Real-time Analysis of Skin Biopsy Specimens Using 2-Photon Fluorescence Microscopy

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Received: 11-May-2022, Manuscript No. pscs-22-80033; **Editor assigned:** 13-May-2022, PreQC No. pscs-22-80033 (PQ); **Reviewed:** 27-May-2022, QC No. pscs-22-80033 (Q); **Revised:** 01-Jun-2022, Manuscript No. pscs-22-80033 (R); **Published:** 08-Jun-2022, doi:10.37532/pscs.22.3.3.1

Introduction

Non Melanoma Skin Cancers (NMSCs) are primarily diagnosed through paraffin section histologic analysis of skin biopsy specimens, which can take several days to weeks before a formal diagnosis is made. Two-Photon Fluorescence Microscopy (TPFM) has the potential to enable point-of-care diagnosis and treatment of NMSC and other dermatologic conditions. Non Melanoma Skin Cancer (NSMC) is the most common type of human cancer, accounting for more cases in the United States each year than all other types of cancer combined. According to Medicare database estimates, approximately 9600 new cases of NMSC are diagnosed each day, with approximately 80% being Basal Cell Carcinoma (BCC) and 20% being Squamous Cell Carcinoma (SCC). Skin biopsy, in which a portion of a suspected lesion is excised, fixed, paraffinized, stained, and mounted on slides before being evaluated by a dermatopathologist, remains the gold standard for diagnosis. From the time of biopsy to diagnosis, this process takes several days, resulting in a delay in treatment and additional clinic visits for definitive care.

Same-day treatments are also associated with higher clinical throughput and a lower risk of wrong-site surgery. While frozen sections can provide same-day treatments, they have variable reliability (83% to 93% concordance rates) due to freezing and disruption artifacts, as well as their reliance on highly skilled technicians and dedicated frozen section laboratories for tissue preparation.

Recent advancements in microscopy and imaging techniques have enabled nondestructive optical sectioning, which allows for the detection and diagnosis of NMSC via noninvasive in-vivo imaging or slide-free histology. For in vivo imaging of NMSC, imaging technologies such as optical coherence tomography, reflectance confocal microscopy, and 2-Photon Fluorescence Microscopy (TPFM) have been investigated and commercialized. While in vivo imaging techniques can provide noninvasive diagnostic information, they are label-free and rely on intrinsic tissue properties (e.g., refractive index, absorptivity, thermoelectricity, and auto fluorescence); thus, they do not directly visualize conventional histological features such as nuclei and stroma. As a result, these images reveal features that differ from traditional histology slides and necessitate extensive retraining for interpretation.

While label-free, slide-free techniques exist, such as photoacoustic remote sensing microscopy and optical coherence tomography, one of the main advantages of slide-free histology is the ability to directly label nuclei and/or stroma with fluorescent stains. Exogenous stains allow for the visualization of features similar to Hematoxylin and Eosin (H&E) and, when combined with computational techniques, can produce virtually stained histology images that closely resemble conventional histology. Deep ultraviolet microscopy, confocal fluorescence microscopy, and TPFM are examples of imaging techniques. Confocal fluorescence microscopy studies of NMSC surgical margins have revealed that these techniques agree well with conventional histology.

TPFM, like confocal fluorescence microscopy, can produce high-resolution, nearly H&E-stained images, but with the added benefit of using near-infrared light, which penetrates deeper into tissue, making it useful for rapid imaging of fresh, irregularly shaped biopsies with minimal preparation. Confocal microscopy, on the other hand, is limited to superficial imaging of the tissue surface and is more easily obscured by debris, which may explain the relatively high rate of excluded samples in some confocal studies. Finally, TPFM with virtual staining can be performed quickly at video rate, for multiple tissue specimens in parallel, and in real-time, allowing for rapid diagnosis.

The study's limitations include a small number of biopsies, a limited number of possible diagnoses, and a single dermatopathologist review. Furthermore, simple diagnoses were made without considering subtypes. To fully assess the ability of TPFM for immediate biopsy evaluation, future studies addressing these limitations and incorporating benign dermatologic conditions representative of typical clinic populations will be required. Finally, future research will be needed to evaluate the use of TPFM in non-biopsy workflows, such as Mohs surgery, which requires comprehensive margin assessment.

TPFM reproduced histological characteristics of NMSC that are present in conventional histology and provided high concordance with paraffin histology on a masked evaluation of a small cohort in this comparative effectiveness research study. While these findings suggest that TPFM imaging has the potential to be a rapid, point-of-care diagnostic tool that does not require extensive sample preparation or retraining for image evaluation, further validation in a larger cohort is required to fully evaluate diagnostic accuracy.

While a discordant pair was present in the study, closer examination revealed that the discrepancy was most likely due to a difference in image plane sampling rather than an error in interpreting the TPFM image. Because paraffinization distorts tissue, sampling errors can occur, and permanent sections may be cut at a different depth or angle than the TPFM image plane on fresh tissue. While the use of en face sections facilitated more accurate co registration in this study, a smaller bread-loaf could be used in a real-world setting where co registration is not required.

In contrast to a previous study of surgical margins using confocal fluorescence microscopy, where nearly one-third of specimens were excluded due to image quality or lack of coregistration, only four samples were rejected or used for training due to TPFM image quality or lack of registration, implying that the ability of TPFM to image deeper into specimens may be advantageous for imaging fresh biopsy tissues with irregular surfaces.

The prototype device is portable, smaller than a cryotome, and requires significantly less operator training than standard tissue processing. This enables point-of-care interpretation of skin biopsies in real time, even in low-resource settings. Furthermore, because TPFM imaging is non-destructive and stains are removed by paraffinization, it does not preclude subsequent histology or immunohistochemistry. The fact that histological evaluation is limited to the tissue surface is a disadvantage of slide-free histology techniques in general. While TPFM allows for deeper imaging than other fluorescent imaging techniques, it is still limited to about 100 microns into tissue. Specimens can be bisected or bread-loafed, as in conventional histology, to expose internal tissue for imaging, eliminating the need for deeper imaging.

Cite this article: Thomas, J. Real-time Analysis of Skin Biopsy Specimens Using 2-Photon Fluorescence Microscopy. J. Plast Surg: Case Stud. 3(3), 2022, 001.