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Effect of staining temperature on topical dual stain imaging of tissue specimens for tumor identification

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Abstract

In the pursuit of reducing re-excision rates in breast conserving surgery, a dual probe specimen staining technique has emerged as a promising approach to identify positive margins during surgery. This approach generally involves staining the tissue with a fluorescent dye targeted to a biomarker of interest, such as a cell surface receptor, and an untargeted counterpart, imaging both dyes and using the two images together to compensate for instrumentation inhomogeneities and non-specific uptake. A growing body of literature suggests that this approach can effectively discriminate tumor and normal tissue in gross fresh specimens in reasonable timeframes. However, the robustness of the staining protocol is still under investigation as all parameters have not been fully evaluated. In this paper, we examine the effect of staining temperature on diagnostic performance. Tumor (overexpressing EGFR) and normal fresh specimens were stained at room temperature or 37 °C and diagnostic performance compared using area under the curve (AUC) from receiver operator characteristic (ROC) analysis. The results suggest that the use of Licor IRDye800CW-labeled anti-EGFR antibody and Licor IRDye680RD-labeled control antibody as the probe pair is not significantly affected by staining temperature, in contrast to our experience with quantum-dot labeled antibodies. The robustness of the technique using these stains is reassuring and simplifies the staining protocol.

Keywords

fluorescence guided surgery; margins; breast conserving surgery; topical

1. INTRODUCTION

The ability to rapidly identify tumor tissue in the margins of resected specimens during surgery is an important aspect of efforts to reduce secondary surgeries caused by incomplete resection of tumor. The challenge of incomplete resection spans many tumor indications, but is particularly prominent in breast conserving surgery due to the reported high rates of re-excision procedures.^{1,2} To address this, we and others have advanced a new tissue staining approach which deploys two spectrally-distinct fluorescent (or Raman-particle-based) stains

used together: A stain targeted to a biomarker of interest in the tumor and an untargeted isotype.^{3–10} After tissue staining, images of each stain are acquired and used either in a difference or ratiometric calculation to produce corrected images which reveal the distribution of biomarkers on the tissue surface. Several papers published over the past 6 years have shown steady progress in optimizing the staining protocol and demonstrated high diagnostic performance (>0.95 AUC) of this technique in distinguishing tumor and normal tissue.⁸ These efforts have also reduced the staining protocol to less than 5 minutes. Current efforts seek to expand the number of targets accessible and further understand the relationship between receptor concentration and reported values.

Prior studies in our lab have all been done using stains at room temperature; however, in a recent experiment examining quantum dots as a label for dual-stain difference imaging, we observed that staining at 37 °C vs. room temperature had a substantial impact on the ability of the technique to discriminate tumor and normal tissue. Herein, we investigate whether staining at this higher temperature could also improve performance of the technique when using the conventional fluorophore-labeled antibodies we have typically used in these studies. Thus, we stained tumor and normal tissues at room temperature and 37 °C and compared these conditions using ROC analysis.

2. MATERIALS AND METHODS

All animal procedures were approved and conducted in accordance with policies by Dartmouth's IACUC. Athymic nude mice were implanted with MDA-MB231 cells in the mammary fat pad. This is a triple negative cell line that has high expression of epidermal growth factor receptor (EGFR). Tumors grew over the course of several weeks and were monitored by pinch measurements. Once tumors reached approximately 5 mm or larger, animals were put on study. Animals were euthanized and the tumor and normal mammary adipose tissue immediately harvested for processing with the staining protocol. Tumors were cut into sections before staining.

The staining protocol has been described elsewhere⁸, though for the study herein, we tested staining temperatures of room temperature (22 °C) and 37 °C. Briefly, harvested tissues were soaked in a 2% BSA blocking solution for 10 minutes. Next, tissues were transferred to the staining solution and stained for 1 minute. This solution consisted of Licor800CW conjugated to cetuximab (200 nM) as the targeted probe and Licor680RD conjugated to donkey anti-rabbit antibody (200 nM) as the untargeted isotype. After the one-minute stain, tissues were washed in PBS, placed on glass slides and imaged with a Licor Odyssey dual channel flatbed scanner. After scanning, tissues were prepared for H&E and IHC of EGFR. Images were normalizing to a calibration drop of the dual stain solution and then the dual stain parameter computed per pixel by: (targeted stain – untargeted stain)/untargeted, as described elsewhere. For each cohort, pixel values in the tissues were used to compute AUC from ROC analysis. These values were used to compare the two temperature groups.

3. RESULTS AND DISCUSSION

Figure 1 shows the EGFR expression level in four tumor cell lines as determined from flow cytometry. The tumor line under investigations herein, MDA-MB231, has a high level of expression of this receptor.

Figure 2(a) provides the computed normalized difference images for five tissue pairs in the room temperature cohort. Tumor tissue is in the left column while normal tissue is in the right. Qualitatively, these images show robust ability to discriminate between tumor and adipose tissue. The corresponding AUC values for the samples shown in Fig. 2(a) are provided in Fig. 2(b). These figures show that the targeted agent alone has no capacity to discriminate tissue. Compensation using the untargeted probe has a dramatic effect on the AUC values, which reach 0.96.

Figure 3 shows the H&E and EGFR-IHC for the images shown in Fig. 1. Tumor cells show high overexpression of EGFR which correlates to the dual stain images in Fig. 2.

Figure 4 presents the mean AUC values for the tissues stained at room temperature and 37 °C. Although both conditions show relatively high AUC, the room temperature shows slightly improved performance, though this difference was not statistically significant from these samples.

4. CONCLUSION

The accumulating evidence for the use of dual stain techniques to assess margin status is promising. When performed with conventional fluorophore labels such as the Licor dyes, this technique generally shows high diagnostic performance in xenograft models (AUC > 0.95) with a reasonable tissue processing time (5 minutes), yet the effect of staining temperature had not been previously examined. In this study, we observed that the Licor-labeled antibodies showed high diagnostic performance regardless of temperature, in contrast to our experience with quantum dot labels, which were highly sensitive to temperature. This robustness is advantageous for translational efforts and provides further validation of this technique.

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Fig. 1.
Flow cytometry for EGFR in four breast tumor lines confirming the high expression in the MDA-MB-231 line used herein.

Fig. 2.
Normalized dual stain difference images of five samples (a) and corresponding average AUC values (b) for the room temperature specimens.

Fig. 3. Pathology (H&E and EGFR-immunohistochemistry) of the tissue samples shown in Fig. 2.

Fig. 4.
Average AUC values for room temperature and 37 °C staining.