In vivo intraoperative confocal microscopy for real-time histopathological imaging of brain tumors

Clinical article

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Object. Frozen-section analysis is the current standard for the intraoperative diagnosis of brain tumors. Intraoperative confocal microscopy is an emerging technology with the potential to visualize tumor histopathological features and cell morphology in real time. The authors report their findings using this new intraoperative technology in vivo with sodium fluorescein contrast during the course of 50 microsurgical tumor resections.

Methods. Eighty-eight regions were visualized with confocal microscopy, and corresponding biopsy samples were examined with routine neuropathological analysis. The tumors studied included meningiomas, schwannomas, gliomas of various grades, and a hemangioblastoma. The confocal microscopic features of each tumor and of various artifacts inherent to the technology were documented. A pathologist working in a blinded fashion reviewed a subset of the images in a further evaluation of the usefulness of the device as a diagnostic tool.

Results. Overall, intraoperative confocal imaging correlated surprisingly well with corresponding traditional histological findings, including the identification of many pathognomonic cytoarchitectural features of various brain tumors. In the blinded study, 26 (92.9%) of 28 lesions were diagnosed correctly.

Conclusions. Further study will be necessary for better definition of the role of intraoperative confocal microscopy as a routine adjunct for intraoperative brain tumor diagnosis.

**Key Words** • brain tumor • confocal microscopy • glioma • meningioma • frozen-section diagnosis • oncology

Histopathology plays an important role in the neurosurgical setting, where diagnosis guides treatment. Intraoperative frozen-section analysis can provide a critical roadmap for the neurosurgical oncologist by establishing a preliminary diagnosis. In some cases, a frozen section can help assess tumor margins, indicate the need for microbial cultures, and suggest a site for final specimen selection. Nevertheless, persistent challenges impede the reliability of intraoperative frozen-section consultation. Specifically, the necessary delay in specimen preparation and interpretation, misdiagnoses due to sampling error, and/or frozen-section artifacts remain ongoing problems. An alternative intraoperative strategy for real-time, in vivo histological analysis without tissue manipulation or resection could be a useful adjunct for both neuropathologists and neurosurgical oncologists.

Intraoperative confocal microscopy adapts conventional confocal microscope technology to provide high-resolution subsurface imaging of living tissue in vivo, allowing visualization of cellular elements and cytoarchitecture. In humans, intraoperative confocal microscopy performed using intravenous fluorescein can distinguish the histological features of some tumor types in vivo. In nonneurosurgical fields, this technology has already been integrated into the tissue selection process for biopsy procedures, where it can limit the number of samples needed for diagnosis. Clinical fields that have adopted its use include gastroenterology, urology, and gynecology.

The technology has now been adapted for neurosurgery in the form of a handheld, 190 × 6.6–mm, sterilizable, 135°-angled, rigid probe that displays images in real time on an attached external monitor at magnifications of up to 1000. A foot switch enables operator control of the focal plane position over a range of 250 μm. In an experimental rodent model, using intravenous fluorescein and topical acriflavine dyes, intraoperative confocal microscopy can detect histological features of glioblastomas, identifying cellular shape and tissue architectural features correspond-
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...ing to tumor infiltration. 11 Our group has reported the feasibility results obtained in 33 patients with an earlier generation of the confocal probe used in the current paper. 10 None of those patients or their data are duplicated in the current study. We now report the first in-depth analysis of the histopathological features observed using intraoperative confocal microscopy for common brain tumors, in an effort to delineate its utility and to suggest future studies.

Methods

This study was conducted at the Barrow Neurological Institute of St. Joseph’s Hospital and Medical Center, with approval from St. Joseph’s institutional review board.

Patient Selection

In 2010, 1253 patients with a brain tumor underwent microsurgical resection at our institution. This report describes findings in 50 patients who were selected for examination with intraoperative confocal microscopy during the course of their resection. Four neurosurgeons (R.F.S., P.N., K.A.S., and N.S.) participated in this study. All patients gave informed consent. Patients younger than 18 years of age, those who were pregnant, and those with a known allergy to fluorescein were excluded. Patients being treated with angiotensin-converting enzyme inhibitors and beta blockers were also not enrolled.

Intraoperative Confocal Microscope

The unit consists of a miniaturized confocal microscope scanner encased in a rigid probe that is connected via a flexible umbilicus to an optical unit and control PC unit. The scanner is based on single-fiber scanning technology in which a single optical fiber acting as both an illumination and detection aperture is used to enact the confocal imaging principle. The fiber is raster-scanned behind a miniature objective lens that projects the scan through a window at the distal end of the probe that is in contact with the tissue. An integrated depth actuator enables the scan to focus to a specific depth in the tissue that is operator controlled in 4-μm steps over a range of 0–250 μm beneath the contact window plane. The scanned field of view is 475 × 475 μm, with a lateral resolution of 0.7 μm and an axial resolution (that is, effective optical slice thickness) of approximately 7 μm. One-megapixel scans are collected at frame rates of 0.8 frames/second. The confocal microscope’s solid-state laser emits a 488-nm violet-blue excitation light, with an incident power of up to 1 mW. Detected light is filtered to the red-violet spectrum via a 605-nm bandpass filter.

Neuropathological Analysis

Biopsy specimens were sharply excised under microscopic visualization at each imaging site by using a combination of scalpels and microspatula. Specimens were immediately marked with tissue ink to indicate superior and inferior surfaces, placed on sterile Telfa (Covidien, Inc.), and divided into specimens for formalin fixation and snap freezing. Intraoperative MR neuronavigation was used to record the location of each specimen in 3D coordinates. Subsequently, specimens were cut into 5-μm sections and stained with H & E for histopathological analysis. Care was taken to ensure accurate orientation of the tissue throughout the handling and staining process so that confocal microscope images and histological sections from the same area could be compared directly. Central neuropathology review was performed by 2 neuropathologists (J.E. and S.W.C.) based on WHO guidelines. When appropriate, special stains were used to establish a definitive diagnosis.

Operative Protocol

Intraoperatively, all patients underwent routine microsurgical resection with StealthStation Treon neuronavigation (Medtronic, Inc.). At the time of tumor exposure, 25 mg of sodium fluorescein was administered intravenously to each patient intraoperatively. Two to 5 minutes after the injection, the surgeon placed the confocal microscope probe on the surface of the tumor. Images were visible in black and white on a nearby video console. The surface of the tumor was washed with sterile saline to eliminate erythrocyte contamination. Total imaging time ranged from 2 to 10 minutes. After each site was studied, its location was recorded using image guidance, and a corresponding biopsy sample was taken for frozen and permanent pathological analysis.

Blinded Study

A neuropathologist who lacked significant prior experience with interpretation of endoscopically obtained confocal images reviewed a tutorial set of matched H & E–stained and confocal images. A set of 28 images without matched H & E–stained images was then reviewed. The images included information about tumor location and enhancement (for example, enhancing mass, left temporal region) but not the final diagnosis. Tumor types included cellular glioma of different grades (9 samples), infiltrating edge of glioma (2), schwannoma (2), meningioma (4), motion/blood artifact (6), treatment effect/reactive process (3), and normal brain (2) (Table 1). The neuropathologist was asked to determine if the image represented cellular tumor. Depending on this determination, the neuropathologist was then directed toward the appropriate list of specific diagnoses from which to choose (Fig. 1).

Results

A summary of all of the tumors analyzed in this paper is included in Table 2.

Tumor Types

Meningiomas. Twenty-four meningiomas were examined intraoperatively. On routine H & E staining, 20 meningiomas were designated as Grade I and 4 as Grade II. Grade I meningiomas included meningothelial (3), transitional (12), and fibrous subtypes, with a total of 30 biopsies from these 20 tumors. The concordance rate in histological diagnosis between confocal imaging and H & E–stained sections was 27 (90%) of 30. Three studies from 3 Grade
I meningiomas did not demonstrate useful or interpretable images due to motion and/or blood artifact (Fig. 2). One of these patients demonstrated interpretable confocal images from another region, so that diagnostic information was obtained in 18 (90%) of 20 patients.

Analysis of confocal images of meningothelial, transitional, and fibrous meningiomas showed histological patterns characteristic of their subtype. Refractile fibers identified on confocal images corresponded with collagen observed on H & E–stained sections (Fig. 3A and B). Psammoma bodies were characterized as spherical, anuclear, whorled structures on both confocal and H & E sections (Fig. 3C and D). Dural invasion by small nests of tumor was visible on confocal images in 1 meningioma, consistent with the matched H & E slide. Overall, the majority of tumor cells did not show evaluable nuclear detail. However, many cells demonstrated probable nuclei, which appeared as dark shadows contrasted against a lighter background of cytoplasm and fluorescein. Five tumors showed cells with intracellular round-to-oval clear centers. These structures appeared to correspond with nuclear pseudoinclusions on H & E sections. Tumor vasculature was highlighted by intravascular fluorescein.

Five biopsy samples from 4 Grade II meningiomas were examined. On H & E sections, these tumors demonstrated 3 or more atypical features according to WHO criteria. All 4 atypical meningiomas demonstrated diffuse growth patterns on both H & E sections, and similar patterns on confocal images. Overall, confocal images of Grade II meningiomas demonstrated a greater population of enlarged atypical cells compared with Grade I meningiomas. One meningioma also appeared hypercellular on both the H & E section and the matched confocal image. Features evident on H & E sections but not observed on confocal matched images included prominent nucleoli and small cell foci.

Schwannomas. Five biopsy samples from 4 tumors were compared. One case demonstrated uninterpretable images due to blood and motion artifact. The remaining 4 biopsies demonstrated fascicles of cells with elongated cytoplasmic processes that correlated with Antoni A regions on H & E sections. Vague nuclear detail could be appreciated and consisted of dark oval intracytoplasmic structures that conformed with cell shape. Neither H & E sections nor confocal images exhibited necrosis, mitotic figures, or significant cytological atypia for any of the schwannomas.

Low-Grade Gliomas. Fifteen biopsied regions from 8 patients with low-grade gliomas were evaluated, including 4 WHO Grade II astrocytomas and 4 WHO Grade II oligodendrogliomas (Fig. 4). Five imaged regions from 5 patients were not interpretable due to blood and motion artifact. Imaging on 2 of these patients produced additional interpretable images from other regions. The remaining 10 visualized regions demonstrated infiltrative and cellular tumor that corresponded to H & E sections. The degree of tumor cellularity on H & E coincided with the imaged region: less cellular tumor on H & E appeared hypocellular on confocal images and moderately cellular tumor on H & E appeared of similar cellularity on confocal images.

Cellular atypia appeared mild to moderate, consistent with matched histological sections. Morphologically, tumor cells of astrocytic origin demonstrated increased pleomorphism compared with those of oligodendrogial origin. Astrocytic cell bodies appeared more elongated than neoplastic oligodendrocytes, which appeared round to slightly oval, mirroring their H & E images. Astrocytomas overall appeared less cellular and more diffuse than oligodendrogliomas. An infiltrating tumor edge, characterized by scattered atypical cells in a paucicellular, variably gray-to-bright background, was often appreciated.

Questions:

Is this image compatible with cellular tumor? If yes, go to question A. If no, go to question B.

A. Is this cellular tumor? If so, which tumor diagnosis best fits the image?
   a. meningioma
   b. glioma
      - astrocytoma
         - favors low grade
         - favors high grade (includes gliosarcoma)
      - oligodendroglioma
         - favors low grade
         - favors high grade
   c. schwannoma

B. The image is most compatible with:
   - infiltrating edge of glioneuronal
   - treatment effect
   - reactive process
   - blood
   - motion artifact
   - normal brain

Fig. 1. Test questions that accompanied the images in the blinded study.

### Table 1: Number of correct diagnoses of 28 confocal images

<table>
<thead>
<tr>
<th>Diagnosis</th>
<th>No. w/ Correct Diagnosis/Total No. of Cases</th>
</tr>
</thead>
<tbody>
<tr>
<td>cellular tumor</td>
<td>15/15</td>
</tr>
<tr>
<td>meningioma</td>
<td>4/4</td>
</tr>
<tr>
<td>schwannoma</td>
<td>2/2</td>
</tr>
<tr>
<td>glioma</td>
<td>9/9</td>
</tr>
<tr>
<td>astrocytoma (total)</td>
<td>4/4</td>
</tr>
<tr>
<td>favored low grade</td>
<td>2/2</td>
</tr>
<tr>
<td>favored high grade</td>
<td>2/2</td>
</tr>
<tr>
<td>oligoastrocytoma†</td>
<td>0/0</td>
</tr>
<tr>
<td>oligodendroglioma (total)</td>
<td>4/4</td>
</tr>
<tr>
<td>favored low grade</td>
<td>1/1</td>
</tr>
<tr>
<td>favored high grade</td>
<td>3/3</td>
</tr>
<tr>
<td>ependymoma</td>
<td>1/1</td>
</tr>
<tr>
<td>glioma, infiltrating edge</td>
<td>2/2</td>
</tr>
<tr>
<td>treatment effect</td>
<td>1/1</td>
</tr>
<tr>
<td>reactive process</td>
<td>2/2</td>
</tr>
<tr>
<td>blood</td>
<td>2/3</td>
</tr>
<tr>
<td>motion artifact</td>
<td>3/3</td>
</tr>
<tr>
<td>normal brain tissue</td>
<td>1/2</td>
</tr>
</tbody>
</table>

* Interpreted by a neuropathologist working in a blinded fashion.
† Although oligoastrocytomas were presented as a diagnostic choice in the blinded study, no images were provided, and correctly, none were selected.

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Perinuclear halos, a fixation artifact often seen in oligodendrogliomas, were not evident on confocal images.

**High-Grade Gliomas.** Twelve high-grade gliomas were imaged for a total of 30 biopsied and imaged regions. High-grade gliomas included 1 anaplastic astrocytoma (1 biopsy), 4 anaplastic oligodendrogliomas (11 biopsies), 3 anaplastic oligoastrocytomas (9 biopsies), and 4 glioblastomas (9 biopsies). The anaplastic astrocytoma demonstrated a moderately cellular tumor consisting of diffusely distributed atypical cells on confocal microscopy. These imaging features were consistent with the H & E findings. Rare multinucleated cells, present on H & E sections, were not identified on confocal images. Interestingly, the background brain parenchyma did not demonstrate significant contrast with fluorescein. On H & E, however, the tumor cells were distributed in a diffuse myxoid matrix, which may have affected the permeability of the fluorescein.

Anaplastic oligodendrogliomas tended to show clear images with excellent clarity. Of the 11 sets of images analyzed, all showed excellent correlation with their H & E sections (Fig. 5). Cellular atypia was evident on confocal images and matched that of H & E images. Morphologically, the neoplastic cells consisted of irregular gray to dark gray cell bodies. On confocal images, tumor cellularity appeared to correlate on all images with the H & E slide. Necrosis was not seen in any H & E or confocal images for this subtype. Five tumors included an infiltrating edge that was visible on confocal images. The infiltrating borders demonstrated less enhancement of the brain parenchyma (presumably reflecting less edema and fluorescein leakage) than regions of cellular tumor; however, cellular cytological features were nonetheless appreciable. In 2 cases perineuronal satellitosis was evident at the infiltrating edge, and corresponded with the matched H & E sections. Microvascular proliferation was not evident on routine pathological investigation or confocal microscopy.

Three anaplastic oligoastrocytomas (9 biopsies), including 2 recurrent tumors, were also imaged. One set of images from the primary nonrecurrent tumor did not show identifiable tumor cells on confocal images, although cellular tumor was seen on the H & E sections. The remaining 8 sets of confocal images demonstrated similar features to...
those seen on the matched histological sections. Tumor cells appeared enlarged, round to oval in shape, and markedly atypical. Processes were not appreciated. Necrosis, identifiable on both confocal and H & E images in 1 biopsied case, appeared as acellular zones of debris.

Of 4 glioblastomas (9 biopsies), 2 were primary tumors (1 gliosarcoma, 1 glioblastoma with oligodendrogial component) and 2 were recurrent tumors. The gliosarcoma demonstrated fascicles of markedly atypical elongated tumor cells, which appeared dark against a bright fluorescent background. Prominent collagen was appreciated adjacent to tumor. A second imaged region also showed necrosis. The glioblastoma with an oligodendrogial component demonstrated markedly atypical tumor cells, with occasionally prominent pleiomorphic nuclei. Acellular zones surrounded by regions of hypercellularity appeared to correlate with pseudopalisading necrosis seen on the matched H & E section. None of the images or matched H & E sections showed microvascular proliferation, although these features were observed in additional tissue submitted for permanent section.

Ependymomas. Two regions were imaged from 1 ependymoma (WHO Grade II) located in the fourth ventricle. The ependymoma demonstrated discrete clusters of dark cell bodies around vessels bearing bright intraluminal fluorescein (Fig. 6). Acellular zones were visible between cell bodies and the vessel wall, consistent with processes seen on H & E. Other areas demonstrated ribbons of nuclei adjacent to acellular regions, possibly representing pseudorosettes as viewed laterally. On both H & E and confocal images, the tumor nuclei demonstrated smooth regular contours and minimal atypia, and the cellularity of the tumor appeared to be similar.

Hemangioblastoma. One patient (1 biopsy, intramedullary) demonstrated confocal images of striking similarity to matched H & E images. Stromal cells of various sizes containing small vacuoles consistent with lipid were present. A rich vascular background was evident on both imaging modalities. Nuclear detail was also appreciated, and consisted of dark gray to black, markedly pleomorphic nuclei (Fig. 7).

Blinded Study

Of the 28 images reviewed, 26 were correctly diagnosed. Consequently, the accuracy rate was 92.9% (Table 1). In 1 case, blood was misinterpreted as infiltrating tumor. Normal thalamus was misinterpreted as infiltrating tumor, although the neuropathologist noted that he could not rule out normal gray matter. All 15 cases of cellular tumor (meningioma, glioma, and schwannoma) and 2 images of an infiltrating edge of glioma were diagnosed correctly. Eleven total cases of glioma were accurately diagnosed. In cases of cellular glioma, the appropriate tumor subtype was selected (that is, astrocytoma, oligodendroglioma, ependymoma). Two reactive lesions were diagnosed correctly. One glioblastoma with treatment effect and no recurrent tumor was accurately diagnosed as treatment effect.

Discussion

In vivo intraoperative confocal microscopy enables vi-
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visualization of histological features in real time as well as the evaluation of cellular and architectural detail at the sub-surface level. Here, we provide the first in-depth analysis of the cellular and architectural characteristics observed in a variety of brain tumors as seen on confocal microscopy.

Routine neuropathological diagnosis depends on excision and processing of tumor tissue, which can be both mechanically and chemically altered during this process. Use of intraoperative confocal microscopy circumvents these limitations with real-time in vivo visualization of tumor cytoarchitecture and microvasculature. This initial analysis of the intraoperative confocal microscope suggests its potential value during the microsurgical resection of both intra- and extraxial brain tumors. This handheld device generates a real-time, fluorescein-contrasted pathological image that, in many instances, may be of sufficient resolution for a neuropathologist to establish a preliminary diagnosis without a frozen-section diagnosis. Tumor heterogeneity and biopsy sampling error remain a considerable source of inaccuracy and a primary cause of glioma undergrading. The ease with which a handheld confocal microscope generates numerous images during the course of tumor exposure and resection may effectively overcome this source of diagnostic imprecision. Furthermore, the ability of the device to scan as much as 500 μm through the visible tissue surface permits analysis of an even broader spectrum of tissue.

Most of the tumors evaluated using intraoperative confocal microscopy demonstrated cellular and architectural characteristics that mirrored those of their described pathological features, and were strikingly similar to matched H & E images from the same tumor: stromal cells in hemangioblastoma demonstrated intracytoplasmic lipid; the WHO Grade II ependymoma demonstrated classic perivascular rosettes; and fascicles of elongated cells were seen in schwannomas. For gliomas, regions of tumor infiltration were discernible for both low- and high-grade tumors, raising the possibility that intraoperative confocal microscopy could one day help neurosurgeons to maximize extent of resection with a modality more easily implemented than intraoperative MR imaging.

Nevertheless, select histological features were less evident on confocal imaging than on matched H & E sections. Specifically, nuclear detail was often difficult to appreciate and, when visible, nuclei were generally vague and lacked crisp detail. Prominent nucleoli were also not appreciated. As expected, familiarization with the device involved a learning curve for both the neuropathologists and neurosurgeons. Initially imaged cases suffered from motion artifact and erythrocyte contamination. In some cases, images had to be acquired with the neuropathologist present in the neurosurgical suite, directly interpreting images and guiding the selection of tumor for potential diagnosis. However, these challenges steadily decreased with experience.

Conclusions

On the whole, our initial experience suggests that in vivo confocal imaging of brain tumors has the potential to change the current paradigm for intraoperative diagnosis. Although the study was limited, blinded review of the 28 images by a single neuropathologist yielded an accuracy rate of 92.9%. Previous studies have reported similar accuracy rates for identification of pathological entities from frozen-section biopsies and H & E sections, ranging from 92% to 99.7%. Mistakes related to sampling error, a common problem associated with frozen-section analysis, could be lessened with real-time examination of specimens. The neuropathologist was able to diagnose cellular tumor accurately in all 15 cases, suggesting that in vivo confocal microscopy could facilitate improved regional selection of highly cellular tissue for histological diagnosis, molecular testing, and tissue banking.

When applied in lieu of frozen-section diagnosis, common confounding factors, such as frozen-section artifact and cautery artifact, also may be avoided. Furthermore, the time to diagnosis could be greatly shortened and the demand on pathology department personnel minimized. Perhaps most important, the speed and sensitivity of such a system could influence neurosurgical decision making, particularly at the presumed margins of a resection cavity. Because all data are digitally acquired and stored, electronic transmission of images to remotely located pathologists could also enable real-time telepathology. Although intraoperative confocal microscopy is still in its infancy, our results suggest that this technology has the potential to revolutionize the way that we approach intraoperative diagnosis.

Disclosure

Zeiss Meditec provided clinical or research support to Dr. Preul for this study (includes equipment or material). The authors report no conflict of interest concerning the materials or methods used in this study or the findings specified in this paper.

Author contributions to the study and manuscript preparation include the following. Acquisition of data: Spetzler, Eschbacher, Martirosyan, Nakaji, Sanai, Smith, Coons. Analysis and interpretation of data: all authors. Drafting the article: all authors. Critically revising the article: all authors. Reviewed submitted version of manuscript: all authors. Approved the final version of the manuscript on behalf of all authors: Spetzler. Statistical analysis: Eschbacher, Nakaji, Sanai, Coons. Administrative/technical/material support: all authors. Study supervision: Spetzler, Eschbacher, Nakaji.

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