Confocal Reflectance Imaging of Head and Neck Surgical Specimens

A Comparison With Histologic Analysis

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Background: Confocal reflectance microscopy (CRM) is an optical method of imaging tissue noninvasively without the need for fixation, sectioning, and staining as in standard histopathologic analysis. Image contrast is determined by natural differences in refractive indices of organelles and other subcellular structures within the tissues. Gray-scale images are displayed in real time on a video monitor and represent horizontal (en face) optical sections through the tissue. We hypothesized that CRM is capable of discerning histologic characteristics of different tissues in the head and neck.

Objectives: To examine the microscopic anatomy of freshly excised head and neck surgical specimens en bloc using CRM and to compare the findings with those generated by conventional histologic analysis.

Design: This was a pilot observational cohort study.

Bone, muscle, nerve, thyroid, parotid, and ethmoid mucosa from human surgical specimens were imaged immediately after excision. Confocal images were compared with corresponding routine paraffin-embedded, hematoxylin-eosin–stained sections obtained from the same tissue.

Results: Characteristic histologic features of various tissues and cell types were readily discernible by CRM and correlated well with permanent sections. However, in all tissues examined, there was less microscopic detail visible in the CRM images than was appreciated in paraffin-embedded histologic sections.

Conclusions: The CRM images revealed cytologic features without the artifacts of histologic processing and thus may have the potential for use as an adjunct to frozen-section analysis in intraoperative consultation.

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Pathologic examination of tissue during a surgical procedure is typically performed by frozen-section preparation and subsequent observation under a light microscope. Studies have appeared frequently in the literature documenting the high accuracy rate of frozen-section analysis. However, frozen sections are technically limited and often more difficult to interpret than paraffin-embedded permanent sections. Frozen-section preparation is also time consuming because of the tissue processing involved (freezing, sectioning, staining of the specimen, difficulties in obtaining an adequate thin section, and the possible need for additional frozen sections). These delays can contribute to increased operative time and cost and could potentially put the patient at risk for additional morbidity.

Confocal reflectance microscopy (CRM) is capable of noninvasive high-resolution imaging of tissue in its native state. A confocal microscope differs from a conventional light microscope because it uses a point source of light, typically a laser, to illuminate a small spot that can be scanned within tissue. By spatially filtering the reflected laser light through a pinhole, an image is created from a thin plane of interest that is in focus. Light from tissue superficial or deep to the focal plane is rejected. In this manner, thin optical sections of biological specimens can be imaged by CRM at resolutions similar to those of conventional light microscopy.

Several studies have demonstrated the use of CRM for imaging human and animal tissues in vivo. To date, the most clinical utility has been demonstrated in ophthalmology, where CRM has been used to image the retina and cornea. Confocal reflectance microscopy has also shown clinical potential in dermatology, where the skin is easily accessible for imaging. It has been used in vivo to monitor response of the skin to laser therapy and to define the margins of basal...
cell cancer during Mohs surgery. Confocal reflectance microscopy has also been reported by our laboratory as a potential in vivo screening tool for the early malignant conversion of oral precancer and to evaluate benign tumors of the parathyroid. In the present article, we describe CRM as a technique for rapid observation of surgically excised, fresh, unfixed, and unstained tissue. A confocal microscope built in our laboratory was used to image normal tissues of the head and neck. Confocal images were compared with corresponding standard histologic sections of the same tissues. The goals of our study were to (1) determine if CRM imaging could resolve the architectural and cellular morphologic patterns normally observed by routine histologic analysis and (2) increase our understanding of the interpretation and analysis of confocal images. This preliminary study on normal tissues serves as the foundation for studying pathologic tissues of the head and neck.

METHODS

TISSUES

Tissue specimens from the head and neck were obtained under a Massachusetts General Hospital protocol approved by the institutional review board for handling of pathologic tissue. Tissue specimens included (1) 3 mandibular bone specimens; (2) 3 skeletal muscle specimens from the floor of the mouth; (3) 5 auriculotemporal nerve specimens; (4) 5 parotid gland specimens; (5) 7 thyroid gland specimens; and (6) 4 pieces of nasal mucosa. Freshly excised tissues were transported immediately to the pathology lab. After adequate frozen sections had been obtained, the remaining tissue was imaged en bloc by CRM. Thus, our confocal imaging did not disrupt the work flow of the surgical pathology laboratory. After imaging, the tissue specimens were submitted for paraffin embedding and permanent-section hematoxylin-eosin staining. Peripheral nerve specimens were processed with Bodian stain specific for myelin.

CONFOCAL REFLECTANCE MICROSCOPE

Details of the optical design of the confocal microscope and images of normal human skin and oral mucosa have been published previously. A near-infrared illumination wavelength of 1064 nm from a neodymium:YAG laser (model CV4; Santa Fe Laser Company, Tucson, Ariz) was used at 30 mW of power at the level of the tissue. A ×30, 0.9–numerical aperture water immersion objective lens provided a measured lateral resolution of 1 mm and a virtual tissue section thickness (axial resolution) of 4 mm. The lateral resolution is the nearest distance between 2 neighboring points or features that can be resolved in the image, and the axial resolution defines the optical section thickness of tissue from which light is detected at any given time. The axial resolution compared favorably with the thickness (typically 5 mm) of paraffin-embedded sections in standard histologic analysis. A tissue holder was developed to secure surgical specimens during imaging and limit the relative motion between the tissue and the objective lens.

Confocal images displayed on a video monitor showed the tissue in the horizontal plane, parallel to the tissue surface. The images were videotaped at 30 frames per second and then digitized using an 8-bit capture board (Pixelpipeline; Perceptics Corporation, Knoxville, Tenn) and image acquisition software on a Macintosh Quadra 840 AV (Apple Computer Inc, Cupertino, Calif). The images were enhanced, primarily to remove electronic noise imparted by videotaping, by scaling with bi-linear interpolation, linear filtering, and contrast adjustment (IP Lab Spectrum, version 3.1; Scanalytics Inc, Fairfax, Va). After enhancement, confocal images were qualitatively correlated with companion routine paraffin sections stained with hematoxylin-eosin.

RESULTS

Confocal images of surgically excised tissue were acquired along with their corresponding permanent hematoxylin-eosin–stained sections. The confocal images were obtained to a depth of up to 400 µm on intact surgical specimens immediately after excision.

Confocal images of bone showed several structures characteristic of Haversian systems. In Figure 1A, a “bullets-eye” structure is seen in the right half of the image surrounding a central bright cylinder. The central bright structure (labeled “V”) is a blood vessel; erythrocytes were seen within the cylinder when the imaging was performed in real time. Encircling this structure were multiple, thin, bright layers that corresponded to the concentric bone lamellae in the histologic preparation (Figure 1B). Several bright star-shaped structures were seen surrounding the central vessel. These corresponded to the nuclei of osteocytes contained within lacunae (Figure 1B).

The confocal image of skeletal muscle (Figure 2A) showed elongated gray bands against a black background. In some areas, elliptical dark structures with central bright dots could be seen wedging between the bands. These structures correspond to the basophilic peripheral nuclei of the skeletal muscle fibers in the histologic section (Figure 2B). Dark lines could be seen at fairly regular intervals running perpendicularly across the gray bands in the confocal image (Figure 2A). The diameter of the muscle fibers was approximately 50 mm in the confocal image, which correlated well with the histologic preparation.

The nerve appeared in the confocal image as a dark tubular structure that contained alternating bright and dark bands (Figure 3A). Axons appeared as dark structures between 2 surrounding layers of bright myelin. In the histologic section of a peripheral nerve stained with Bodian stain specific for myelin (Figure 3B), fascicles of myelinated nerve fibers in longitudinal section were seen surrounded by connective tissue stroma. The epineurium, composed of loose connective tissue, could also be seen as a dark layer surrounding the nerve in the confocal image.

Confocal images of the parotid gland sample (Figure 4A) appeared to be predominately composed of gray round structures separated by dark winding bands. The round structures correspond to the secretory alveoli, consisting of serous cells arranged around a central small lumen, which was barely visible, and striated ducts (Figure 4B). The dark background in the confocal image corresponds to the connective tissue septae that divide each lobule. Several large, bright polygonal cells could be seen interspersed within the gland, which correlated to fat cells in the histologic section (Figure 4B).

The confocal image of normal thyroid tissue (Figure 5A) revealed multiple dark round structures encircled by tightly spaced bright dots. When the optical section was moved through the intact tissue, it became apparent that these structures were spherical. These
spherical structures correspond to thyroid follicles containing colloid, and the surrounding bright dots represent nuclei of the follicular cells. Interfollicular connective tissue was barely visible, and parafollicular C cells of the thyroid were also difficult to distinguish. In the histologic section, follicles of varying sizes were lined by flattened follicular cells and colloid with focal dense areas, which correspond to inspissated colloid (Figure 5B).

The confocal images of nasal mucosa (Figure 6) showed a pseudostratified columnar ciliated epithelium containing columnar cells, goblet cells, and basal cells. Figure 6A shows a vertical section similar to a standard histologic study (Figure 6B) because the excised mucosal tissue was folded 90° from its surface and placed in contact with the objective. The cilia, as well as the basal bodies (centrioles) from which they arose, appeared bright. Interestingly, when imaging was performed in real time on the excised en bloc tissue, active ciliary motion was observed. Focally, goblet cells were identified between columnar cells by the presence of mucin in each, which appeared as individualized, round, and well-circumscribed bright structures. The nuclei of the basal and columnar cells appeared gray. Scattered, small, bright round cells, which appeared morphologically to be lymphocytes, were seen percolating through the epithelium. The lamina propria consisted of gray connective tissue, fibroblasts with bright nuclei, and blood vessels.

In this study, we investigated the use of CRM as an alternative to conventional histologic analysis to noninvasively visualize and distinguish several different tissues. For each specimen, characteristic histologic features of various tissues and cell types were readily discernible by CRM and correlated well with routine histologic findings. In most tissues, there was less histologic and cytologic detail in the confocal images than was seen in the

**Figure 1.** Comparison images of normal bone. A, The confocal image shows a Haversian system containing a central canal with surrounding concentric lamellae (L). The multiple bright spots are osteocytes (arrows) contained within lacunae. A Volkmann canal (V) is seen piercing the Haversian canal at a right angle (objective lens ×30). B, In the conventional histologic image, a Haversian canal (C) is seen in the center. Densely stained osteocytes (arrows) are seen as well as multiple empty lacunae. Concentric lamellae (L) are seen encircling the Haversian canal (hematoxylin-eosin, objective lens ×40). The white scale bar in panel A and the bracketed scale bar in panel B indicate 100 µm.

**Figure 2.** Comparison images of normal skeletal muscle. A, In the confocal image, fibers are seen as parallel, horizontal, gray bands with peripheral bright nuclei (N) and dark transverse striations (arrows) (objective lens ×30). B, In the conventional histologic image, several skeletal muscle fibers show peripheral nuclei (N). Multiple transverse striations (arrows) are also seen (hematoxylin-eosin, objective lens ×40). The white scale bar in panel A and the bracketed scale bar in panel B indicate 100 µm.
histologic sections. Some microscopic structures were more easily visualized with CRM than with histologic analysis, such as transverse striations in skeletal muscle (Figure 2A) and adipose cells in the parotid gland (Figure 4A); others were more difficult to visualize with CRM, such as the parafollicular cells of the thyroid (Figure 5A).

Variation in brightness of structures imaged by CRM is a function of the light-scattering properties of the tissues. Image contrast is due to local natural variations in refractive indices of various tissue substructures. Lipids, such as the phospholipids making up myelin, have a high refractive index and scatter light, making the myelin appear as bright layers surrounding dark axons in the confocal image (Figure 3A). Similarly, the cytoplasm of fat cells (Figure 4A) in the parotid gland appears bright owing to the high lipid content. Structures with similar refractive indices appear dark, such as the homogeneous colloid substance of the thyroid follicle (Figure 5A), which is composed of a glycoprotein complex called thyroglobulin. Axons in a peripheral myelinated nerve (Figure 3A), which are composed of numerous neurofilaments, microtubules, and mitochondria, also appear dark relative to their myelin sheath. An in-depth understanding of the factors that contribute to image contrast (ie, tissue optics) is still lacking, and these factors will need to be investigated further.

One potential advantage of CRM is the elimination of the drawbacks associated with tissue processing. For example, assessing the adequacy of tumor removal during a composite resection of the oral cavity is limited by the ability to evaluate bone margins. Bone is usually not evaluated by frozen-section analysis since the calcified tissue cannot be sectioned with a microtome. Pathologic evaluation of bone requires that it first be decalcified, which can take days, and so this procedure is not practical for intraoperative use. With CRM, rapid imaging of bone is possible with-
out processing (Figure 1A), albeit at a limited depth, and may be used to provide immediate feedback to the surgeon. To view the lipid substance of tissues in frozen section, special staining procedures must be used (eg, oil red O), and paraffin-embedded tissue cannot be stained for fat, since the fixative solvent removes it. With CRM, the parotid gland can be imaged without dissolution of lipid (Figure 4A). Other artifacts in frozen-section analysis include enlargement of cells due to the freezing process, shrinkage (causing empty spaces or separation of tissue layers), distortion during excision, folds or wrinkles, uneven staining, precipitate particles, and degeneration of cells structure including shrinkage of nucleoli.4

However, confocal imaging may introduce its own artifacts, which are primarily optical effects and are not related to tissue processing or alteration. Optical artifacts may include (1) bright or dark spherical disks on the tissue surface due to bubbles or dirt in the immersion medium; (2) bright dots in the center of nuclei due to back-reflection of the illuminating cone of light from the underlying spherical nuclear membrane; and (3) interference fringes or “aliasing effects” from closely spaced structures within the tissues.8

Additional challenges to confocal imaging remain. Imaging with CRM can be performed to approximately a depth of half a millimeter, which limits imaging of invasive tumors en bloc or in vivo. However, the surgical margin is available at the surface of either resected tissue samples or in the surgical field. Also, this depth of imaging is more than adequate to quickly scan tissue sectioned for frozen

Figure 5. Comparison images of normal thyroid tissue. A, The confocal image shows multiple follicles of varying sizes lined by bright follicular cells (short arrows) and filled with dark colloid (C). There is scant interfollicular connective tissue. Insipissated colloid (long arrow) is also visible with confocal microscopy (objective lens ×30). B, The conventional histologic image shows that the thyroid follicles are lined with a simple cuboidal epithelium (short arrows) or follicular cells. The follicles contain a homogenous material called colloid (C). Within the colloid, insipissated colloid (long arrow) can be seen, which occurs commonly with aging (hematoxylin-eosin, objective lens ×40). The white scale bar in panel A and the bracketed scale bar in panel B indicate 100 µm.

Figure 6. Comparison images of normal nasal mucosa. A, The confocal image shows a pseudostratified columnar ciliated epithelium containing columnar cells, goblet cells, and basal cells resting on a basement membrane. The cilia (long arrow) appear bright, as do the basal bodies (centrioles) from which they arise. A sharp distinction is apparent between the epithelium (e) and the submucosa (sm) containing serous glands (s). Focally, goblet cells are identified between columnar cells by the presence of mucin, which appears as individualized, round, and well-circumscribed bright structures. Scattered small bright round lymphocytes (short arrow) are seen in the submucosa (objective lens ×30). B, In the conventional histologic image, the nasal mucosa appears as ciliated, pseudostratified columnar epithelium (e). Within the epithelium are numerous interspersed goblet cells. The epithelium rests on a prominent basement membrane, and the underlying submucosa (sm) contains many serous (s) and mucous glands. Overlying the epithelium are numerous cilia (long arrow) (hematoxylin-eosin, objective lens ×40). The white scale bar in panel A and the bracketed scale bar in panel B indicate 100 µm.
analysis prior to embedding to ensure proper sampling of the lesion. Confocal reflectance microscopy does not involve the use of stains, but scattering agents may be useful to increase contrast and highlight structures in the tissues. Confocal images may be difficult for the untrained clinician to interpret, similar to ultrasound images, and additional training may be required. In the present study, only formalin-fixed paraffin-embedded, hematoxylin-eosin–stained sections were imaged with CRM. Studies should be performed to characterize different tumors in the head and neck by CRM and to determine how and to what extent disease alters tissue optics.

Since confocal imaging can easily be performed in vivo or on fresh surgically excised tissues (ex vivo), there are numerous potential applications for CRM as an intraoperative imaging tool or as an adjunct to standard surgical tissue histopathologic analysis. Intraoperatively, a large area of tissue could be scanned in a time-efficient manner with CRM, possibly improving the accuracy of margin selection and also increasing the chance of identifying tumor “skip areas.” Identifying the surgical margin in vivo could provide quick feedback to the surgeon and decrease the need for multiple frozen sections. Confocal reflectance microscopy may also be useful for tissue identification and differentiation intraoperatively. For example, live confocal imaging of blood flow in the oral mucosa is striking, and vessels can easily be distinguished from nerves in the lamina propria.

Confocal reflectance microscopy could also provide a means of examining tissue specimens prior to cutting for frozen-section analysis, possibly to quickly differentiate mucosa from submucosa to ensure that a specimen is properly embedded—a judgment often difficult to make with the unaided eye. Studies have shown that a large number of errors in frozen-section analysis are due selection of nonrepresentative tissue (ie, sampling error). Obtaining multiple frozen sections has been suggested as a way to reduce errors in sampling, but this further prolongs the surgical procedure and increases the cost incurred by the patient.

It is unlikely that confocal imaging will replace frozen-section analysis, but its ability to image patients in vivo or excised tissues without histologic processing makes CRM useful. It probably has the greatest potential as an adjunct to frozen-section analysis, providing an additional armamentarium to the pathologist and surgeon for intraoperative consultation. This new imaging technique, if proven effective, may in some instances reduce the need for multiple frozen sections, thereby decreasing the “wait time” for the surgeon, total operative time, cost, and the risk of morbidity to the patient.

In conclusion, the present work demonstrates that intact, fresh surgical tissue can be rapidly imaged by CRM with high resolution and contrast such that microscopic anatomy can be delineated. Confocal images of freshly excised surgical specimens were compared with paraffin-embedded, hematoxylin-eosin–stained sections. We were able to distinguish the histologic characteristics of various tissue types with CRM and document good correlation between CRM and conventional histologic analysis. An in-depth correlation of confocal images to standard histopathologic findings of both normal and diseased tissue is essential to further validate this diagnostic imaging modality.

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