

Title: Investigation of Mohs surgical margins using two photon fluorescence microscopy
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1. Purpose of the Study:

In a previous protocol (STUDY00003085, PI: Giacomelli) we performed preliminary two photon fluorescence microscopy (TPFM) imaging of surgical excisions and tissue biopsy specimens at both UPMC and Rochester Dermatologic Surgery (RDS), an outpatient clinic which has a reliance agreement under which the RSRB provides IRB supervision. In another ongoing study at RDS (STUDY00006388, PI: Giacomelli), we are imaging diagnostic biopsies from patients with suspected skin cancer to provide immediate assessment of cancer status. Collectively we have imaged several hundred skin cancer and biopsy specimens, demonstrating that TPFM can be used to image and detect skin cancer in a dermatological surgery clinic and that TPFM can be performed on diagnostic tissues.

This new study proposes to extend the previous line of research to skin cancer detection on surgical margins during Mohs surgery. At present, patients undergoing Mohs surgery for basal cell carcinoma (the most common skin cancer) undergo excisions followed by lengthy delays while tissue

is cryosectioned, stained and then evaluated to guide subsequent excision. This study proposes to evaluate margins immediately after excision during Mohs surgery using TPFM, potentially saving an hour or more of treatment time per patient.

2. Background:

Mohs surgery. Basal cell carcinomas (BCC) of the skin are treated each year than all other forms of cancer combined. Although slow growing and seldom fatal, they typically occur on sun-exposed skin such as the face where treatment can be difficult and costly. Mohs surgery, where the tumor is removed in stages using frozen section analysis (FSA) to evaluate each excision before performing the next obtains the highest cure rates and minimizes resection of healthy tissue. However, Mohs surgery is expensive and labor-intensive because each excision must be cryosectioned for analysis while the patient waits with an open surgical wound before the next round of excision or closure of surgical wounds can occur. The lengthy delays during Mohs surgery have motivated the search for faster, more efficient means to evaluate margins.

Two Photon Fluorescence Microscopy (TPFM). TPFM is a nondestructive optical microscopy technique that is widely used in the life sciences for imaging of live tissue. In previous research conducted at BIDMC in Boston, we conducted studies to develop TPFM imaging protocols for skin, breast, and prostate cancer. In reference [1] we developed a system and protocol for imaging breast and other surgical specimens. In reference [2] we validated the use of this protocol to image breast tissue and verified that fluorescent imaging had no effect on conventional histology, IHC or FISH assays. In reference [3], we performed preliminary investigations of Mohs surgical specimens showing that fluorescent imaging may be potentially valuable for guiding surgery. In reference [4], we performed more extensive imaging of excised skin cancer specimens, and further demonstrated

that two photon imaging did not interfere with subsequent histology or immunohistochemistry in dermatologic specimens. Our findings are consistent with that of other groups investigating fluorescent histology as a means of evaluating surgical pathology [5]. Finally, concurrent with previous work we conducted a clinical study imaging diagnostic prostate specimens from 40 patients using the two photon imaging protocol proposed here, demonstrating that diagnosis was similar on rapid two photon imaging performed immediately after excision as compared to subsequent histology of the same specimens [6].

In a large study of 127 discarded excisions left over after Mohs surgery, two Mohs surgeons not involved in data collection obtained very high agreement with frozen sections when evaluating TPFM images (preliminary manuscript attached). In total, there were 14 disagreements due to tissue co-registration and sample preparation which do not reflect true diagnostic accuracy and 8 specimens (7%) where 1 reviewer diagnosed TPFM differently than frozen sections and one reviewer agreed with frozen sections. **There were zero co-registered samples where both reviewers were incorrect.** Thus, TPFM was highly reliable for the diagnosis of BCC on Mohs margins.

Previous studies under supervision of the RSRB: Under STUDY00003085, we imaged 21 biopsy specimens from Mohs surgery patients that were not required for diagnosis using TPFM and demonstrated that BCC could be readily visualized. These results were published recently in JAMA Dermatology [7]. Under STUDY00006388 we are routinely imaging diagnostic biopsy specimens from patients prior to Mohs surgery. While this study is still running, we have not encountered problems imaging BCC and have had no adverse events due to TPFM imaging of diagnostic tissues at RDS.

Rochester Dermatologic Surgery (RDS). RDS is an outpatient dermatologic surgery clinic located in Victor NY and owned by Dr. Sherrif Ibrahim, co-investigator on this protocol. Dr. Ibrahim splits his surgical practice between UPMC and RDS. To facilitate collaboration between the lab of Michael Giacomelli, located at UPMC and Dr. Ibrahim's practice at both locations, the RSRB typically acts as the IRB for studies jointly between UPMC and RDS. In this case, we request a reliance agreement.

3. Administrative Organization

The research location is Rochester Dermatologic Surgery in Victor, NY.

4. Study Design

4.1. Study Population and Recruitment:

This study proposes to image surgical margins from patients undergoing Mohs surgery at RDS. Mohs surgery is an outpatient procedure conducted in a single visit with only local anesthesia. During the visit, Dr. Ibrahim or another physician under his supervision will explain to patients being treated for basal cell carcinoma (BCC) that they are eligible to participate in a clinical study and provide them with a consent form that contains additional information. If interested, he or another study member will obtain consent (see **Consent procedure and documentation below**).

The study population will be adult patients undergoing Mohs surgery at RDS. This study will be powered to reject the null hypothesis that the true sensitivity and specificity of TPFM for the evaluation BCC margins is less than 85% at a confidence level of 95%. If the true sensitivity and specificity are each 95%, and 38% of patients have a positive surgical margin, then 123 patients would be required. Furthermore, to account for screen fails and patients for which all data is not available during analysis, we request an additional 12 patients. Thus, we anticipate an enrollment of 135 patients.

4.2. Study Activities:

This study is an interventional study of skin cancer that will use TPFM to evaluate surgical margins followed by standard of care FSA for confirmation. This study will image routine skin cancer margins from the practice of Dr. Ibrahim at RDS and image them using TPFM to determine if TPFM can accurately detect BCC on surgical margins. To protect patients, following TPFM imaging, patients will receive standard of care evaluation using cryosectioned H&E slides, thus **this design will ensure that all patients will be adequately treated and that TPFM cannot deny any patient complete treatment.**

Under Dr. Ibrahim or another physician under his supervision's direction, individual excisions from consenting patients will be selected, fluorescently labeled (protocol described below, already approved in STUDY00006388) and diagnosed using the two photon microscope at RDS. A full image of the tissue will also be digitally recorded. Following imaging, specimens will be cryosectioned as per normal procedure. In the event of definitive BCC on margins, Dr. Ibrahim or another physician under his supervision may choose to immediately excise the specific area of BCC rather than wait the additional 30-60 minutes for FSA. If no BCC is found, or if TPFM images are equivocal, the FSA images will be consulted first. As a result of this design, the last excision (which must always be negative or else surgery is continued) will always be confirmed on FSA, thus ensuring standard treatment.

In STUDY00003085, we deployed and tested a compact two photon microscope engineered for use in a dermatologic clinic at RDS. In testing, this device could rapidly perform 3D imaging of entire Mohs margin specimens in approximately 10 minutes or less. In normal practice, specimens may wait 20 minutes or more in queue for cryosectioning or post cryosectioning evaluation, so the delay for study activities will fit into existing delays built into clinical workflows. Thus, TPFM imaging will not significantly prolong surgery.

Following conclusion of surgery there will be no further study-related contact with patients.

Following treatment, the results of the TPFM evaluation of each Mohs excisions will be compared to the results from FSA to assess the overall accuracy of TPFM. To perform this comparison, we will record two photon fluorescence images of each surgical margin as well as a white light image for orientation. We will further record images of FSA histology in the event that TPFM images disagree with FSA for comparison.

Information Retained

This study will record basic demographic information, microscopic images recorded from TPFM and FSA, and the results of diagnostic procedures performed using FSA (for example, the presence of cancer on the surgical margin).

Each patient's name, RDS case number and basic demographic information (age, race, sex and surgery location) will be recorded in URM's RedCap. To enable deidentification of image data, each patient will be assigned an index number that will be stored in RedCap. The diagnosis based on TPFM and conventional microscopic images will also be recorded under the same deidentified index number. Signed consent forms with the patient's name and index number will be stored in locked cabinets at RDS. Following completion of the study, consent forms will be stored for 3 years and will then be destroyed. In general, we expect that no medical record review will be required and patients will be able to state their age, race and surgery location during the consent process. However, we will record the RDS case number (which can be used by RDS staff to look up specific procedures) in the event that information (e.g. FSA slide images) must be retrieved on a later date. Thus, we are also requesting permission to retrieve demographic, procedure information (e.g. location) or slide images from RDS medical records using the case number.

Access to information

Access to the RDS case number in RedCap (the only PHI recorded electronically) will be restricted to personnel assisting with data collection and consenting using RedCap record permissions. Study personnel will not directly access RDS computers or records, and will instead request that Dr. Ibrahim or one of his clinic staff provide information if a medical record must be reviewed. In this way, Dr. Ibrahim will ensure that case numbers are translated into other information only as required. Signed consent forms (the only place patient names are recorded) will be locked by RDS staff, and only study personnel involved in consenting patients will have access to consent forms. Thus, Dr. Ibrahim and his staff, who already have access to patient information as part of their professional duties, will serve as gate keepers and ensure that access to patient names and medical records are performed only as approved in this protocol.

Individual TPFM or FSA images will be stored at URM on an offline data server in the Giacomelli lab (URM 5-8145). Images will be stored without PHI and will be identified only by the index number. Thus, all PHI will remain in RedCap or at RDS where patient records would anyway be stored. Following completion of the study, deidentified TPFM and FSA images will be stored indefinitely and will be shared with the scientific community consistent with NIH policies on data sharing.

Diagnostic Tissue Staining Protocol and Noninterference With Histology

TPFM is a nondestructive optical imaging technique that can be performed on fresh tissue without fixation, embedding or microtome cutting. This imaging protocol uses dilute fluorescent dyes to label tissue analogously to hematoxylin and eosin staining in conventional H&E histopathology. Fluorescent dyes will be applied to the tissue specimen *ex vivo* hence the fluorescent dyes will never come in contact with the patient and do not pose a direct risk to patient health.

Unlike conventional histopathology in which stains are chosen that are visible to the human eye, fluorescent microscopy uses fluorescent dyes that have much weaker absorption and are not visible

the human eye on histology slides. Our protocol performs staining with a solution containing one of the reagents acridine orange (AO) at less than 1 millimolar (~20x lower concentration than typical histology stains), or SYBR Green (SG) at less than 100 micromolar (~200x lower concentration) and sulforhodamine 101 (SR101) at less than 1 millimolar concentration (~20x lower concentration than typical histology stains), for up to 3 minutes treatment dissolved in 70-100% ethanol. AO is a fluorescent stain occasionally used in blood smears to identify infectious organisms. SG is a newer DNA label that has improved specificity, but otherwise performs similar functions and fluoresces at identical wavelengths to AO. SR101 is a dye that is chemically related to eosin that stains stroma and fluoresces red. These stains provide nuclear and stromal fluorescent contrast analogous to hematoxylin and eosin staining in conventional histopathology. None of these agents are routinely used to label skin tissue during routine histopathology and therefore represent a change in procedure. However, because neither agent is visible to the human eye on histology slides at the low concentration used, labeling is not apparent on conventional visible light microscopy. Furthermore, **all agents only temporarily label human tissue, and are removed from tissue by routine histological processing solvents such as ethanol as used during paraffinization or staining during cryosectioning [4].** Simple soaking in standard histological processing solvents (ethanol or xylene) extracts all labels. During testing with fresh tissue conducted in the Department of Pathology at UPMC, all reagents were removed to undetectable levels during standard paraffinization and prior to conventional staining on a commercial tissue processor. In testing at RDS, FSA performed on TPFM samples was visually identical to unstained samples.

In addition to verifying that the reagents are extracted by routine histological processing, we have further conducted testing to demonstrate noninterference with IHC. Specimens were stained at the concentrations used in this protocol with AO/SR101 and then submitted for routine IHC (ER, PR, MART1, HER2). SG was stained at concentrations used in this protocol and submitted for routine IHC (MART1, BEREPA, and pancytokeratin as would be used for dermatology specimens). No difference was observed, and no fluorescence was visible. **These results are consistent with our finding that all three agents are removed during paraffinization and not physically present after paraffinization.**

Investigational Device Exemption not required: The TPFM system is exempt from the IDE requirements Per 21 CFR 812.2 because it is noninvasive, does not require an invasive sampling procedure, does not introduce energy into subjects, and will not be used as a diagnostic without confirmation from an established diagnostic. Therefore, no IDE is provided.

5. Inclusion and Exclusion Criteria

Patients 18 years or older being treated for basal cell carcinoma of the skin with Mohs surgery at Rochester Dermatologic Surgery will be eligible to participate. As RDS is a small clinic that rarely treats non-English speaking patients, translation services are not typically available. Therefore, as we cannot ensure informed consent in this case, patients that are unable to read an English-language consent form will be excluded from the study.

6. Recruitment Methods

Dr. Ibrahim or another staff member under his supervision will inform patients meeting the inclusion criteria that they are eligible to participate in a clinical study while discussing treatment with them prior to surgery.

7. Informed Consent:

We will use a written consent form to document informed consent. The consent form is attached to this protocol submission. Signed consent forms will be stored on file at RDS.

Consent procedure and documentation

The patient will be informed that images will be acquired of their surgical excision and that these images may be used to guide treatment. The patient will be informed that regardless of choosing to participate in the study, final treatment status will be determined according to standard of care. Finally, a consent form will be provided to the patient that includes information summarizing the study. If the patient agrees to participate, Dr. Ibrahim or someone under his supervision will answer any questions, record their signature, and record their demographic information. Consenting will be performed shortly before the patient's surgery.

8. Risks and Benefits:

Risks from TPFM imaging. This imaging protocol involves staining freshly excised pathology specimens with dilute solutions of fluorescent contrast agents. These agents are not routinely used and differ from the standard postoperative histology protocol. However, this is no more than minimum risk because (1) the dye concentrations are low compared to standard concentrations used for dyes such as hematoxylin and eosin, (2) all dyes are highly soluble in histological processing agents (e.g. alcohol and xylene) and can be removed during routine processing (3) Fluorescent label concentration decreases exponentially below the tissue surface, and therefore normal sectioning removes most or all of the exposed tissue. Furthermore, we have conducted an investigation with the Department of Pathology at BIDMC that verifies that no interference is observed on pathology specimens (as described in reference 2) and then replicated this investigation with the Department of Pathology at UPMC and confirmed that all agents are completely removed using their standard tissue processing protocol. We have further tested IHC on treated specimens and found no interference or residual fluorescent signal. All imaging will be performed at the surgeon's discretion, and while interference or complications from fluorescent imaging have never been observed, if any are suspected, imaging can be halted, and the fluorescent labels washed out with ethanol (a standard reagent used during staining of cryosectioned tissues) or simply sectioned off to expose unlabeled tissue.

Risks to patient treatment. The design of this study prevents undertreatment by concluding every procedure with standard of care FSA histology. Thus, complete tumor resection will always be confirmed using the standard method regardless of TPFM sensitivity. However, if TPFM demonstrates poor specificity, there is a risk that additional tissue could be removed that would not otherwise be (overtreatment). This is unlikely given our preliminary data in which BCC could be readily distinguished from healthy tissue and because the appearance of BCC is typically very apparent on both TPFM and FSA, but to further guard against this possibility, Dr. Ibrahim and Dr.

Sipprell will wait for FSA confirmation before further excisions unless they determine that BCC is both obvious on TPFM and the excision of additional tissue would not have a significant impact on the patient's cosmetic outcome or reconstruction.

Risks to privacy. In order to reduce the risk to patient privacy image data will be deidentified before removing it from the clinic. PHI will be stored in RedCap and at RDS, where patient files would anyway be kept. Thus, this study poses not significant increase in risk to the patient's privacy.

Benefits. Positive margins may be more rapidly diagnosed for patients in the study, resulting in faster treatment and less time spent with open surgical wounds. At present, patients with positive margins for BCC must wait approximately 1 hour mid-surgery per stage of excision whereas TPFM imaging can be done in a few minutes. The development of new methods for real-time imaging of surgical pathology would have major impact on the health care by improving the efficiency of Mohs surgery. In comparison to the benefits, the risks are minimal since the investigation will maintain the standard of care for final margin confirmation and in case of any ambiguity in TPFM imaging. Consequently, the risk to benefit assessment is strongly in favor of benefit.

9. Costs for participation

There will be no additional costs to participate in this study beyond normal treatment costs.

10. Payment for participation

There will be no payment for participation.

11. Subject Withdrawals

This is a single visit study with no follow up. Thus subject withdrawal is unlikely, but could happen if a patient were unable to receive therapy during the clinic visit. In this case, the subject would be withdrawn from the study and no data collected.

12. Data Analysis:

The primary goal of this study is to evaluate the ability of TPFM to evaluate Mohs surgical margins during treatment of BCC. Consequently, analysis will compare the margin status as determined by TPFM to that determined on subsequent FSA. Analysis will be performed for each stage of excision and lumped for all stages, but because not all surgeries have more than 1 stage, most likely analysis of stages 2 and higher will not be well-powered.

Secondary goals will investigate the source of disagreements between FSA and TPFM, which are expected to include both limitations of FSA (poor tissue coverage, freezing artifacts, etc.) as well as TPFM (poor tissue flatness in the image plane, problems with staining or contrast, etc.). This analysis will use images of FSA and TPFM images to determine the source of disagreement, providing both better insight into the overall sensitivity of the technique as well as limitations that could be addressed through technical improvements in microscope hardware.

A final analysis will look at excisions where TPFM enabled faster correction of positive margins. The time savings for patients will be measured, and the benefit to patients estimated.

13. Data and Safety Monitoring Plan

Data and Safety Monitoring Entities

The operating surgeon will provide safety monitoring during study activities. The study PI will provide data and safety monitoring outside of the clinic.

Patient Safety Monitoring

Dr. Ibrahim will monitor imaging as part of treatment and report any suspected alterations of care to the study team and the RSRB.

Protection of patient privacy

All image data will be coded at the time of consent so as to protect identity of the patient. Consent forms and demographic information will be stored in the clinic.

Data Security

Research materials obtained from patients will include fluorescent images, digital copies of histology slides and basic demographic information (age, sex, race) for enrollment. Each dataset will be deidentified and computerized using the protocol –assigned ID number in the URM RedCap database.

Storage of deidentified image data will occur at URM using a secure server not connected to the internet. Deidentified and digital histological data will be shared with the clinical and research communities as appropriate, and we will invite the pathology community to evaluate or use deidentified image data as scientifically appropriate. PHI such as demographic data will be stored at URM in RedCap.

PI Oversight Plan

We have attached a separate PI oversight plan.

14. References:

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