Immediate ex-vivo diagnosis of pituitary adenomas using confocal reflectance microscopy: a proof-of-principle study

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OBJECTIVE The objective of this study was to evaluate the feasibility of using confocal reflectance microscopy (CRM) ex vivo to differentiate adenoma from normal pituitary gland in surgical biopsy specimens. CRM allows for rapid, label-free evaluation of biopsy specimens with cellular resolution while avoiding some limitations of frozen section analysis.

METHODS Biopsy specimens from 11 patients with suspected pituitary adenomas were transported directly to the pathology department. Samples were immediately positioned and visualized with CRM using a confocal microscope located in the same area of the pathology department where frozen sections are prepared. An H & E–stained slide was subsequently prepared from imaged tissue. A neuropathologist compared the histopathological characteristics of the H & E–stained slide and the matched CRM images. A second neuropathologist reviewed images in a blinded fashion and assigned diagnoses of adenoma or normal gland.

RESULTS For all specimens, CRM contrasted cellularity, tissue architecture, nuclear pleomorphism, vascularity, and stroma. Pituitary adenomas demonstrated sheets and large lobules of cells, similar to the matched H & E–stained slides. CRM images of normal tissue showed scattered small lobules of pituitary epithelial cells, consistent with matched H & E–stained images of normal gland. Blinded review by a neuropathologist confirmed the diagnosis in 15 (94%) of 16 images of adenoma versus normal gland.

CONCLUSIONS CRM is a simple, reliable approach for rapidly evaluating pituitary adenoma specimens ex vivo. This technique can be used to accurately differentiate between pituitary adenoma and normal gland while preserving biopsy tissue for future permanent analysis, immunohistochemical studies, and molecular studies.

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Intraoperative frozen section analysis is often used during pituitary surgery to establish a diagnosis and to assess margins. Frozen section analysis requires tissue processing and has inherent limitations, such as preparation time, freezing and sectioning artifact, and degradation or loss of tissue for permanent specimen studies.16,17 Each of these limitations is particularly relevant in cases of pituitary microadenomas, because only a small amount of tissue is available for analysis and surgical decision making may depend on the intraoperative diagnosis.

Confocal reflectance microscopy (CRM) is an imaging modality that generates cellular resolution images of biopsy specimens without the need for tissue processing, sectioning, or staining. Tissue samples can be transported directly from the operating room to the confocal microscope, and varying depths of the specimen can be analyzed by changing the focal plane of the microscope. CRM therefore allows real-time analysis of cellular morphology and tissue architecture within a biopsy specimen, and it can provide rapid analysis of the tissue while preserving...
the entire specimen for future permanent-section staining, molecular analysis, and biobanking. This technology is currently used in several fields other than neurosurgery to rapidly assess biopsy specimens and guide surgical decision making.\(^2,6,7,14,15\)

In this study, we evaluated the utility of CRM for rapid assessment of pituitary specimens in the immediate ex vivo period as a proof-of-principle study. To our knowledge, this is the first description of CRM for this application in the medical literature to date.

**Methods**

**Biopsy Specimens**

Patients with CNS tumors undergoing surgery at our institution between February 2014 and November 2015 were prospectively enrolled in our study. This study was approved by the IRB of St. Joseph’s Hospital and Medical Center, in Phoenix, Arizona. Fresh human biopsy specimens were collected in the operating room and transported to the Department of Pathology, where both CRM and diagnostic frozen sections were performed, when applicable. CRM images were collected by team members with training in confocal microscopy (K.Y.G., J.G., and M.I.Y.). Eleven patients who underwent transnasal transphenoidal surgery for suspected pituitary adenoma during this period were included in the study.

**CRM Imaging**

For CRM imaging, our methodology has been previously described.\(^5\) Briefly, biopsy specimens were placed in ice-cold saline in uncoated no. 1.5 glass-bottom dishes (MatTek Corp.). No tissue processing or staining was performed prior to CRM imaging. CRM imaging was performed with a Zeiss inverted LSM 710 laser-scanning confocal microscope (Carl Zeiss AG). Images were acquired with a magnification of 20×/0.8 NA (numerical aperture) air objective and a magnification of 40×/1.2 NA water immersion objective. CRM images were acquired by raster-scanning the sample with a 633-nm diode laser and collecting reflected photons of the same incidence wavelength. The confocal aperture was set to 1 Airy unit. All imaging. The laser and gain values were set to fill the dynamic range of the photomultiplier tube, and the frame size was set to sample at the Nyquist sampling rate (i.e., cycles per unit distance). Images were collected in an 8-bit format. Simulated low-magnification images were generated by using the Zeiss tiling function, which produces a large-volume image by stitching together multiple highly resolved and magnified individual images. After CRM, biopsies were marked and processed for standard histopathological assessment with H & E staining. Frozen sections were performed when an intraoperative diagnosis was required. CRM images were assessed for pathognomonic features and compared with the corresponding H & E images. All image processing was performed using linear functions within ImageJ (US NIH).

**Specimen Analysis**

All biopsy specimens from the 11 patients were compared on the basis of their CRM, frozen section, and H & E–stained features by a neuropathologist (J.M.E.), who noted distinguishing features for descriptive purposes. For the blinded portion of the study, 16 representative CRM images from the 11 cases were selected by the neuropathologist (J.M.E.): 7 images of normal adenohypophysis and 9 images of pituitary adenoma. These images were presented in a blinded fashion to a second dedicated neuropathologist (S.W.C.), who had no prior knowledge of the cases.

**Results**

**Descriptive Features**

CRM images of pituitary adenoma demonstrated sheets of cells with prominent nuclei and brightly reflective cytoplasm. Increased cellularity, tissue architecture, vascularity, and stroma were all readily visualized and were similar to the matched H & E–stained slides (Fig. 1). CRM images of normal pituitary gland showed smaller, more uniform nuclei, as well as scattered small lobules of pituitary epithelial cells, consistent with matched H & E–stained images of normal gland (Fig. 2). Pituitary adenoma subtypes included 4 gonadotroph adenomas, 2 corticotroph adenomas, 1 prolactinoma, and 1 alpha-subunit secreting adenoma; 3 biopsy specimens were from normal pituitary gland.

**Blinded Review**

Fifteen (94%) of the 16 CRM images were correctly identified as either normal pituitary adenohypophysis or pituitary adenoma by the second neuropathologist, who had no prior knowledge of the cases. This neuropathologist incorrectly labeled 1 image of normal gland as adenoma.

**Discussion**

With the increasing trend toward personalized medicine, the importance of patient-specific tumor analysis and molecular characterization of biopsy specimens will continue to expand. This movement requires not only optimized tumor specimens for analysis but also research dedicated to the genetic and molecular characteristics of individual patient biopsy specimens. Indeed, the characterization of pituitary adenomas on the basis of genetic and molecular characteristics, and the link between these markers and clinical behavior, has grown exponentially within the past decade.\(^3,8,11,18\) Given the recent advances in our knowledge in these fields, biopsy specimen preservation and biobanking for research studies are arguably more important now than at any time in the past.

The current standard for intraoperative diagnosis is frozen section analysis, which is limited by prolonged processing time, freezing and sectioning artifact, and degradation or loss of tissue for permanent specimen studies.\(^16,17\) When an intraoperative diagnosis is required and only a small biopsy specimen is available, such as in the case of a pituitary microadenoma, frozen sectioning may jeopardize the ability to perform definitive molecular or genetic evaluation in the future. The ability to obtain an...
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Intraoperative diagnosis with CRM without sacrificing a biopsy specimen circumvents this problem and facilitates definitive diagnostic studies and further molecular characterization.

Confocal microscopy is an imaging modality that generates high-resolution images by spatially filtering visualized photons through a pinhole. This technique allows for the “optical sectioning” of a specimen, wherein various depths of a biopsy sample can be visualized without its physical manipulation. The use of confocal microscopy has also been coupled with the use of fluorescent dyes to achieve rapid histopathological diagnosis in numerous fields, including gastroenterology, dermatology, gynecology, and ophthalmology. Furthermore, confocal microscopy has been used intraoperatively with intravenous dyes to visualize tumors of the CNS in vivo. In contrast, CRM utilizes incident light, rather than high-energy fluorescence, and it provides a label-free assessment of biopsy specimens without tissue manipulation. We recently reported on the utility of CRM for assessment of the cellularity of high-grade glioma specimens prior to biobanking, as well as on the preservation of molecular integrity within biopsy specimens with this technique. The goal of the current study was to expand upon the utility of this emerging technology and to outline its particular advantages for use in the diagnosis of pituitary adenoma specimens.

Several key aspects of pituitary adenoma histopathology allow for its definitive diagnosis on frozen-section and permanent histology. Pituitary adenomas grow as sheets of proliferative cells and demonstrate increased cellularity compared with sections of normal pituitary gland (Figs. 1 and 2). These key features were readily observed on CRM after specimens were transferred directly from the operating room. Although CRM does not reach the level of cellular resolution and tissue contrast observed with H & E staining, features necessary for diagnosis of normal pituitary versus adenoma were apparent to both neurosurgeons and neuropathologists. The ability of CRM to reveal the diagnosis rapidly and preserve the entire specimen for further permanent sectioning and molecular analysis prompted us to perform this study and report these findings.

In this proof-of-principle study, we tested a second neuropathologist with no prior knowledge of the cases and limited CRM experience (S.W.C.). He was able to correctly distinguish between pituitary adenoma and normal gland in 15 (94%) of 16 CRM images. One image of normal gland was labeled as adenoma in this portion of the study; however, the remainder of the images were correctly identified. It is important to note that this study did not address the sensitivity and specificity of CRM for pituitary adenoma diagnosis, and future studies should be designed to address these outcomes.

Although our results demonstrate the utility of this novel technology, the study does have limitations. The main limitation is that all 11 specimens were evaluated prospectively by the senior author (J.M.E.) and 16 representative images were selected for blinded review by the second neuropathologist (S.W.C.). While this study design demonstrates the utility of CRM for intraoperative adenoma diagnosis, it does not provide a direct comparison with frozen sectioning. Future blinded studies of CRM compared with frozen sectioning for intraoperative pituitary adenoma assessment are warranted.

The study also did not assess the time to diagnosis for intraoperative CRM. Although our recent experience using CRM for this application demonstrates a substantial savings in time compared with frozen sectioning, diagnosis by CRM is associated with a learning curve, and analysis of early specimens required substantially more time than specimens later in our series. This learning curve precluded our ability to include time to diagnosis as an outcome in this study. Future studies of CRM for pituitary adenoma should include this outcome measure when comparing this technology to frozen sectioning.

Conclusions

CRM is a simple and reliable method for the rapid evaluation of pituitary adenoma specimens ex vivo and offers several advantages over the current practice of frozen sectioning. This technique can accurately distinguish between pituitary adenoma and normal gland, while preserving the entire specimen for further permanent analysis, immunohistochemical studies, and molecular studies. This technology may have an increasing role in pituitary adenoma surgery as the need for genetic and molecular characterization of individual tumors increases.
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References


Disclosures

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Author Contributions

Conception and design: Eschbacher, Mooney, Georges, White, Little, Nakaji. Acquisition of data: Eschbacher, Mooney, Georges, Yazdanabadi, Goehring, Coons. Analysis and interpretation of data: Eschbacher, Mooney, Preul, Nakaji. Drafting the article: Eschbacher, Mooney, Georges, Nakaji. Critically revising the article: Nakaji. Reviewed submitted version of manuscript: Eschbacher. Administrative/technical/material support: Goehring, Preul. Study supervision: Eschbacher.

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