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Topical dual-stain difference imaging for rapid intra-operative tumor identification in fresh specimens

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Abstract

Assessing tumor margin status during surgery is critical to ensure complete resection of cancer tissue; however, current approaches are ineffective and often result in repeat surgery. We present a novel optical imaging approach for margin assessment using topical application of two fluorescent stains, one targeted to a tumor biomarker and the other a non-targeted reference, to freshly excised specimens. Computing a normalized difference image from fluorescence images of the targeted and untargeted stains suppresses the confounding effects of non-specific uptake. Applying this approach in excised breast tumor models produced promising tumor-to-normal tissue contrasts that were significantly higher than single-targeted-stain imaging.

The extent to which cancer tissue is completely removed during primary surgery is a critical prognostic indicator of local recurrence and overall patient survival. However, current techniques deployed to identify and remove cancer cells during surgery are terribly inadequate, often relying on a combination of visual inspection, palpation, and co-registered pre-operative imaging. While the shortcomings of the standard-of-care are recognized for resection of all types of solid tumors, incomplete resection during breast conserving surgery has emerged as a particularly prevalent problem [1], with involved or close margins identified post-surgery in 20 - 40% of patients. This diagnosis usually triggers immediate follow-up surgery, resulting in elevated risk of morbidity, undo patient stress, increased cost, and a reduced probability of a positive outcome. Studies have reported re-excision rates as high as 57% [2-3], representing an enormous mental and physical cost for patients and the health care system. Thus, an urgent need exists for a new technique which integrates into the clinical workflow and is capable of rapidly identifying margin status during surgery.

The current approaches to improve tumor resection for breast conserving surgery, such as frozen section analysis (FSA) and touch prep cytology have been shown to reduce rates of involved margins during breast conserving surgery, though have inherent limitations [1, 4-5]. FSA is an undesirably long procedure which uses tissue that cannot be reliably re-analyzed post-operatively with pathological staining, and touch prep cytology is limited to

cells on the surface of the tissue specimen, precluding identification of sub-surface tumor tissue. Other imaging approaches, such as ultrasound and specimen radiography, have demonstrated promise but can have limited sensitivity to certain pathologies found in breast.

Optical techniques for surgical guidance using near-infrared (NIR) light have been the focus of broad efforts in the research community for more than a decade. These approaches can be extremely sensitive, molecularly specific, and facilitate visualization of subsurface tumor tissue. Studies have reported promising results for techniques using both intrinsic [6-7] and extrinsic optical contrast [8-9], with the latter often deployed to enable imaging of fluorescent probes targeted to tumor biomarkers inaccessible with intrinsic techniques (such as up-regulation of receptors). In principle, *in vivo* approaches which mark residual tumor tissue within the patient's cavity are most consistent with the surgical objective; however, introducing diagnostic imaging molecules with proven safety profiles is an enormous regulatory challenge. Furthermore, despite advances in the development of molecular imaging contrast agents, vascular dynamics and non-specific uptake pose additional challenges for the diagnostic capacity of *in vivo* fluorescence guided surgery. While these efforts may eventually produce an effective clinical standard, a rapid, wide-field molecular imaging technique which circumvents the regulatory requirements for systemically administered contrast agents by analyzing excised specimens could have a significant impact on breast cancer resection in the near-term.

Topical application of a fluorescently labeled targeted agent to excised specimens, followed by removal of unbound agent by rinsing, is an attractive alternative to approaches which require administering diagnostic contrast agents to humans.[10] Although this approach is conceptually simple, non-specific uptake in both tumor and normal tissue is a challenging problem which limits diagnostic performance. Adipose tissue is particularly adept at absorbing and retaining stains, resulting in poor tumor-to-adipose contrast. Thus, suppressing the confounding effects of non-specific uptake is a pivotal criterion for developing effective topical staining approaches for margin status assessment.

In this study, we report on a new imaging approach for identifying margin status in freshly excised tissue specimens which mitigates the effects of non-specific uptake and eliminates patient safety concerns of *in vivo* contrast agents. This technique involves incubating fresh tissue specimens in a solution of two fluorescently labeled stains, one stain targeted to a molecular tumor biomarker, and the other a non-targeted counterpart stain, then rinsing the tissue and imaging fluorescence from both stains simultaneously. Provided the tissue transport kinetics of the stains are nearly identical, except for binding, computing the normalized difference between images of the two stains produces an image that emphasizes the difference in the amount of each stain that remains in the tissue after rinsing. This difference is directly related to specific binding of the targeted stain.

We investigated the feasibility of dual-stain difference imaging using breast cancer tumor xenografts from five athymic nude mice. All procedures were reviewed and approved by the Institutional Animal Care and Use Committee at Dartmouth College. Mice were pre-treated with 0.72mg 17-BEstradiol pellets (Innovative Research of America, Sarasota, FL) implanted subcutaneously four days prior to inoculation with one million MCF7 cells

transfected with HER2/neu[11] in two sites. Tumors grew for between 28 and 32 days before surgical imaging.

The dual-stain cocktail was composed of two NIR fluorescent stains, one targeted to the receptor of interest, in this case HER2, a transmembrane protein that is part of the epidermal growth factor receptor family, and a non-targeted counterpart. HER2 is overexpressed in 25-30% of breast cancer tumors[12] and has become a primary therapeutic target in the subpopulation of patients with elevated expression. Thus, numerous molecules with high affinity for HER2 are available. This study used the anti-HER2 antibody Trastuzumab (Genentech, molecular weight (MW) = 145kD) conjugated to Licor IRDye800-CW (LI-COR Biosciences, Lincoln, NE, MW=1.2kD) for the targeted stain and Immunoglobulin (IgG, Jackson ImmunoResearch, MW=160kD) conjugated to Licor IRDye680-RD (MW=1kD) for the untargeted counterpart. These stains were chosen to have nearly equivalent molecular weight so that their kinetics in the tissue were as similar as possible. For tissue staining, both stains were mixed together in a solution of PBS, 0.1% Tween 20, and 1% bovine serum albumin (BSA) such that each was at a concentration of 100 nM (by protein).

Once tumor diameters reached approximately 7 mm, animals were anesthetized and sacrificed by cervical dislocation, followed immediately by excision of the tumor and normal mammary adipose tissues. Since subcutaneous tumors are often encapsulated in a membrane, the tumor was divided into three specimens before being processed for imaging. The specimen processing and imaging protocol is outlined in Fig. 1. First, the tumor and adipose specimens were incubated in a blocking solution of PBS with 2% BSA, as is commonly done for immunohistochemistry, for 10 minutes. This was followed by a 10 minute soak in 2 ml of the dual-stain solution. Specimens were then transferred to a 50 ml solution of PBS and 0.1% Tween20 for a 5 minute rinse. Immediately following the rinse, the tumor and adipose specimens were transferred to a glass slide and scanned in a Li-Cor Pearl fluorescence imaging system designed to image these two fluorescent dyes. This dual-channel imaging system acquired a 22-bit fluorescence image for each channel (i.e. for each fluorescent stain). In addition to the tissue specimens, a sample of the stock dual-stain incubation solution in a covered optical well plate was placed in the imaging field for normalization. Finally, tumor specimens were processed to confirm elevated HER2 expression using immunohistochemical staining. All tumors had a uniform staining profile for HER2 showing elevated levels of the receptor.

Each dual-channel tissue scan yielded two images, one of the fluorescence emission of the targeted stain, and the other of the non-targeted stain. Image processing began by subtracting the median of a region selected from the background (non-tissue) in each image. The images from each channel were then normalized to an average intensity value from the stain calibration sample included in the imaging field. This yielded normalized images for the targeted and untargeted stains. Before computing the normalized difference image, a mask determined by a threshold of the untargeted image was applied to both targeted and untargeted images such that only pixels with measurable fluorescence (at least 10-fold higher than background noise) were used in the calculation. These two images were then used to calculate the dual-stain difference image as:

$$I_{Difference} = (I_{targeted} - I_{untargeted}) / I_{untargeted}$$

Finally, a negative constraint was applied to $I_{Difference}$, which implicitly interprets regions with a higher concentration of untargeted (vs. targeted) stain as having no receptor binding. Tumor-to-normal tissue contrasts were computed as the ratio between the mean of the intensity in the tumor to the mean in the adipose tissue.

Representative images for three of the ten tumors and normal adipose tissue are presented in Fig. 2. For each sample, images of the untargeted, targeted and difference staining techniques are shown. Qualitative inspection suggests that the dual-stain difference approach provides significant improvements in tumor-to-normal differentiation over single agent targeted staining, even after the stained tissues have been rinsed in a PBS/Tween20 solution. This is evident in the image series' in Fig. 2 (a) and (b). These images show modest enhancement in uptake between tumor and normal tissues with the targeted stain, while the prevalence of significant normal tissue uptake minimizes the overall contrast between tissue types. The differencing method, however, effectively suppressed the non-specific uptake of targeted stain in the normal adipose tissue, elevating tumor-to-adipose contrast significantly.

Figure 2 (c) presents an image series for the sample which produced the least tumor-to-normal tissue contrast in the difference image (and the only specimen with a contrast below 1). Examining the untargeted and targeted stain images indicates that this case displayed particularly high uptake in the adipose tissue as compared to the tumor. The differencing technique mitigated this to a large extent, but not enough to produce positive tumor-to-adipose contrast. Despite this result, differencing produced enhanced tumor-to-normal contrast greater than 1 for the nine other tissue specimens.

Difference images for the seven specimens not included in Fig. 2 are provided in Fig. 3 (a) and confirm a consistent trend of normal tissue suppression. Contrast values for each stain and the difference images are reported in Fig. 3 (b), results which confirm the significant increase in tumor-to-normal contrast using the dual-stain technique. The average increase in tumor-to-normal contrast for all samples was 4.9 as compared to single-agent targeted staining. In many cases, the differencing approach elevated contrasts from near or below 1 for the single stain images to 2 - 4. Contrasts greater than 3 were observed for seven out of ten samples using the differencing approach, whereas only one sample achieved a contrast greater than 2 in the single stain images.

We extended the analysis to examine the diagnostic potential of the technique using ROC curves. Even with the relatively small sample size of this preliminary data set, ROC analysis can reveal information more relevant to the objective of the imaging technique. Area under the curve (AUC) results computed from empirical ROC curves (based on the mean values in each tissue region, all specimens considered independent) are included in Fig. 3, and indicate significant improvements in diagnostic potential for the dual-stain approach. Differences in AUC values between dual-stain (AUC = 0.96) and untargeted images (AUC = 0.61), and dual-stain and targeted images (AUC = 0.66) were statistically significant

(critical ratio, $z = 2.71$ and 2.54 , respectively), as determined using the method reported by Hanley and McNeil[13].

These results suggest that referencing targeted topical stain uptake with an untargeted agent may overcome the severe non-specific uptake barrier for rapid margin assessment in fresh tissue. This dual-stain approach emphasizes the differences in the rinsing kinetics of the two stains, and thus reports specific target binding. This is consistent with recent reports demonstrating that dual-tracer imaging *in vivo* has the capacity to account for tracer kinetics to provide more specific molecular information [14-15]. These promising new approaches leverage the unique multi-reporter detection capabilities enabled by optical imaging to suppress the effects of reporter transport mechanisms such as blood flow, vessel perfusion, or in the case of topical staining, diffusion. Other approaches aimed at eliminating transport effects, such as the use of activatable probes, also effectively reduce non-specific signal; however, these are limited in their capacity to exploit receptor-based drug targets commonly expressed in cancer.

In this proof-of-principle study, tumor and normal adipose tissues from the same mouse were analyzed as separate specimens and tissue contrast calculated based on average values for each specimen. However, inspection of the images shown in Figs. 2 and 3 reveals that the heterogeneity within both tumor and normal tissue would challenge the identification of tumor tissue in a mixed specimen. Thus, while the current results are encouraging, tumor-to-adipose tissue contrast will likely need to be increased to provide a truly diagnostic platform. Improvements may well be identified by optimizing the solvents used in staining and rinsing solutions, stain concentration, incubation times, agitation, affinity of the stain to the receptor, and the molecular weights and charge structures of the stains.

The most critical assumption applied in dual-stain difference imaging is that both stains follow identical transport kinetics in the tissue specimen, with the exception of specific binding. The accuracy of the technique in identifying molecular targets, such as HER2, depends on the degree to which the stains follow similar transport kinetics. While the similarity between the molecular structure of IgG and Trastuzumab stains are quite similar, their kinetic behavior during topical application and washing of fresh tissue has not been compared explicitly. Deviations from this criterion will manifest as degraded diagnostic performance, and are likely the primary source of tissue misclassification in this study. The extent to which new stain pairs which meet this criterion can be identified, developed and validated could determine whether this technique will be successful in improving clinical outcomes.

A complete optimization program must also consider practical requirements to minimize the disruption of the clinical workflow. Maintaining the tissue processing procedures within surgical time constraints is a particular concern for this application. Although the 25 minute processing time (plus 60 s. for imaging/image processing) used for this pilot study is less than the typical times required to prepare pathology slides, it is likely unreasonably long for tissue assessment during surgery. We expect that this will be reduced by adjusting the stain and rinse solutions and eventually automating the tissue processing procedure. The development of dual-channel fluorescence imaging systems optimized for this application

and capable of video rate imaging will be straightforward. Finally, NIR stains that penetrate tissue readily may enable visualization of sub-surface tumors to identify close margins.

Although this study focused on examining tumor-to-adipose tissue contrast, fibro-glandular tissue is another major normal tissue type encountered during breast conserving surgery. Due to the small scale of mouse models, tumor-to-fibro-glandular contrast was not examined here. However, our observations indicate that adipose tissue is particularly adept at absorbing these stains, resulting in significant non-specific signals. While imaging the skin is not relevant to clinical applications in breast surgery, skin specimens for each mouse were included in the imaging field in this study (not shown) and showed very little non-specific uptake resulting in exceptionally high tumor-to-skin contrast. Cataloging the response of dual-stain difference imaging in a variety of tissue types is an important objective for ongoing studies.

The ability of receptor status to diagnose tumor tissue remains an unanswered question with major implications for the approach described here. The heterogeneity in cellular phenotypes within tumors poses a significant challenge for receptor-based molecular imaging in this application. Using multi-target stains to label several molecular abnormalities simultaneously could improve sensitivity and specificity metrics and is a logical next step in further development of this approach.

Molecular imaging provides little useful information unless non-specific uptake is mitigated. The topical dual-stain approach presented here for the first time largely accounts for the diffusion of stains into excised tissue to reveal tumor tissue based on molecular markers. While this study has focused on breast tumors, the approach is readily transferrable to any cancer surgery requiring margin status assessment. Provided optimization efforts establish the technique as diagnostically useful, this approach could facilitate rapid, wide-field assessment of tumor margin status in the operating room and thus has the potential to reduce the rate of repeat surgery and increase overall survival.

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Fig. 1.
Procedure for processing tissue specimens for dual stain difference imaging.

Fig. 2.

Fluorescence image overlays for three tumor/adipose samples ([a]- [c]). The columns correspond to images of untargeted and targeted stains, and the dual-stain difference images. Significant enhancement in tumor-to-adipose contrast is observed in the difference images for (a) and (b). The example in (c) provided the least contrast of all 10 specimens, but still shows suppression of the significant non-specific uptake in the adipose tissue. Note: Overlay threshold is 5% of maximum value.

Fig. 3.

(a) Dual-stain difference images for all specimens not shown in Fig. 2. (b) Box plot of tumor-to-adipose contrast for each stain alone and the difference technique (N=10). AUC values using ROC analysis for each imaging technique are reported under the x-axis.