $4 \times \pi \times \text{area}/\text{perimeter}^2$
• Ellipse fit: Measurement values based on an ellipse fit of the ROI
  – Major axis length
  – Minor axis length
  – Major axis angle
• Feret’s diameter: Largest diameter of the ROI
• Aspect ratio: major axis length/minor axis length
• Roundness: \((4 \times \text{area})/(\pi \times \text{major axis}^2)\)
• Solidity: area/convex area

**Color Descriptors**  Color descriptors were measured in two complementary color spaces, RGB and YUV. RGB is the default color space used during image capture and processing. The YUV color space was additionally selected for image capture because it is modeled after human color perception[39–42]. Grey representations of each color channel are displayed in Figure 5.7. For each of the 6 components of the two color spaces (R, G, B, Y, U, V) the equivalent grey descriptor measurements were performed on the ROI. These measurements were defined as the inner color descriptors. A similar set of measurements is then performed on a modified rectangle centered at the original coordinates of the ROI. This rectangle has a width and height dimension that are 3 times the original ROI but is modified by subtracting out the original area in the center where the ROI is located. These measurements were the outer color descriptors.

**Phase II: Object Analysis**

In the object analysis phase, whole slide ROI statistics and calculations are performed taking into account more than a single ROI object. Three classes of measurements are performed Local Area Descriptors, ROI Classification, Cancer Cluster Identification.
Figure 5.7: Color Channels used for analysis.
Local Area Descriptors  For the local area descriptors the 10 closest objects were selected and summary statistics are calculated for the area, perimeter, circularity, Feret’s diameter and grey standard deviation.

- Minimum distance
- For the closest 10 objects
  - Standard deviation of the area
  - Minimum area
  - Maximum area
  - First quartile area
  - Third quartile area
  - Mean area
  - Median area

In addition to the above descriptors, the following additional local area descriptors are calculated.

- The radius that encompasses the 10 closest objects
- The distance to the closest object
- For a radius of 50 \( \mu \text{m} \)
  - Fraction of area within ROIs: \( \frac{\text{Area of ROIs}}{4 \times \pi \times 50^2} \)
  - The number of ROIs

ROI Classification  Each ROI consists of an outline along with 179 descriptors. Each of these measurements was classified using linear discriminant analysis (LDA). LDA was selected because of its widespread utility and performance in several classification problems[43–50] and the general ease of prediction of unknown data by using a nearest neighbor analysis in LDA-transformed space. The LDA classifier is previously trained based on an 8 slide training set consisting of positive and close margins samples as
diagnosed by permanent section analysis. For this analysis, the LDA classifier was trained to identify three classes of objects consisting of cancer cells, normal cells, debris and artifacts. Classification of each category was performed by a 10-nearest neighbor voting algorithm in LDA-transformed space, which was populated by the training set.

**Cancer Cluster Identification**  LDA classification yields individual outlines which were defined as being cancerous, normal, or debris. Due to the lack of architecture on the IC specimen, a manual analysis of IC primarily makes use of individual cancer cells on the specimen. One of the pitfalls of looking at any one individual cancer cell is that it may have visual characteristics which are hard to discern from leukocytes or epithelial cells. Therefore the higher the number of cancer-like cells that are clustered near each other, the greater the probability that a given cell indeed came from a positive margin. Thus, it is more beneficial to show to the end-user the largest cancer clusters first. To achieve this, for each cell that has been classified by LDA as cancer, a 200 µm radius search is performed and the number of other LDA-classified cancer cells within the search radius is calculated. This list is sorted by decreasing average circularity of the cluster and the top 25 clusters that are greater than 5 cancer cells in size is selected for presentation and evaluation by pathologist (Figure 5.8). Should a given IC specimen be confirmed to have cancer cells, then the surgeon can be instructed to shave additional tissue from the cavity.

### 5.3.4 Statistical Analysis

Statistical analysis was performed using the R statistical computing environment version 2.13.0. Raw values were examined to elicit sources of maximal variance for data classification studies. Multivariate linear discriminant analysis (MASS package, was used to create the training set for IC object classification. Recursive feature elimination was performed with the caret package. For error estimation, the errorest function was used in the ipred package. Cross-validation was used to provide estimates of variable performance for classification and for the determination of which variables provided the greatest prognostic significance.
**Figure 5.8:** Example identification of top 25 identified “cancer” regions (ordered from top left to right by row) based on described circularity metric. Positive cancer cells are present in clusters 1, 2, 3, 4, 6, 7, 18, 21.
### Table 5.4: LDA Training Set Summary

<table>
<thead>
<tr>
<th>Manual Object Classification</th>
<th>Training Set Size</th>
<th>Training Set Proportion</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cancer</td>
<td>184</td>
<td>12%</td>
</tr>
<tr>
<td>Cell</td>
<td>416</td>
<td>28%</td>
</tr>
<tr>
<td>Junk</td>
<td>893</td>
<td>60%</td>
</tr>
<tr>
<td>Total:</td>
<td>1493</td>
<td></td>
</tr>
</tbody>
</table>

### Table 5.5: LDA leave-one-out cross validation performance estimation.

<table>
<thead>
<tr>
<th>Predicted Class</th>
<th>Cancer</th>
<th>Cell</th>
<th>Junk</th>
<th>Accuracy</th>
</tr>
</thead>
<tbody>
<tr>
<td>True Class</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cancer</td>
<td>155</td>
<td>15</td>
<td>14</td>
<td>84%</td>
</tr>
<tr>
<td>Cell</td>
<td>33</td>
<td>361</td>
<td>22</td>
<td>87%</td>
</tr>
<tr>
<td>Junk</td>
<td>24</td>
<td>27</td>
<td>842</td>
<td>94%</td>
</tr>
</tbody>
</table>

### 5.4 Results

#### 5.4.1 Classifier Training

Classifier training was performed on the 8-slide dataset as previously described (Table 5.1 & 5.2). A manual search was performed on the digitized slides and selected objects were identified as belonging to one of three possible classes: cancer, normal, and debris (Figure 5.9). The labeling process yielded 184 cancer, 416 cell, and 893 junk objects (Table 5.4).

This set of manually identified objects was then used as the training set for an LDA classifier. The classifier training yielded two linear discriminants, LD1 and LD2 each having a proportion of trace, a measure of how much of the between-class variance is explained, of 78% and 22% respectively. The LDA-transformed representation of the training set is plotted in Figure 5.10. LDA classifier performance was analyzed with a leave-one-out cross validation yielding an overall accuracy of 91%. Individual class performance was 84%, 87% and 94% for cancer, cell and junk objects, respectively. A contingency table of these results are shown in Table 5.5.
Figure 5.9: Representative examples of cancer, normal and debris on imprint cytology specimens.
Figure 5.10: Class separation in LDA-transformed space showing Cancer (red), Normal Cell (Green), and Junk/Debris (Blue) clustered separately. 80% normal probability ellipses for each class is overlaid.
Table 5.6: LDA accuracy evaluation by descriptor used.

<table>
<thead>
<tr>
<th>Descriptor Used</th>
<th>LDA Classification Accuracy</th>
<th>Performance Improvement</th>
</tr>
</thead>
<tbody>
<tr>
<td>Worst Case Random Variable</td>
<td>60%</td>
<td>–</td>
</tr>
<tr>
<td>Grey</td>
<td>69%</td>
<td>9%</td>
</tr>
<tr>
<td>Shape</td>
<td>72%</td>
<td>12%</td>
</tr>
<tr>
<td>Local Area</td>
<td>65%</td>
<td>5%</td>
</tr>
<tr>
<td>Inner Color</td>
<td>83%</td>
<td>23%</td>
</tr>
<tr>
<td>Outer Color</td>
<td>75%</td>
<td>15%</td>
</tr>
<tr>
<td>All</td>
<td>91%</td>
<td>31%</td>
</tr>
</tbody>
</table>

LDA Performance by Class of Descriptors Used

To evaluate the respective contributions of each set of descriptors to the overall classifier performance, an evaluation of classification accuracy was surveyed by omitting sets of descriptors used during the LDA training phase. Grey, shape, local area, inner color and outer color descriptors individually were used to train an LDA classifier to distinguish between the classes described above. This was compared to a dummy classification where the training was done against the random variable and the full classification set, which uses all the combined variables. Accuracy estimation was performed by a 10-fold cross-validation and is displayed in Table 5.6. LDA-transformed plots are in Figure 5.11.

LDA Variable Importance by Object Class

An analysis of the respective contribution to the overall accuracy of the classifier was performed by evaluation of each class as a binary classification. Recursive feature elimination was performed on each LDA binary classifier, which involves the sequential elimination of variable by decreasing importance. Rankings of variable importance by selected class are presented in Table 5.7.
Figure 5.11: LDA-transformed plots of the training set with only individual classes of descriptors used and also with all descriptors combined.
Table 5.7: Variable Importance by individual and combined classes.

<table>
<thead>
<tr>
<th>Importance Ranking</th>
<th>Cancer</th>
<th>Non-Cancer Cell</th>
<th>Debris/Artifact</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>B_INTEGRATEDDENSITY</td>
<td>R_MEAN</td>
<td>R_MEDIAN</td>
</tr>
<tr>
<td>2</td>
<td>U_INTEGRATEDDENSITY</td>
<td>R_MEDIAN</td>
<td>R_MEAN</td>
</tr>
<tr>
<td>3</td>
<td>INTEGRATEDDENSITY</td>
<td>R_MIN</td>
<td>R_MIN</td>
</tr>
<tr>
<td>4</td>
<td>Y_INTEGRATEDDENSITY</td>
<td>U_MAX</td>
<td>U_MAX</td>
</tr>
<tr>
<td>5</td>
<td>AREA</td>
<td>OUTER_R_MEAN</td>
<td>U_INTEGRATEDDENSITY</td>
</tr>
<tr>
<td>6</td>
<td>G_INTEGRATEDDENSITY</td>
<td>V_MIN</td>
<td>ELLIPSEMINOR</td>
</tr>
<tr>
<td>7</td>
<td>ELLIPSEMINOR</td>
<td>DLSD</td>
<td>B_INTEGRATEDDENSITY</td>
</tr>
<tr>
<td>8</td>
<td>LOCAL_Median_AREA_C10</td>
<td>OUTER_R_MEDIAN</td>
<td>AREA</td>
</tr>
<tr>
<td>9</td>
<td>SOLIDITY</td>
<td>Y_STDDEV</td>
<td>V_MIN</td>
</tr>
<tr>
<td>10</td>
<td>R_INTEGRATEDDENSITY</td>
<td>Y_MIN</td>
<td>SOLIDITY</td>
</tr>
<tr>
<td>11</td>
<td>V_INTEGRATEDDENSITY</td>
<td>STDDEV</td>
<td>U_MEDIAN</td>
</tr>
<tr>
<td>12</td>
<td>ELLIPSEMAJOR</td>
<td>G_STDDEV</td>
<td>OUTER_G_INTEGRATEDDENSITY</td>
</tr>
<tr>
<td>13</td>
<td>OUTER_B_INTEGRATEDDENSITY</td>
<td>U_MEDIAN</td>
<td>OUTER_Y_INTEGRATEDDENSITY</td>
</tr>
<tr>
<td>14</td>
<td>OUTER_Y_INTEGRATEDDENSITY</td>
<td>R_STDDEV</td>
<td>OUTER_B_INTEGRATEDDENSITY</td>
</tr>
<tr>
<td>15</td>
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<td>U_MEDIAN</td>
<td>OUTER_R_MEDIAN</td>
</tr>
<tr>
<td>16</td>
<td>FERETMINIMUMDIAMETER</td>
<td>B_MAX</td>
<td>OUTER_R_MEDIAN</td>
</tr>
<tr>
<td>17</td>
<td>OUTER_G_INTEGRATEDDENSITY</td>
<td>MIN</td>
<td>FERETMINIMUMDIAMETER</td>
</tr>
<tr>
<td>18</td>
<td>OUTER_U_INTEGRATEDDENSITY</td>
<td>ELLIPSEMINOR</td>
<td>OUTER_R_MEDIAN</td>
</tr>
<tr>
<td>19</td>
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<td>V_MEDIAN</td>
<td>OUTER_R_INTEGRATEDDENSITY</td>
</tr>
<tr>
<td>20</td>
<td>LOCAL_Q1_AREA_C10</td>
<td>OUTER_R_SKEWNESS</td>
<td>INTEGRATEDDENSITY</td>
</tr>
</tbody>
</table>
Table 5.8: Automated analysis performance with testing subset.

<table>
<thead>
<tr>
<th>Sample Number</th>
<th>Diagnosis</th>
<th>Margin Status</th>
<th>Automated Margin Status</th>
<th>Positive Fields</th>
</tr>
</thead>
<tbody>
<tr>
<td>17</td>
<td>IDC</td>
<td>Positive</td>
<td>Negative</td>
<td>0</td>
</tr>
<tr>
<td>18</td>
<td>IDC</td>
<td>Negative</td>
<td>Positive</td>
<td>Medial (8)</td>
</tr>
<tr>
<td>24</td>
<td>IDC</td>
<td>Negative</td>
<td>Positive</td>
<td>Anterior(6)</td>
</tr>
<tr>
<td>26</td>
<td>IDC</td>
<td>Positive</td>
<td>Negative</td>
<td>0</td>
</tr>
<tr>
<td>32</td>
<td>IDC</td>
<td>Positive</td>
<td>Positive</td>
<td>Superior(3)</td>
</tr>
<tr>
<td>41</td>
<td>Lobular</td>
<td>Positive</td>
<td>Positive</td>
<td>Inf(20), Sup(25), Deep(25)</td>
</tr>
<tr>
<td>44</td>
<td>IDC</td>
<td>Positive</td>
<td>Negative</td>
<td>0</td>
</tr>
<tr>
<td>58</td>
<td>Lobular</td>
<td>Positive</td>
<td>Negative</td>
<td>0</td>
</tr>
</tbody>
</table>

5.4.2 System Validation on Testing Set

Validation of the analysis system was performed using an 8 case testing subset, listed in Table 5.3, consisting of approximately 6 margin faces per case (46 IC specimens). The LDA classifier, created from the training set, was used to identify objects of class cancer. Identified cancer cells were then clustered for presentation to the user as described earlier. The performance of the final user presentation was quantified by the actual number of positive cancer fields presented to the user (Table 5.8).

5.5 Discussion

The requirement of an automated margin analysis tool is acute considering the relatively high rate of second surgeries due to positive margin status. An automated margin analysis tool has been developed which is able to leverage whole slide scanning microscopes with automated computational analysis to provide a solution for intraoperative margin determination. Analysis was performed on pre-labeled training data which was primarily analyzed using resampling techniques until a robust system was obtained. Subsequent validation consisted of performing an analysis on a true margin dataset that was independent of the training set, to give a sense of true system performance.
5.5.1 Training Set

The training set was based on an 8 slide positive/close margin subset, which was manually identified for cells belonging to the class of either cancer cells, normal cells, or junk/debris objects (Figure 5.9). After an exhaustive search, final class proportions were 12%, 28% and 60% for the classes respectively (Table 5.4). Because these slides were derived from a true cauterized margin, a disproportionate selection of junk/debris objects over other class types occurs. Subsequent analysis must then take into account that the minimum overall accuracy performance of classifying random data with such a class structure is equivalent to the largest class proportion, or 60%.

10-fold cross validation of the overall LDA performance yielded approximately 91% accuracy. To further understand the individual class performance, a leave-one-out cross validation was performed and a contingency table was created to understand the relative performances of the LDA discriminator for each class respectively (Table 5.5). For our three classes, individual class accuracy is estimated to be greater than 84%, which is indicative that a high degree of discrimination is present between all three classes. This is further validated by the LDA discrimination plot whereby each training object is transformed by the linear combination of the LDA components and plotted (Figure 5.10). Two-dimensional normal probability distributions at the 80% probability level indicate the highest separation occurring with the junk/debris class. The lowest degree of separation occurs between the cancer cell and normal cell classes. This is most likely due to the cancer cell and normal cell classes having related biological origins, while the junk/debris class does not. Looking at the contribution of each linear component (LD1, LD2) to the class separation, it is evident LD1 accounts primarily for the separation of the junk/debris class from the rest, while LD2 primarily separates out the two biologically derived classes from each other.

Observable Characterization

To evaluate the physical characteristics which lead to the highest degree of classification performance, an analysis of the overall LDA classification accuracy (10-fold cross validation) in response to selected observables was performed (Table 5.6). The baseline accuracy for this analysis was obtained by training an LDA classifier on ran-
domized data with equivalent class probabilities. This yielded a 60% classification accuracy, which basically implies always guessing the largest class, to maximize classification performance. Therefore, the relative improvements in classifier performance were defined relative to a 60% classification accuracy baseline. These descriptor classes, in order from highest to lowest performance improvement, are inner color (23%), outer color (15%), shape (12%), grey (9%) and local area (5%).

Colorimetric information (RGB and YUV color spaces) from both the nuclear area (inner color) of the object and the surrounding area (outer color) provides the highest classification performance, and therefore variable importance. This is reflected visually with cell-like objects, such as cancer cells and normal cells generally having a dark nuclear area with a lighter cytoplasmic surrounding, while the junk/debris is more variable and often is within much darker stained surroundings. Furthermore, in normal cells, a tight clustered and extremely dark (almost black) nuclear appearance is observed, while a much larger, diffuse and lighter stained purple nucleus is present for cancer cells.

Shape information is additionally important primarily in the separation of the junk/debris class from the biologically derived classes as shown in Figure 5.11b. The shape characteristics of cell-like objects on imprint cytology specimens tend to have a round nuclear configuration, while it is expected that a junk/debris object would not. Thus a high degree of separation is observed when looking at shape alone. Additionally, it is noted that a degree of separation between the cancer class and the normal cell class occurs with this discrimination. This can be explained due to cancer cells having larger nuclei than normal cell types.

To understand the important observables which best separate each of the classes of objects on an imprint cytology specimen, a recursive feature elimination approach was used to determine the variables that impart the highest classification accuracy. To gain insight into each of the best observables that separate each class, a single versus all binary classification approach was used (Table 5.7).

Looking at the top 5 observables for cancer discrimination, there is a high importance in looking at the integrated density (Area × Mean Color Value) along with the area of these object types. Thus a high degree of information with regards to classification
performance is gained from knowing the cell area along with the degree of colored area. In the case of normal cells, emphasis is shifted primarily to the red color in both the nucleus and cytoplasm. Finally, for discrimination of junk/debris the red color is also particularly important along with the luminance density. The similar importance of the red color metrics on both normal cells and junk/debris implies that a separation between the red intensity populations is present. For a normal cell such as a lymphocyte, a low intensity red nuclear characteristic is usually present, while the more diffuse nature of the a junk/debris object would have a brighter red profile.

5.5.2 Testing Set

Validation of system properties was performed on retrospective set of positive margins as determined by permanent section pathology or previous identification by pathologist as a positive section. This set was independent of the original training data and served as an initial validation of performance. Permanent section analysis resulted in 6 positive margins and 2 negative margins as listed in Table 5.8. The 2 negative margin samples were determined as having positive status by manual imprint cytology evaluation by pathologist.

Based on the 2 imprint cytology specimens that were evaluated manually by pathologist, there was 100% agreement between an automated approach and manual interpretation. Overall analysis of the 8 case validation set, was able to determine 6 positive imprint cytology specimens corresponding to 4 cases. Therefore 50% of positive cases were identified using the automated imprint cytology analysis approach, which may translate to a reduction in the number of second surgeries required.

It is noted that this performance analysis approach may under-report the true capability of the analysis system to determine a positive imprint cytology specimen because it may not be the case that the imprint cytology slide accurately represents the positive margin surface. Factors such as incorrect removal of the cauterized surface, along with the inherent random sampling of IC could create cases where a positive margin existed but the imprint cytology specimen was unable to identify cancer cells. A more precise validation of imprint cytology performance, would require the manual assessment of the validation set for comparative purposes. Furthermore, the identification
Figure 5.12: Locations of identified positive clusters on validation sample 18.
of positive margins across an entire slide is represented in a positive/negative fashion. A single slide may contain multiple clusters of cancer cells that may be missed during a casual manual analysis, which may prove useful for model verification (Figure 5.12).

Although, this analysis is based on permanent section pathology, it is noted that in samples 18 and 24 of our validation set, margin status was not correctly identified by permanent section analysis. The presence of cancer cells at the margin interface as determined by imprint cytology can only occur in the case of a positive margin status and consequently permanent section analysis may under-report true positive margins. This is most likely due to the low resolution tissue sampling inherent in the section process. Therefore a full assessment of imprint cytology performance would require the analysis of both positive and negative margin specimens.

5.6 Conclusions

The evolution of high speed slide scanning technology along with the increased ubiquity of computing resources enables a new class of automated analysis tools in the clinic. A system for automatically determining margin status is presented which is conservatively estimated to detect approximately 50% of positive imprint cytology specimens from breast surgical margins.

The wide spread availability of this technology can drastically reduce the number of second surgeries required thereby resulting in increased prognostic outcomes, improved quality of life, better cosmesis and the lowering of health care costs. It is expected that further improvements in imprint cytology slides can improve the sampling capabilities of the method and thereby improve the number of positive margins that can be detected intraoperatively.

5.7 Acknowledgments

The contents of Chapter 5 are part of a manuscript in preparation, Manuel E. Ruidíaz, Andrew C. Kummel; Decision Support System for Tumor Margin Determination during Breast Conservation Therapy. The dissertation author was the primary
5.8 References


[38] Maurice Norton Miller and Herbert Upham Williams. *Students' histology; a course of normal histology for students and practitioner of medicine*. W. Wood & company, 1898.


Chapter 6

Development of a Sensitive Bead-Based Assay for Enhanced Monoclonal Antibody Detection

6.1 Abstract

Monoclonal antibodies are increasingly used in the treatment of cancer due to their enhanced targeting and immune system stimulation properties. Dosage guidelines typically do not account for personal cancer load or metabolism, thereby possibly affecting treatment outcome or causing unwanted side effects. The requirement for an assay that can quickly and precisely measure the concentration of the monoclonal antibody in a serum sample of a patient during therapy is unmet. A bead-based assay with peptide antigen mimetics has been developed to rapidly determine the concentration of antibody drug present in serum specimens with high sensitivity. Alemtuzumab (anti-CD52) and rituximab (anti-CD20) antigen mimetic peptides, as discovered by phage display, were synthesized on 10 µm TentaGel resin beads using conventional solid phase peptide synthesis techniques. The beads were modified to allow for multiplexing and microfluidic handling via fluorescent labeling and magnetic functionalization. The antigen-displaying fluoromagnetic particles were incubated with spiked serum samples which allowed free antibody to be captured. Primary antibody detection was performed on
alemtuzumab while rituximab detection was used to compensate for non-specific serum binding to the beads. After washing, the beads were incubated with a fluorescently tagged secondary label for detection by flow cytometry. (Results) A fast, low cost, specific assay has been developed with several key techniques which allows detection at low concentration (0.1 µg/ml) of spiked samples. Primary to achieving this detection limit was the implementation of a compensation scheme where two antigen mimetic peptides behave linearly ($R^2 = 0.996$) which enables the calculation of the zero response of the antigen mimetic peptide of interest (alemtuzumab antigen mimetic) while measuring the zero response of the compensatory antigen mimetic peptide (rituximab antigen mimetic) during primary assay measurement. This reduces fluorescence response variation due to variations present due to sample preparation, storage and different patients because of the equivalent interactions these effects have on the compensatory beads. The developed assay is therefore robust against serum variation and enables a lower limit of detection.

### 6.2 Introduction

Monoclonal antibodies are a growing class of therapeutics used in the treatment of various cancers and auto-immune disorders [1–6]. Traditional monoclonal dosage regiments typically do not take into account the specific tumor load or physiology of the patient [7–9] therefore it is hypothesized that an improper dosage may occur. Patients with a high intrinsic tumor load may have a low dosage relative to the amount of cancer that is present in that patient. Conversely, a patient with a lower tumor load, may be overdosed. Typical techniques used for detection of drugs from human serum samples are almost always difficult to perform and often have a high cost that makes it prohibitive to perform on every patient [10–13]. Therefore, the need for a quick, low cost, assay to reliably determine the levels of monoclonal antibody drugs is unmet [11, 13]. Herein, we describe a system to quickly determine the level of monoclonal antibody levels in human serum samples. Several techniques have been integrated into the assay to account for variable non-specific binding of different patient serums. Additionally the assay platform has been engineered for compatibility with automated sample handling systems.
6.3 Experiment

6.3.1 Bead Preparation

Alemtuzumab and rituximab antigen mimetic peptides sequences were generated by phage-displayed peptide library screening and synthesized on 10µm Tentagel beads as described previously [14]. Automated handling and multiplexing capability were enabled on the bead platform by the trapping of nanoparticles within the polystyrene matrix of the bead. For automated handling capability, magnetic nanoparticles can be incorporated to enable handling of beads by magnetic field thereby eliminating the requirement for centrifugation during the assay and enabling control by automated means. For multiplexing of multiple bead types, fluorescent nanoparticles with different emission wavelengths can be similarly incorporated within the beads allowing them to be identified by dependent on which emission wavelength is detected.

A two phase process is used to incorporate the dye and/or the magnetic material which involves the mixture of two separate solutions, a swelling solution and an incorporation solution. **Swelling Solution:** 5 mg of peptide-beads were dissolved in 500µl N-N-dimethylformamide (DMF) in a microcentrifuge tube followed by sonication in a Branson 2510 bath sonicator (Branson Ultrasonic Corp., Danbury, CT) at room temperature until beads were visibly dissolved and dispersed. **Incorporation Solution:** A 500µl aliquot of DMF was placed into a microcentrifuge tube. For magnetization, 200µl of 6.5 ± 3.0nm iron oxide (FeO) magnetic nanoparticles (MagNP) dissolved in heptane (Fluka Chemika, Buchs, Switzerland) was added creating a DMF/heptane-MagNP dual phase system. A small hand-held neodymium magnet was positioned below the microcentrifuge tube causing the MagNP in the heptane phase to migrate to the DMF phase. The heptane phase was remove and discarded. The resultant DMF-MagNP is placed into a bath sonicator until MagNP were dispersed into the DMF.

For fluorescence multiplexing, 3µl of 40 nm red (alemtuzumab-binding beads) or dark red (rituximab-binding beads) fluorescent FluoSpheres carboxylate-modified microspheres (Invitrogen Corp., Carlsbad, CA) was added to the DMF solution. This was followed by sonication at room temperature until the beads were dispersed. The 500µl incorporation solution was transferred to the 500µl swelling solution tube. The
combined solution was sonicated for 10 minutes to assure dispersion and incorporation of the nanoparticles within the polystyrene bead matrix. The beads in solution were centrifuged for 2 minutes at 14,000rpm (Microfuge 18 Centrifuge, Beckman Coulter, Mount Holly, NJ). The resultant supernatant was discarded and the pellet was resuspended in phosphate buffered saline (PBS) (Invitrogen Corp., Carlsbad, CA). Sonication and centrifugation followed by resuspension with PBS was repeated two additional times and finally resuspended with PBS with 0.05% sodium azide (Teknova, Hollister, CA) and stored at 4°C. Bead characterization is detailed in Figure 6.1.

### 6.3.2 Monoclonal Antibody Measurement Protocol

BD Falcon 96-well polystyrene V-bottom plate wells (Becton, Dickinson and Company, Franklin Lakes, NJ) were coated with a 3% solution of IgG/protease free Bovine Serum Albumin (BSA) (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA) in PBS with 0.05% sodium azide to reduce non-specific binding to the well surfaces. The remaining BSA in the wells was aspirated and discarded. To each well, a primary antibody incubation solution was added which consisted of 100µl of 3% BSA solution, 10µl of PBS with a specific concentration of monoclonal alemtuzumab antibody as obtained from the UCSD cancer center pharmacy (Genzyme, Cambridge, MA), 10µl of human serum, purchased from Innovative Research (Novi, MI) and Valley Biomedical
Inc (Winchester, VA), with an “unknown” concentration of monoclonal antibody and 3\(\mu\)l of a 1:1 mixture of 5mg/ml alemtuzumab-binding beads and 5mg/ml rituximab-binding beads in PBS. Samples were incubated for 1hr on a rocking platform shaker at room temperature. The samples were washed 3 times by magnetic capturing of the beads on the well bottom and replacing the supernatant with 100\(\mu\)l of the 3% BSA-azide solution. The samples were incubated with 0.5\(\mu\)l of 2mg/ml Alexa Fluor 488 conjugated Protein G (Invitrogen, Carlsbad, CA) in PBS with 99.5\(\mu\)l of 3% BSA-azide solution for 1 hr on shaker at room temperature. The samples were washed 3 times with 3% BSA-azide solution and finally resuspended in 100\(\mu\)l of 3% BSA-azide. Beads were then measured on a BD FACSCalibur (Becton, Dickinson and Company, Franklin Lakes, NJ) and the data was analyzed and gated using FlowJo (Treestar, Ashland, OR) software. Limit of detection was performed using the protocol described previously [14].

6.4 Discussion

6.4.1 Compensation for Non-specific Binding

Fluorescence analysis of 10 different serums samples on 2 time points separated by 15 weeks demonstrated an 8 fold variation in the alemtuzumab fluorescence at zero concentration, indicating a time dependent non-specific background response in addition to the serum dependent background response changes (Figure 6.2). Therefore a technique that can independently determine the level of non-specific background response for the alemtuzumab was developed by including an additional bead with a peptide for an antibody (rituximab) known not to be present in the sample of interest. As shown in Figure 6.2, the non-specific binding on the peptides for the two antibodies is highly correlated so it is a reasonable basis for developing a method for non-specific background response determination in a large range of samples. The increase in background fluorescence of the alemtuzumab-binding peptide was determined to be linearly related \((R^2 = 0.996)\) to the increase in the background fluorescence of the rituximab-binding peptide (Figure 6.2). This enables a method of zero determination of alemtuzumab
Figure 6.2: Non-specific background fluorescence values of 10 serum samples showing time dependent and serum dependent response. Changes in alemtuzumab-binding bead fluorescence are correspondingly exhibited in the rituximab-binding bead fluorescence. Black points are at week 0, grey points are at week 15.

samples without having to obtain samples that do not have alemtuzumab.

6.4.2 Monoclonal Antibody Measurement Assay

“Unknown” serum samples were created as a surrogate for clinically relevant serum samples that would be obtained during ongoing monoclonal antibody therapy. To determine the fluorescence response for a given serum with an unknown alemtuzumab concentration, three different spike concentrations of alemtuzumab was added to three separate samples in addition to the unspiked sample. These four samples were performed in 4 replicates. Alemtuzumab-binding bead and rituximab-binding bead fluorescence response was measured by flow cytometry. For each sample, the fluorescence intensity of the rituximab-binding beads was used to subtract the non-specific binding of the corresponding alemtuzumab-binding beads. The resultant compensated curve was used to determine the original “unknown” concentration of alemtuzumab in the sample (Figure 6.3).
Figure 6.3: The above linear relationship is the uncompensated alemtuzumab-binding bead fluorescence response. Using the negative background response compensation rituximab-binding beads, a compensated alemtuzumab-binding bead fluorescence response is calculated below. The “unknown” alemtuzumab concentration was $0.5 \mu g/ml$ and the measured alemtuzumab concentration is $0.83 \pm 0.044 \mu g/ml$ (Standard Deviation).
A determination of the limit of detection was performed on a serum sample with 7 spiked alemtuzumab concentrations 0, 0.1, 0.5, 1, 2, 4, 8 µg/ml on 3 different days in quadruplicate and was determined to be 0.5 µg/ml, as determined by the lower 95th percentile above zero. An ELISA based assay limit of detection based on a 6 samples performed over 4 different days was determined to be 1 µg/ml [14].

Three key advantages of this assay platform are exploited to successfully detect antibody at low concentration: (1) The ability to determine the level of background non-specific binding of the antibody of interest by determining the background level of another peptide known to have zero specific binding activity, (2) the presence of the polyethylene glycol (PEG) subgroup inherent in the Tentagel bead provides the platform with the low background necessary to provide detection at physiologically relevant concentrations, and (3) the porous nature of the Tentagel bead structure allows for multiple binding sites to be available to the antibody thereby allowing a cooperative binding effect which further increases the sensitivity of the assay.

6.5 Conclusions

A promising platform for low concentration monoclonal antibody detection has been presented which is able to compensate for donor serum, experimental, time based non-specific background variation. The versatility of the platform allows for the incorporation of magnetic and fluorescent nanoparticles which impart multiplexing and automated handling capability. As currently implemented, the protocol calls for a 1:10 dilution of the serum to be analyzed. Therefore the beads are physically reporting an order of magnitude reduced concentration of antibody. With further enhancement it is believed that the sensitivity of the assay may be improved at least 5 fold.

6.6 Acknowledgements

Chapter 6 in full, is a reprint of the material as it appears in Development of a Sensitive Bead-Based Assay for Enhanced Monoclonal Antibody Detection in Journal of Materials Research 2011. Manuel E. Ruidiaz, Natalie Mendez, Ana B. Sanchez,
Bradley T. Messmer and Andrew C. Kummel. The dissertation author was the primary investigator and author of this paper.

6.7 References


