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RESEARCH ARTICLE

Effect of Formalin Fixation for Near-Infrared Fluorescence Imaging with an Antibody-Dye Conjugate in Head and Neck Cancer Patients

Shrey Kapoor,[1] Guolan Lu,[1] Nynke S. van den Berg,[1] Giri Krishnan,[1] Jacqueline Pei,[1] Quan Zhou,[1] Brock A. Martin,[2] Fred M. Baik,[1] Eben L. Rosenthal,[1] Naoki Nishio [1,3]

[1]Department of Otolaryngology-Head and Neck Surgery, Stanford University School of Medicine, Stanford, CA, USA

[2]Department of Clinical Pathology, Stanford University School of Medicine, Stanford, CA, USA

[3]Department of Otorhinolaryngology, Nagoya University Graduate School of Medicine, 65, Tsurumai-cho, Showa-ku, Nagoya, Aichi, 466-8550, Japan

Abstract

Purpose: This study evaluated the effect of formalin fixation for near-infrared (NIR) fluorescence imaging of an antibody-dye complex (panitumumab-IRDye800CW) that was intravenously administered to patients with head and neck squamous cell carcinoma (HNSCC) scheduled to undergo surgery of curative intent.

Procedures: HNSCC patients were infused with 25 or 50 mg of panitumumab-IRDye800CW followed by surgery 1–5 days later. Following resection, primary tumor specimens were imaged in a closed-field fluorescence imaging device, before and after formalin fixation. The fluorescence images of formalin-fixed specimens were compared with images prior to formalin fixation. Regions of interest were drawn on the primary tumor and on the adjacent normal tissue on the fluorescence images. The mean fluorescence intensity (MFI) and tumor-to-background ratios (TBRs) of the fresh and formalin-fixed tissues were compared.

Results: Of the 30 enrolled patients, 20 tissue specimens were eligible for this study. Formalin fixation led to an average of 10 % shrinkage in tumor specimen size ($p < 0.0001$). Tumor MFI in formalin-fixed specimens was on average 10.9 % lower than that in the fresh specimens ($p = 0.0002$). However, no statistical difference was found between the TBRs of the fresh specimens and those of the formalin-fixed specimens ($p = 0.85$).

Conclusions: Despite the 11 % decrease in MFI between fresh and formalin-fixed tissue specimens, the relative difference between tumor and normal tissue as measured in TBR remained unchanged. This data suggests that evaluation of formalin-fixed tissue for assessing the accuracy of fluorescence-guided surgery approaches could provide a valid, yet more flexible, alternative to fresh tissue analysis.

Trial Registration: NCT02415881

Key words: Formalin fixation, Near-infrared fluorescence imaging, Head and neck cancer, Antibody-dye conjugate, Anti-EGFR antibody

Shrey Kapoor and Guolan Lu contributed equally to this work.
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Correspondence to: Naoki Nishio; *e-mail:* naokin@med.nagoya-u.ac.jp

Introduction

Surgical resection of the primary tumor plays an essential role for cancer treatment, and incomplete surgical resections,

including positive margins, are directly correlated with poor prognosis [1–3]. Fluorescence-guided surgery has emerged as a novel intraoperative technique that provides surgeons real-time guidance during surgery [4, 5]. Recent clinical introduction and evaluation of fluorescently labeled imaging agents, such as the anti-epidermal growth factor receptor (EGFR) antibodies or the anti-vascular endothelial growth factor (VEGF) antibody, have enabled us to differentiate the tumor regions from the surrounding normal tissues during surgery and demonstrated feasibility of obtaining a tumor-free margin resection in oncologic surgery [4, 6–9]. Conducted immediately after tumor resection, *ex vivo* assessments of resected fresh tumor specimens allowed us to predict the closest regions of tumor tissues for both deep and peripheral margins [10, 11]. Moreover, the use of these antibody-dye conjugates allows us to evaluate the delivery and distribution of systemically injected antibodies [12]. Recently, we have reported that antibody-dye conjugate can be used as a surrogate to measure the interpatient and intratumoral heterogeneity of antibody distribution in HNSCC at an unprecedented resolution, resulting in a deeper understanding of the antibody distribution within patient tumors [13].

Recent studies show the structured workflow of the surgical process of the primary tumor and lymph node specimens including an intraoperative setting and pathological process such as formalin fixation [9, 14]. However, there is no clinical data to assess the impact of formalin fixation on the fluorescence signal in resected human tissues during the pathological process. In order to accurately assess the surgical results using a fluorescently labeled tumor-specific imaging agent, it is crucial to understand the changes in the fluorescence intensity of the tumor specimens during the formalin fixation and how this process would affect the differentiation between tumor tissues and adjacent normal tissues. Moreover, to apply the fluorescent-based molecular imaging into common use in the clinical setting, the pathological process for the resected specimens should be optimized and standardized to ensure consistent results. Here, we evaluated the effect of formalin fixation for near-infrared (NIR) fluorescence imaging of an antibody-dye complex (panitumumab-IRDye800CW) that was intravenously administered to patients with head and neck squamous cell carcinoma (HNSCC) scheduled to undergo surgery of curative intent.

Materials and Methods

Study Design

The protocol for this clinical study was approved by the Stanford University Institutional Review Board (IRB 35064) and the FDA and is listed on clinicaltrials.gov as NCT02415881. Informed consent was obtained from all individual participants included in the study. The research was conducted in full accordance with FDA's ICH-GCP guidelines, the Helsinki Declaration of 1975 and its amendments, and the laws and regulations of the USA.

Between August 2017 and February 2019, 30 patients were enrolled in the study and infused with 25 or 50 mg of panitumumab-IRDye800CW 1–5 days prior to surgery. Following resection, primary tumors were imaged with a near-infrared fluorescence imager on the day of the surgery and following formalin fixation.

Ex Vivo Fluorescence Image Analysis of the Primary Tumor

The workflow for the analysis of fluorescence imaging of the whole specimen is illustrated in Fig. 1. In brief, following excision and surgeon's gross examination of the tissue specimen, the specimen was imaged with the closed-field fluorescence imaging device (Pearl Triology; or IGP-ELVIS; LI-COR Biosciences Inc., Lincoln, NE, USA), which enables the measurement of the fluorescence intensity and tissue area in the specimens and allows for imaging in a controlled environment, including elimination of ambient light [10, 15]. Thereafter, specimens were transferred to pathology for formalin fixation overnight or over the weekend (Friday to Monday) per standard of care. Specimens were fixed in 10 % neutral buffered formalin for 1–4 days at ambient/room temperature in sealed, opaque plastic containers with limited light exposure. Specimens were fixed in a volume of formalin equal to or greater than the volume of tissue in the sample. Directly before grossing of the primary tumor specimen, the formalin-fixed tissue specimen was re-imaged in the closed-field fluorescence imaging device.

The fluorescence images of formalin-fixed specimen were compared with images prior to formalin fixation. Regions of interest (ROIs) were drawn on the primary tumor and in the adjacent normal tissue using a fluorescence imaging device–integrated software (Image Studio; LI-COR Biosciences, Inc.) as described previously by us [16]. Briefly, mean fluorescent intensities (MFI) were determined as follows:
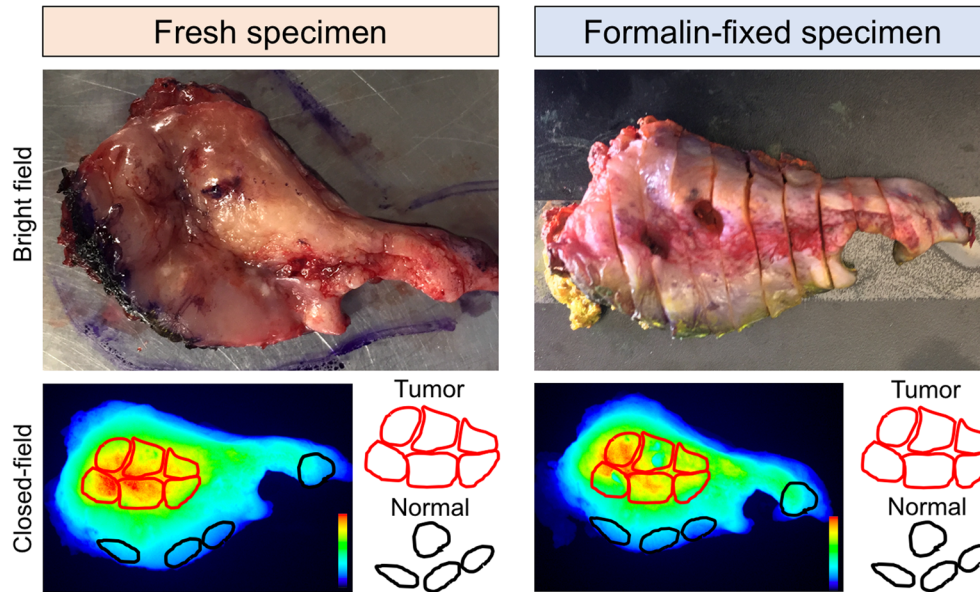
$$\text{Tissue MFI} = \frac{\{(MFI1*Area1)+(MFI2*Area2)+\ldots+(MFIn*Arean)\}}{\{Area1+Area2+\ldots+Arean\}}$$

Tumor-to-background ratios (TBRs) were calculated as follows:

$$\text{TBR} = (\text{primary tumor MFI})/(\text{adjacent non−tumor MFI})$$

Statistical Analysis

Descriptive statistics and figures were obtained using GraphPad Prism (Version 6.0c, GraphPad Software, La

Fig. 1. Workflow of the primary tumor analysis. Following excision of the tissue specimen, the specimen was imaged in a closed-field fluorescence imaging device. Hereafter, the tissue specimen was transferred to pathology for formalin fixation and re-imaged in the fluorescence imaging device. To determine the mean fluorescence intensity (MFI) and the tumor-to-background ratio (TBR), multiple regions of interest (ROIs) were drawn on the primary tumor and the adjacent normal tissue using the fluorescence imaging device–integrated software. Tissue MFIs were then calculated by dividing the sum of the measured fluorescence intensities by the sum of the areas. The TBR was calculated by dividing the primary tumor MFI by the adjacent normal tissue MFI.

Jolla, CA, USA). MFI and TBR of the primary tumor between fresh and formalin-fixed conditions were analyzed with a paired t test. Differences of change rate of MFI and TBR between the overnight fixation group and the over the weekend fixation group were analyzed with a Mann–Whitney U test. The variance in signal was defined as the coefficient of variance (CV), which is the standard deviation divided by mean signal intensity and describes the heterogeneity of the signals [17]. Pearson correlation analysis among fluorescence signals was used to explore the relationship between the fresh condition and the formalin-fixed condition. All data are presented as means or means ± standard deviation (SD), and a two-sided p value of 0.05 or less was considered statistically significant.

Results

Patient Characteristics

Of the 30 enrolled patients, tissue specimens of 10 patients had to be excluded due to difficulty of matching the pre- and post-fixation whole specimens caused by the different imaging angles or by their complicated three-dimensional tumor anatomy. Characteristics of the remainder 20 patients are shown in Table 1. The mean time difference between infusion of drug and the start of surgery was 43 h (range 17–120) and the average formalin fixation time of the tissue specimens was 29 h (range 17–94). The most common

primary tumor site was the tongue (45 %), followed by the buccal mucosa (20 %) and retromolar trigone (15 %).

Tissue Shrinkage of the Primary Tumor After Formalin Fixation

We assessed the areas of the primary tumor tissue specimens directly after resection and following formalin fixation to determine using the fluorescence imaging device–integrated software if tissue shrinkage had occurred (Fig. 2a). On average, following formalin fixation, the tissue decreased 9.9 % (range 0–17) in size ($p < 0.0001$, Fig. 2b).

Table 1. Patient characteristics

Number of patients (N)	20
Age (year) (mean (range))	66.7 (47–85)
Gender, males (N (%))	9 (45 %)
Body weight (kg) (mean (range))	71.2 (41–96)
Primary tumor site (N (%))	
Tongue	9 (45 %)
Buccal	4 (20 %)
Retromolar trigone	3 (15 %)
Maxilla	1 (5 %)
Sinus	1 (5 %)
Skin	1 (5 %)
Glottis	1 (5 %)
Time of infusion-to-surgery (h)	42.5 (17–120)
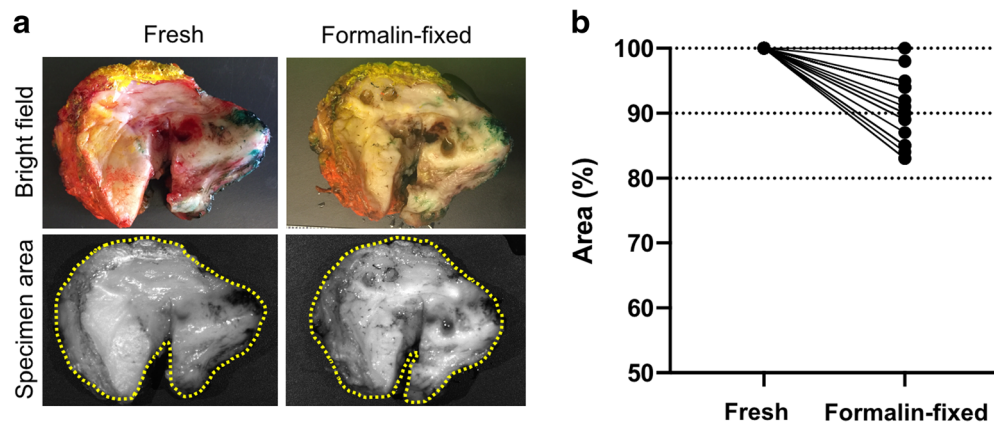
Fig. 2. Tissue specimen shrinkage following formalin fixation. **a** Illustrating the whole specimen area on the closed-field image using the fluorescence imaging device–integrated software. **b** Before and after formalin fixation plot demonstrating tissue shrinkage.

Changes of Tumor MFI and TBR After Formalin Fixation

Figure 3 shows the primary tumor MFI and TBR for all 20 evaluated tissue specimens. A high-interpatient variability for MFIs was found between fresh and formalin-fixed tissue samples (CV = 0.66 and 0.68 for fresh and formalin-fixed specimens, respectively). Low interpatient variability was found for TBRs in fresh (CV = 0.31) and formalin-fixed (CV = 0.35) tissue specimens with an average TBR of 4.4 (range 2.3–8.5). Comparing fresh tissue MFI to the MFI of the formalin-fixed tumor tissue specimens resulted in a strong correlation (Supplemental Figure 1; $R^2 = 0.93$). Findings were similar for TBR (Supplemental Figure 1, $R^2 = 0.97$).
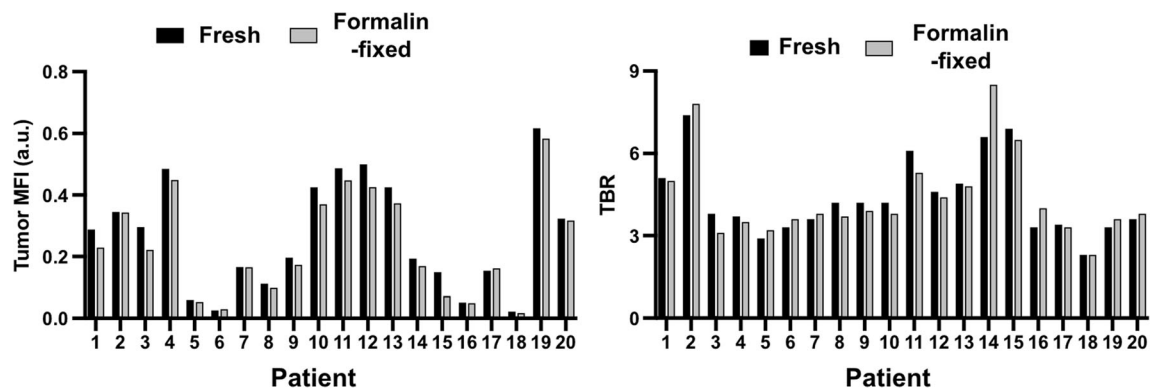
Figure 4a demonstrates a representative case in which a decrease in primary tumor MFI was seen following the formalin fixation process. The MFI of tumor in formalin-fixed specimens was on average 10.9 % lower than that of the fresh specimens ($p = 0.0002$, 0.27 ± 0.18 a.u. vs. 0.24 ± 0.17 a.u.). No statistically significant difference was found

for TBRs when comparing fresh and formalin specimens ($p = 0.85$, 4.4 ± 1.4 vs. 4.4 ± 1.6).

To evaluate if the fluorescence signal remains stable over the number of scans, we did additional experiments by imaging the same piece of tissue 10 times. As shown in Supplemental Figure 2, we measured the signal decrease of the tissue compared to the first time of imaging and only found up to 6 % decrease over the 10 times, suggesting that repetitive imaging might only contribute to a small part of the fluorescence decrease for the resected tissue specimens.

Time Effect of Formalin Fixation for Tumor MFI and TBR

To evaluate the effect of the time of formalin fixation on tumor MFI and TBR, we compared the overnight fixation group ($n = 16$, mean; 20 h (range 17–22)) to the specimens that were formalin fixed over the weekend ($n = 4$, mean; 67 h (range 40–94)) with no significant differences in tumor MFI and TBR found (Fig. 5; $p = 0.75$ and $p = 0.38$, respectively).

Fig. 3. Patient by patient comparison of MFI and TBR of fresh vs. formalin-fixed tumor specimens. Lower interpatient variability was found for TBRs in fresh and formalin-fixed tissue specimens than that for tumor MFIs.
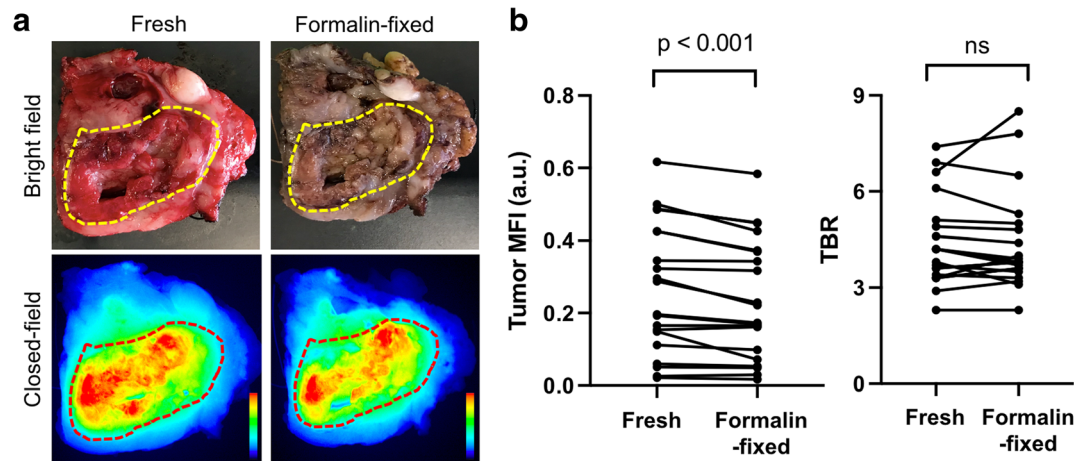
Fig. 4. Before and after the plot of changes in tumor MFI and TBR. **a** A representative case in which a decrease in primary tumor MFI was seen following the formalin fixation process. **b** The MFI of tumor in formalin-fixed specimens was on average 10.9 % lower than the fresh specimens ($p = 0.0002$). However, no statistically significant difference was found for TBRs when comparing fresh and formalin-fixed specimens ($p = 0.85$).

Discussion

There is a lack of clinical data regarding the impact of formalin fixation on the changes of fluorescence signals in resected tissue specimens during the pathological process. With the rise of clinical trials investigating novel and/or repurposed fluorescently labeled imaging agents (some of which are already in phase 2/3), this is of considerable importance as a majority of tissue analyses are currently being performed on formalin-fixed tissue specimens [4, 18–20]. The current study demonstrates that after systemic administration of a fluorescently labeled antibody, an average 11 % decrease of fluorescence intensity was found in the resected primary tumor specimens after the formalin

fixation. However, no significant differences in TBR were seen between the fresh and formalin-fixed specimens. Moreover, the fluorescence signals in the resected tumor specimens remained stable within 1–4 days after formalin fixation. These results suggest that although after the formalin fixation a decreased MFI is seen, it does not affect their relative differences between the tumor and the surrounding normal tissue as measured by TBR, demonstrating the rigidness of the model.

Formalin, a formaldehyde solution buffered to a neutral pH, is the most prevalent tissue fixative used for morphological preservation as a standard pathological process, and it forms cross-linkages between peptides and forms hydroxymethyl groups on reactive amino acid side chains [21].
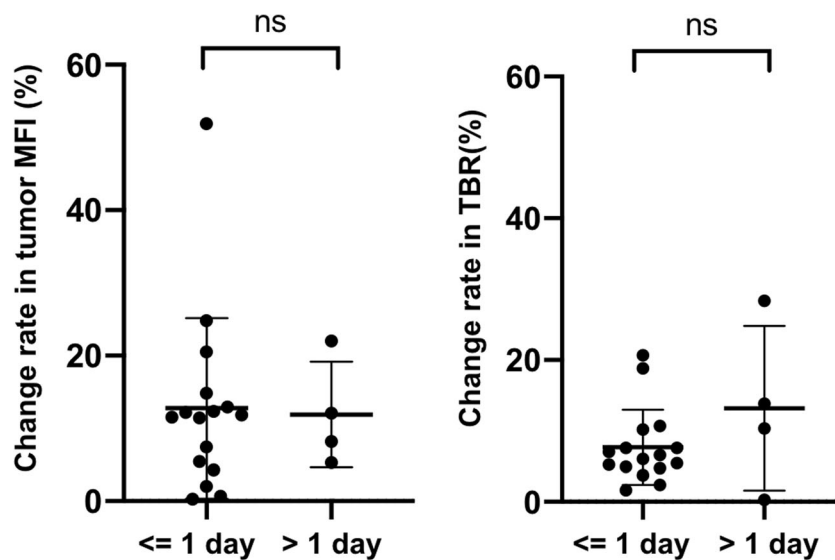

Fig. 5. Boxplots of changes in tumor MFI and TBR separated by formalin fixation time. No significant differences were found between the overnight fixation group and the over the weekend fixation group in tumor MFI and TBR ($p = 0.75$ and $p = 0.38$, respectively).

Human specimens are usually fixed overnight with fixation times varying from 12 to 48 h and are influenced by factors such as tissue components, specimen size, and temperature [22, 23]. Tissue specimen shrinkage of about 10 % was found between fresh and formalin-fixed tissues consistent with previous literature [24, 25], with no difference found if the specimen was fixed overnight or over the weekend. The soaking of the tissues in formalin could result in clearance of remaining fluorescence imaging agents from circulation (*i.e.*, blood vessels) and the exposure to ambient light may also reduce the fluorescence signal over time [26]. Our study demonstrated an average 11 % decrease in MFI between the fresh and formalin-fixed specimens, but no effect on TBR was found suggesting that the signal decrease is similar for tumor tissue as well as the surrounding normal tissue.

By using the closed-field fluorescence imaging devices, quantitative and objective evaluation of the tissue specimens was achieved due to the controlled imaging environment, which eliminated ambient light and fixed camera-tissue distance [15]. Back-table fluorescence assessment on fresh tissue specimens enables surgeons to predict the closest deep and peripheral margins and to detect secondary primary tumor during surgery [10, 11, 17]. However, despite fluorescence imaging being near-real-time when using such a device, specimen assessments for factors such as sensitivity and/or specificity, and negative and positive predictive values of the imaging agent used are generally better calculated on formalin-fixed tissue due to logistical challenges and constraints when wanting to perform such analysis in the operating room. With the current study, it was demonstrated *ex vivo* formalin-fixed specimen analysis can be used as a valid alternative to fresh tissue specimen analysis. By fixating the tissue specimens in a state as close to the original as possible, we can connect intraoperative surgical situation (*in vivo*) and pathological evaluation after resection (*ex vivo*) and apply the near-infrared fluorescence imaging with an antibody-dye conjugate into a clinical use in surgical oncology.

Conclusion

Despite the 11 % decrease in MFI between fresh and formalin-fixed tissue specimens, the relative difference between tumor and normal tissue as measured in TBR remained unchanged. This data suggests that evaluation of formalin-fixed tissue for assessing the accuracy of fluorescence-guided surgery approaches could provide a valid, yet more flexible, alternative to fresh tissue analysis.

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