

Ex Vivo Confocal Microscopy: A Diagnostic Tool for Skin Malignancies



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PRACTICE POINTS

- Confocal microscopy is an imaging tool that can be used both in vivo and ex vivo to aid in the diagnosis and management of cutaneous neoplasms, including melanoma, basal cell carcinoma, and squamous cell carcinoma, as well as inflammatory dermatoses.
- Ex vivo confocal microscopy can be used in both reflectance and fluorescent modes to render diagnosis in excised tissue or check surgical margins.
- Both in vivo and ex vivo confocal microscopy produces images with cellular resolution with a main limitation being depth of imaging.

Confocal microscopy is an imaging tool that uses a laser system to image the skin noninvasively with cellular resolution. With both in vivo and ex vivo systems, tissue can be imaged rapidly without damaging the sample. Herein, we discuss the capabilities of the ex vivo confocal microscope and its implementation for treatment and management of cutaneous malignancies.

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Skin cancer is diagnosed in approximately 5.4 million individuals annually in the United States, more than the total number of breast, lung, colon, and prostate cancers diagnosed per year.¹ It is estimated that 1 in 5 Americans will develop skin cancer during their lifetime.² The 2 most common forms of skin cancer are basal cell carcinoma (BCC) and squamous cell carcinoma (SCC), accounting for 4 million and 1 million cases diagnosed each year, respectively.³ With the increasing incidence of these skin cancers, the use of noninvasive imaging tools for detection and diagnosis has grown.

Ex vivo confocal microscopy is a diagnostic imaging tool that can be used in real-time at the bedside to assess freshly excised tissue for malignancies. It images tissue samples with cellular resolution and within minutes of biopsy or excision. Ex vivo confocal microscopy is a versatile tool that can assist in the diagnosis and management of skin malignancies such as melanoma, BCC, and SCC.

Reflectance vs Fluorescence Mode

Excised lesions can be examined in reflectance or fluorescence mode in great detail but with slightly varying nuclear-to-dermis contrasts depending on the chromophore that is targeted. In reflectance mode (reflectance confocal microscopy [RCM]), melanin and keratin act as endogenous chromophores because of their high refractive index relative to water,^{4,5} which allows for the visualization of cellular structures of the skin at low power, as well as microscopic substructures such as melanosomes, cytoplasmic granules, and other cellular organelles at high power. Although an exogenous contrast agent is not required, acetic acid has the capability to highlight nuclei, enhancing the tumor cell-to-dermis contrast in RCM.⁶ Acetic acid is clinically used as a predictor for certain skin and mucosal membrane neoplasms that blanch when exposed to the solution. In the case of RCM, acetic acid increases the visibility of nuclei by inducing the compaction of chromatin. For the acetowhitening to be effective, the sample must be soaked in the solution for a specific amount of time, depending on the concentration.⁷ A concentration between 1% and 10% can be used, but the less concentrated the solution, the longer the time of soaking that is required to achieve sufficiently bright nuclei.⁶

The contrast with acetic acid, however, is quite weak when the tissue is imaged en face, or along the horizontal surface of the sample, due to the collagen in the dermal layer, which has a high reflectance index. This issue

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is rectified when using the confocal microscope in the fluorescence mode with an exogenous fluorescent dye as a nuclear stain. Fluorescence confocal microscopy (FCM), results in a stronger nuclear-to-dermal contrast because of the role of contrast agents.⁸ The 1000-fold increase in contrast between nuclei and dermis is the result of dye agents that preferentially bind to nuclear DNA, of which acridine orange is the most commonly used.^{5,8} Basal cell carcinoma and SCC tumor cells can be visualized with FCM because they appear hyperfluorescent when stained with acridine orange.⁹ The acridine orange-stained cells display bright nuclei, while the cytoplasm and collagen remains dark. A positive feature of acridine orange is that it does not alter the tissue sample during freezing or formalin fixation and thus has no effect on subsequent histopathology that may need to be performed on the sample.¹⁰

High-Resolution Images Aid in Diagnosis

After it is harvested, the tissue sample is soaked in a contrast agent or dye, if needed, depending on the confocal mode to be used. The confocal microscope is then used to take a series of high-resolution individual en face images that are then stitched together to create a final mosaic image that can be up to 12×12 mm.^{6,11} With a 200- μ m depth visibility, confocal microscopy can capture the cellular structures in the epidermis, dermis, and (if compressed enough) subcutaneous fat in just under 3 minutes.¹²

The images produced through confocal microscopy have an excellent correlation to frozen histological sections and can aid in the diagnosis of many epidermal and dermal malignancies including melanoma, BCC, and SCC. New criteria have been established to aid in the interpretation of the confocal images and identify some of the more common skin cancers.^{5,12,13} Basal cell carcinoma samples imaged through fluorescence and reflectance in low-power mode display the distinct nodular patterns with well-demarcated edges, as seen on classical histopathology. In the case of FCM, the cells that make up the tumor display hyperfluorescent areas consistent with nucleated cells that are stained with acridine orange.

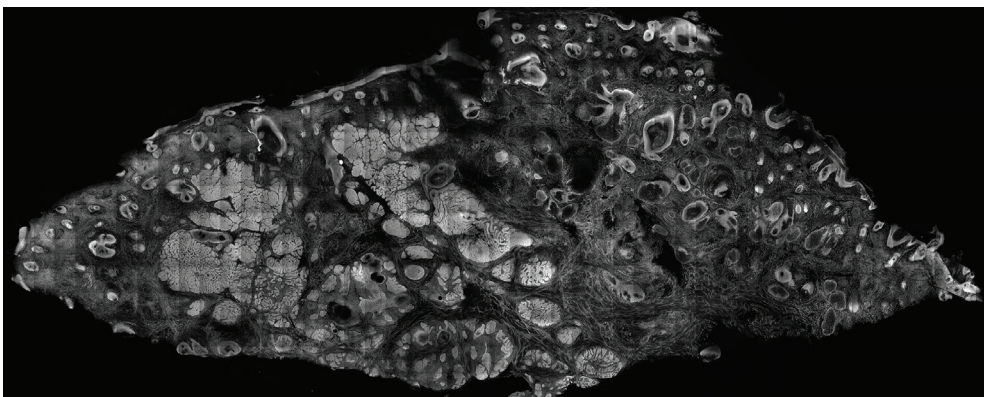
The main features that identify BCC on FCM images include nuclear pleomorphism and crowding, peripheral palisading, clefting of the basaloid islands, increased nucleus-to-cytoplasm ratio, and the presence of a modified dermis surrounding the mass known as the tumoral stroma^{5,12} (Figure).

In addition to fluorescence and a well-defined tumor silhouette, SCC under FCM displays keratin pearls composed of keratinized squames, nuclear pleomorphism, and fluorescent scales in the stratum corneum that are a result of keratin formation.^{5,13} The extent of differentiation of the SCC lesion also can be determined by assessing if the silhouette is well defined. A well-defined tumor silhouette is consistent with the diagnosis of a well-differentiated SCC, and vice versa.¹³ Ex vivo RCM also has been shown to be useful in diagnosing malignant melanomas, with melanin acting as an endogenous chromophore. Some of the features seen on imaging include a disarranged epithelium, hyperreflective roundish and dendritic pagetoid cells, and large hyperreflective polymorphic cells in the superficial chorion.¹⁴

Comparison to Conventional Histopathology

Ex vivo confocal microscopy in both the reflectance and fluorescence mode has been shown to perform well compared to conventional histopathology in the diagnosis of biopsy specimens. Ex vivo FCM has been shown to have an overall sensitivity of 88% and specificity of 99% in detecting residual BCC at the margins of excised tissue samples and in the fraction of the time it takes to attain similar results with frozen histopathology.⁹ Ex vivo RCM has been shown to have a higher prognostic capability, with 100% sensitivity and specificity in identifying BCC when scanning the tissue samples en face.¹⁵

Qualitatively, the images produced by RCM and FCM are similar to histopathology in overall architecture. Both techniques enhance the contrast between the epithelium and stroma and create images that can be examined in low as well as high resolution. A substantial difference between confocal microscopy and conventional hematoxylin and



Ex vivo confocal image of a nodular basal cell carcinoma using acridine orange as a contrast agent. Note the well-demarcated basaloid tumor islands in the dermis.

eosin-stained histopathology is that the confocal microscope produces images in gray scale. One way to alter the black-and-white images to resemble hematoxylin and eosin-stained slides is through the use of digital staining,¹⁶ which could boost clinical acceptance by physicians who are accustomed to the classical pink-purple appearance of pathology slides and could potentially limit the learning curve needed to read the confocal images.

Application in Mohs Micrographic Surgery

An important clinical application of ex vivo FCM imaging that has emerged is the detection of malignant cells at the excision margins during Mohs micrographic surgery. The use of confocal microscopy has the potential to save time by eliminating the need for tissue fixation while still providing good diagnostic accuracy. Implementing FCM as an imaging tool to guide surgical excisions could provide rapid diagnosis of the tissue, expediting excisions and reconstruction or the Mohs procedure while eliminating patient wait time and the need for frozen histopathology. Ex vivo RCM also has been used to establish laser parameters for CO₂ laser ablation of superficial and early nodular BCC lesions.¹⁷ Other potential uses for ex vivo RCM/FCM could include rapid evaluation of tissue during operating room procedures where rapid frozen sections are currently utilized.

Combining In Vivo and Ex Vivo Confocal Microscopy

Many of the diagnostic guidelines created with the use of ex vivo confocal microscopy have been applied to in vivo use, and therefore the use of both modalities is appealing. In vivo confocal microscopy is a noninvasive technique that has been used to map margins of skin tumors such as BCC and lentigo maligna at the bedside.⁵ It also has been shown to help plan both surgical and nonsurgical treatment modalities and reconstruction before the tumor is excised.¹⁸ This technique also can help the patient understand the extent of the excision and any subsequent reconstruction that may be needed.

Limitations

Ex vivo confocal microscopy used as a diagnostic tool does have some limitations. Its novelty may require surgeons and pathologists to be trained to interpret the images properly and correlate them to conventional diagnostic guidelines. The imaging also is limited to a depth of approximately 200 μm ; however, the sample may be flipped so that the underside can be imaged as well, which increases the depth to approximately 400 μm . The tissue being imaged must be fixed flat, which may alter its shape. Complex tissue samples may be difficult to flatten out completely and therefore may be difficult to image. A special mount may be required for the sample to be fixed in a proper position for imaging.⁶

Final Thoughts

Despite some of these limitations, the need for rapid bedside tissue diagnosis makes ex vivo confocal microscopy

an attractive device that can be used as an additional diagnostic tool to histopathology and also has been tested in other disciplines, such as breast cancer pathology. In the future, both in vivo and ex vivo confocal microscopy may be utilized to diagnose cutaneous malignancies, guide surgical excisions, and detect lesion progression, and it may become a basis for rapid diagnosis and detection.¹⁹

REFERENCES

1. Siegel RL, Miller KD, Jemal A. Cancer statistics, 2016 [published online January 7, 2016]. *CA Cancer J Clin*. 2016;66:7-30.
2. Robinson JK. Sun exposure, sun protection, and vitamin D. *JAMA*. 2005;294:1541-1543.
3. Rogers HW, Weinstock MA, Feldman SR, et al. Incidence estimate of nonmelanoma skin cancer (keratinocyte carcinomas) in the US population, 2012. *JAMA Dermatol*. 2015;151:1081-1086.
4. Welzel J, Kästle R, Sattler EC. Fluorescence (multiwave) confocal microscopy. *Dermatol Clin*. 2016;34:527-533.
5. Longo C, Ragazzi M, Rajadhyaksha M, et al. In vivo and ex vivo confocal microscopy for dermatologic and Mohs surgeons. *Dermatol Clin*. 2016;34:497-504.
6. Patel YG, Nehal KS, Aranda I, et al. Confocal reflectance mosaicing of basal cell carcinomas in Mohs surgical skin excisions. *J Biomed Opt*. 2007;12:034027.
7. Rajadhyaksha M, Gonzalez S, Zavislan JM. Detectability of contrast agents for confocal reflectance imaging of skin and microcirculation. *J Biomed Opt*. 2004;9:323-331.
8. Karen JK, Gareau DS, Dusza SW, et al. Detection of basal cell carcinomas in Mohs excisions with fluorescence confocal mosaicing microscopy. *Br J Dermatol*. 2009;160:1242-1250.
9. Bennàssar A, Vilata A, Puig S, et al. Ex vivo fluorescence confocal microscopy for fast evaluation of tumour margins during Mohs surgery. *Br J Dermatol*. 2014;170:360-365.
10. Gareau DS, Li Y, Huang B, et al. Confocal mosaicing microscopy in Mohs skin excisions: feasibility of rapid surgical pathology. *J Biomed Opt*. 2008;13:054001.
11. Bini J, Spain J, Nehal K, et al. Confocal mosaicing microscopy of human skin ex vivo: spectral analysis for digital staining to simulate histology-like appearance. *J Biomed Opt*. 2011;16:076008.
12. Bennàssar A, Carrera C, Puig S, et al. Fast evaluation of 69 basal cell carcinomas with ex vivo fluorescence confocal microscopy: criteria description, histopathological correlation, and interobserver agreement. *JAMA Dermatol*. 2013;149:839-847.
13. Longo C, Ragazzi M, Gardini S, et al. Ex vivo fluorescence confocal microscopy in conjunction with Mohs micrographic surgery for cutaneous squamous cell carcinoma. *J Am Acad Dermatol*. 2015;73:321-322.
14. Cinotti E, Haouas M, Grivet D, et al. In vivo and ex vivo confocal microscopy for the management of a melanoma of the eyelid margin. *Dermatol Surg*. 2015;41:1437-1440.
15. Espinasse M, Cinotti E, Grivet D, et al. 'En face' ex vivo reflectance confocal microscopy to help the surgery of basal cell carcinoma of the eyelid [published online December 19, 2016]. *Clin Exp Ophthalmol*. doi:10.1111/ceo.12904.
16. Gareau DS, Jeon H, Nehal KS, et al. Rapid screening of cancer margins in tissue with multimodal confocal microscopy. *J Surg Res*. 2012;178:533-538.
17. Sierra H, Damanpour S, Hibler B, et al. Confocal imaging of carbon dioxide laser-ablated basal cell carcinomas: an ex-vivo study on the uptake of contrast agent and ablation parameters [published online September 22, 2015]. *Lasers Surg Med*. 2016;48:133-139.
18. Hibler BP, Yélamos O, Cordova M, et al. Handheld reflectance confocal microscopy to aid in the management of complex facial lentigo maligna. *Cutis*. 2017;99:346-352.
19. Rajadhyaksha M, Marghoob A, Rossi A, et al. Reflectance confocal microscopy of skin in vivo: from bench to bedside. *Lasers Surg Med*. 2017;49:7-19.