Intraoperative tissue analysis is essential in guiding diagnosis and treatment decisions in the neurosurgical setting. Frozen tissue histology is the most widely used approach for intraoperative tissue analysis, but its limitations (for example, sampling bias, waiting time, and freezing artifacts) are frequently encountered and widely recognized. Advances in optical imaging have expanded the tools available to both the neurosurgeon and the neuropathologist, and their implementation in the neurosurgical operating room has the potential to facilitate, and also refine, intraoperative diagnosis, treatment decisions, and surgical resections.

Confocal microscopy is one of these valuable tools, as it can provide high-resolution imaging of tissue morphology, cytoarchitecture, and intracellular elements at various depths within a tissue sample. The application of confocal imaging into a handheld endomicroscope with in vivo utility has spurred the exploration of this technology in the management of brain tumors intraoperatively. When combined with intravenous and topical fluorophores, real-time information about brain neoplasms at the cellular level can be gathered in the operating room. In this review, we provide a background on intraoperative confocal devices and fluorophore technology, describe the application of this technology in both animal models and human studies, and discuss the future directions and potential applications of confocal endomicroscopy in neurosurgery.

Technical Considerations: Confocal Endoscopes and Fluorophores

Laser scanning confocal microscopy (LSCM) is an optical fluorescence imaging modality used for imaging thick in vivo and ex vivo tissues. In LSCM, a specific wavelength laser is raster scanned across a tissue, and fluorophores within the tissue are excited. Photons emitted from the excited fluorophores pass through an objective and then a confocal aperture (pinhole) that spatially restricts photons emitted from above and below the point of focus. This allows generation and visualization of optical sections, which are images of thin tissue planes generated without physical sectioning of the examined tissue. Tissue sections from various depths within the tissue can be visualized by translating the imaging objective along a vertical axis. Recently, this technology has been miniaturized into clinically available endomicroscopy systems.
Clinical confocal endomicroscopes generate contrast when coupled to appropriate fluorescent agents. Fluorescent agents with neurosurgical use include fluorescein, indocyanine green (ICG), and 5-aminolevulinic acid (5-ALA). Of these, the first two are FDA approved in the US for nonneurosurgical applications, and the third is under research evaluation. In Europe and many other countries, formal approval has been obtained for 5-ALA. Specific fluorophore properties and clinical uses are outlined in Table 1. Fluorescein and ICG generally contrast tissue architecture similarly to H & E staining. ICG can be used to visualize deeper structures within tissue due to its infrared excitation-emission, which travels further through tissue without scattering. 5-ALA, on the other hand, provides tumor-specific contrast that is more similar to immunohistochemistry. It is preferentially concentrated in neoplastic cells and converted to the endogenous fluorophore protoporphyrin IX intracellularly, which provides fluorescent contrast of neoplastic cell cytoplasm. 5-ALA is best excited with ultraviolet (UV) light and emits far-red photons, which can travel further through tissue without scattering. This makes appropriate coupling to excitation/emission filters particularly important for minimizing detection of autofluorescence generated by UV light and collection of far-red photons specific to 5-ALA.

Clinically available confocal endomicroscopy systems contain lasers with precise excitation wavelengths and dichroic filters for detecting appropriate emission wavelengths. The experimental use of these systems for neurosurgical applications is gaining in popularity as they allow real-time visualization of tissue cytoarchitecture in the operating room. Currently, Optiscan and Cellvizio produce commercially available laser scanning confocal endomicroscopes. These systems both use laser scanning systems coupled to optical fibers. The Optiscan device is available in a 488-nm and infrared excitation platform. Cellvizio produces single-laser and dual-laser systems containing 488-nm and 560-nm excitation. Both of these manufacturers use specific dichroic mirrors for filtering excitation and emission photons. Technical specifications of these 2 systems and available probes are listed in Table 2.

**Table 1: Fluorophore properties**

<table>
<thead>
<tr>
<th>Fluorophore</th>
<th>Excitation (nm)</th>
<th>Emission (nm)</th>
<th>Route of Administration</th>
<th>Localization</th>
<th>Tested in Animals</th>
<th>Tested Clinically</th>
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</thead>
<tbody>
<tr>
<td>fluorescein</td>
<td>494</td>
<td>521</td>
<td>intravenous</td>
<td>extracellular</td>
<td>yes</td>
<td>yes</td>
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<tr>
<td>ICG</td>
<td>778</td>
<td>820</td>
<td>intravenous</td>
<td>extracellular</td>
<td>yes</td>
<td>no*</td>
</tr>
<tr>
<td>5-ALA</td>
<td>410</td>
<td>635 &amp; 704</td>
<td>oral</td>
<td>intracellular</td>
<td>yes</td>
<td>yes</td>
</tr>
</tbody>
</table>

* Indocyanine green has been used clinically for visualization of vasculature but not with LSCE.
Laser scanning confocal endomicroscopy

### TABLE 2: Confocal endomicroscope and probe properties

<table>
<thead>
<tr>
<th>System</th>
<th>Probes</th>
<th>Wavelength Detected (nm)</th>
<th>Lat Resolution (μm)</th>
<th>Axial Resolution (μm)</th>
<th>FOV (μm)</th>
<th>Working Distance (μm)</th>
<th>Distal Tip Diameter (mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Optiscan/Pentax ISC-1000 confocal endomicroscope</td>
<td>488 &amp; 790</td>
<td>0.7</td>
<td>7</td>
<td>475 × 475</td>
<td>250</td>
<td>5.0</td>
<td></td>
</tr>
<tr>
<td>Cellvizio S</td>
<td>488 &amp; 660</td>
<td>5</td>
<td>20</td>
<td>600 × 500</td>
<td>0</td>
<td>1.5</td>
<td></td>
</tr>
<tr>
<td>HD</td>
<td>488 &amp; 660</td>
<td>2–3</td>
<td>20</td>
<td>240 × 240</td>
<td>30–80</td>
<td>1.8</td>
<td></td>
</tr>
<tr>
<td>Z</td>
<td>488 &amp; 660</td>
<td>30</td>
<td>30</td>
<td>600 × 500</td>
<td>70</td>
<td>0.94–1.8</td>
<td></td>
</tr>
<tr>
<td>MinoO</td>
<td>488 &amp; 660</td>
<td>1</td>
<td>3</td>
<td>240 × 240</td>
<td>30</td>
<td>2.6–4.2</td>
<td></td>
</tr>
<tr>
<td>AlveoFlex</td>
<td>488 &amp; 660</td>
<td>5</td>
<td>100</td>
<td>600 × 500</td>
<td>0</td>
<td>1.4</td>
<td></td>
</tr>
</tbody>
</table>

this phenomenon, which was through direct expression of eYFP in tumor cell lines. Tumor cells were readily identified with this strategy through LSCE, and even single tumor cells migrating from the main tumor into normal tissue could be visualized. This demonstrates the potential for improved visualization of neoplastic cells with LSCE through advances in fluorescence-labeling techniques (discussed below).

In a divergence from animal models of glioma, Peyre et al. published a recent study examining the ability of LSCE and ICG to identify histological properties of meningiomas ex vivo in an animal model. This study used a LSCE (Optiscan) to examine tumor specimens ex vivo for evidence of brain invasion in 2 animal models of aggressive meningioma (a transgenic mouse model and a xenograft model). Laser scanning confocal microscopy reliably differentiated between histological subtypes of meningioma in the transgenic model and was able to detect invasion of Virchow-Robin spaces and brain parenchyma (with the addition of ICG), demonstrating the potential application of LSCE in meningioma surgery for improving the detection invasive disease.

### Human Studies

Alongside studies of LSCE in animal models, this technology has been explored in the study of human brain tumors. To date, 5 reports have been published utilizing LSCE in either ex vivo or in vivo human studies.

In 2010, Schlosser et al. reported the first use of LSCE, which they termed neurolaser microscopy, in human brain tissue. This study examined 9 patients with glioblastoma (GBM) and compared LSCE with conventional histopathology in ex vivo brain tissue samples. LSCE was used intraoperatively immediately following excision of the brain tumor specimen, and, thereafter, the specimen was sent for conventional histopathological examination (that is, H & E, periodic acid Schiff, silver-impregnation, and/or immunohistochemical staining). Acriflavine hydrochloride was applied topically to ex vivo tissue samples prior to LSCE examination.

With this technique, Schlosser et al. were able to identify all WHO microscopic criteria for GBM diagnosis (that is, cell number/density criteria, cell pleomorphism, mitotic figures, microvascular proliferation, and pseudopalisading necrosis) using LSCE ex vivo. Laser scanning confocal endomicroscopy also identified additional features seen in GBM, such as apoptotic figures in perinecrotic palisading tumor cells, giant cells, and fibrillary tumor matrix/blood vessels in select specimens. Notably, however, not all WHO criteria were identified in all 9 patients included in this study. While cell density and cell pleomorphism criteria were met in all 9 patients, microvascular proliferation was only visualized in 4 of 9 patients, and mitosis was only visualized in 2 of 9 patients. These findings may not be a limitation of LSCE, however, since this study was not designed to directly compare LSCE diagnosis to H & E diagnosis, and more tissue samples were collected for H & E staining than for LSCE in these patients. Furthermore, excellent agreement between LSCE and H & E–stained images was demonstrated for numerous tissue samples collected from the same tumor resection block in various patients in this study. The limitations of the study included the use of topical acriflavine hydrochloride, as well as the ex vivo application of LSCE technology: Although topical acriflavine readily distinguishes superficial cell borders and their nuclei for LSCE analysis, mutagenesis with this agent has been demonstrated in cell culture, and it is not currently used in vivo in the CNS. Furthermore, while the ex vivo approach can be justified for testing the feasibility of LSCE tissue analysis, the use of LSCE for guiding tissue biopsy and maximizing extent of resection requires an in vivo application of this technology. Notably, one other study examined ex vivo application of LSCE in human brain tumors, with similar results. Additionally, at least 3 studies examining LSCM for ex vivo analysis of brain tumors have been reported with promising results.

Sanai et al. expanded upon the application of LSCE in 2011 when they used this technology with intravenous fluorescein to examine a variety of brain tumor subtypes in vivo at our institution. In their prospective feasibility study, any patient with an intracranial mass undergoing craniotomy for biopsy and/or resection was considered eligible, and 33 patients with a variety of lesions (for example, meningiomas, oligodendrogiomas, astrocytomas, and metastases, among others) were enrolled. Following surgical exposure of the tumor and intravenous fluorescein administration, LSCE images were acquired over the course of approximately 10 minutes. Following image acquisition, tissue biopsies were obtained at the imaging site and stained with H & E (as well as other stains, when applicable) for histological analysis and comparison.
Utilizing this approach, specific confocal features of high-grade glioma (specifically, neovascularization, dense cellularity, and irregular cellular phenotypes) were readily identified in vivo and confirmed histologically. Notably, it was reported that these features were readily apparent to both the surgeon and the neuropathologist intraoperatively, allowing for integration of this information into the operative plan. Additionally, specific cellular features of other tumor subtypes, including low-grade glioma, neurocytoma, hemangioblastoma, and meningioma, were identified on LSCE images, demonstrating the potential for LSCE to intraoperatively diagnose and guide treatment decisions for these lesions.

Following this feasibility analysis, a prospective study of LSCE and intravenous fluorescein was performed at our institution to determine the accuracy and reliability of real-time intraoperative diagnosis of a variety of brain tumors in vivo.7 Fifty patients were included in the study in which image acquisition and tumor biopsy specimens were obtained in a similar fashion to the aforementioned study. This was followed by a blinded assessment of 2 neuropathologists’ ability to properly diagnose tissue specimen based on intraoperative LSCE images. Tumors in this study included 24 meningiomas, 12 high-grade gliomas, 8 low-grade gliomas, 4 schwannomas, 1 hemangioblastoma, and 1 ependymoma. Features identified on confocal imaging were extensively described for these tumors and their subtypes utilizing LSCE and H & E comparison slides. Interestingly, in the blinded portion of this study, 26 (92.9%) of 28 LSCE images were correctly diagnosed, including 9 of 9 glioma images and 2 of 2 infiltrating glioma edge images. This provides evidence for the role of in vivo LSCE for intraoperative diagnosis and also demonstrates its potential for in vivo identification of infiltrating tumor edge and maximizing extent of resection in infiltrative tumors.

While initial results from studies utilizing LSCE with intravenous fluorescein are encouraging, technical limitations in visualization have been recognized. Specifically, the ability to visualize nuclear morphology, cytoplasm characteristics, and nuclear-to-cytoplasm ratios is limited when using fluorescein as a contrast agent.35 Administration of 5-ALA offers one potential solution to this limitation, as it provides intracellular fluorescent signal preferentially in tumor cells. The capacity of 5-ALA for macroscopic fluorescence in high-grade gliomas and its utility in resection of these tumors has been demonstrated and extensively discussed elsewhere.29 Its utility in low-grade glioma surgery, on the other hand, was previously limited due to the lack of macroscopic fluorescence in these cells.9,41 Interestingly, despite the lack of macroscopic fluorescence in low-grade gliomas, microscopic fluorescence has been observed,9,15 and in a recent study of LSCE paired with 5-ALA for low-grade glioma, visualization of fluorescently labeled tumor cells was achieved.34

This study by Sanai et al. examined 10 patients with newly diagnosed low-grade glioma and evaluated the ability of LSCE to detect intraoperative 5-ALA fluorescence in vivo in these patients. While macroscopic tumor fluorescence was not present in any patient, microscopic fluorescence with LSCE was present in 10 of 10 patients (Fig. 1). Furthermore, in 6 of the 10 patients, microscopic fluorescence was visualized at the margins of the resection cavity after a gross-total resection was thought to be achieved—this finding suggests that there may be a significant role for intraoperative LSCE and 5-ALA imaging in achieving full extent of resection in patients with low-grade glioma in the future. The Barrow 5-ALA Intraoperative Confocal (BALANCE) study is a Phase IIIa, multicenter, randomized, placebo-controlled trial that is currently underway to evaluate this approach (ClinicalTrials.gov ID no. NCT01502280).

**Future Directions**

Despite the successful application of LSCE in vivo to date, much work remains to fully incorporate this technology into the neurosurgical operating room. We believe that future advances will come in 3 main realms: fluorescent and nonfluorescent technology for imaging neoplasms in vivo; confocal endoscope technology for improving imaging quality, depth, and ease of use; and improved incorporation of LSCE into the neurosurgery and neuropathology workflow.

Fluorescein and ICG are primarily visualized through the enhanced permeability and retention effect, which means that fluorophores are preferentially taken up by tumor tissue due to increased breakdown and leakage in the blood-brain barrier.5-ALA, on the other hand, provides detection of neoplastic cells through intracellular fluorescence, but its ability to demarcate tumor margins is somewhat subjective, partly due to difficulty interpreting levels of fluorescence near tumor boundaries.19,24,47 All 3 of these currently available fluorescent agents also have limited circulation time and readily diffuse into and out of interstitial space.41 Intraoperative confocal microscopy combined with molecular imaging probes specific for tumor biomarkers could play a significant role in future neurosurgical applications of LSCE.

Molecular probes that have been investigated for use in brain neoplasms can be divided into 3 functional categories: peptides, antibodies, and nanoparticles. The efficacy and applicability of these probes is based on their selectiveness for tumor tissue, resistance to photo bleaching and autofluorescence (by using near-infrared probes), and capacity to be safely administered to patients.32

Peptide probes have been used to label multiple cellular targets because of their high affinity and specificity for intracellular proteins. One promising example of this is a peptide biomarker for the cell-adhesion glycoprotein, integrin αvβ3. Integrin αvβ3 is expressed in tumor cells and plays a role in tumor growth, angiogenesis, and metastasis.16 Its expression is low in normal cells but high in tumors cells and tumor vessels, and fluorescently labeled near-infrared peptide probes have been used to specifically target integrin αvβ3 in glioblastomas and medulloblastomas in vivo in animal models.4,10,37,26,69 These studies demonstrate the ability to specifically tag tumor cells with fluorescent peptide probes and help differentiate between neoplasm and normal brain tissue. However, heterogeneous expression of integrins in nontumor regions makes the specificity of the probes an ongoing
issue. Other promising peptide probes include those targeted toward cysteine cathepsin (GB119) and epidermal growth factor receptor (EGFR), but use of these probes for guiding neurosurgical resection or tissue sampling intraoperatively must still be explored.

Antibodies have also been developed to target brain tumor molecular targets. A 2010 study used single-domain antibodies (sdAb) targeted to anti-insulin-like growth factor-binding protein–7 (IGFBP7), which accumulates in the basement membranes of glioblastoma endothelium. Due to their small size and nanomolar affinity, IGFBP7 antibodies displayed rapid accumulation to human and animal GBM vessels after systemic injection. Anti-IGFBP7 sdAbs have advantages compared with other predominantly peptide-based strategies, including high specificity to the target, and the appropriate pharmacokinetic characteristics for imaging applications, such as PET scan.

Antibodies conjugated to fluorophores have also been used to target vascular endothelial growth factor receptor–1 (VEGFR-1) in a transgenic mouse model of medulloblastoma. Results of using this conjugated antibody demonstrated preferential binding to tumor tissue and microscopic delineation of tumor margins using a confocal microscopic in vivo. Further studies incorporating this strategy and using a miniaturized microscope intraoperatively have exciting potential.

Nanoparticles are organic or inorganic particles of nanometer size that have also been demonstrated to provide intraoperative imaging of brain neoplasms. Nanoparticle strategies have been used to demarcate tumor margins and have been incorporated into multimodal imaging strategies by conjugating near-infrared fluorochromes (quantum dots) and traditional imaging molecules (fluorescein and ICG) with MRI-detectable iron oxide or polymer-based cores. Magneto-optical nanoparticles permit visualization of tomographic landmarks on MRI for surgical planning while subsequently allowing for intraoperative fluorescent visualization of tumor boundaries. Although these imaging probes provide a precise method of demarcating the brain-tumor interface, further experimentation in clinical models is needed to establish the safety profile and long-term effects before translation into the clinical setting can proceed. Notably, confocal microscopy techniques that do not require the use of exogenous fluorophores have been described and show exciting potential.

In addition to advances in tumor-specific fluorophores, the future success of LSCE depends upon advances in confocal endoscope technology. Future LSCE technology must facilitate deeper tissue imaging, greater detection of weak fluorescent signals, and faster acquisition than what is currently available. Current LSCE systems have 1–2 mm of imaging depth in the brain, but advances in fluorophores and available detection spectra in endoscopes will improve these imaging properties and allow for more useful incorporation of LSCE into the neurosurgical operating room. Systems that have tunable filters for specific fluorophores with intelligent algorithms for multispectral imaging are being developed to enhance fluorescence detection.

Lastly, the implementation of LSCE requires changes in neurosurgical and neuropathological training, as well as the neurosurgical workflow. Intraoperative fluorescence-guided surgery provides real-time information that can overcome the issues of standard frozen-section pathology; however, interpretation of LSCE-generated images requires a shift from interpreting routine H & E–stained tissue. This transition requires neurosurgeons and/or neuropathologists to be trained in interpreting LSCE images, which would facilitate both intraoperative diagnosis and tumor resection by eliminating frozen-section processing time and decreasing sampling error. Initial studies have already documented the ability of neuropathologists to be trained to interpret confocal images. In the absence of trained neuropathologists at a given institution, LSCE lends itself to tele-pathology through the real-time transmission of digital images. Although the use and incorporation of LSCE technology requires significant change in practice up front, the potential for this technology to improve the neurosurgical approach to brain neoplasms is extensive.
Conclusions

Laser scanning confocal endomicroscopy has great potential to improve the way we approach and treat brain neoplasms in the neurological operating room. Despite the wide array of neurosurgical applications of LSCE, the in vivo applications of this technology remain limited in both animals and humans, and there is a need for future high-impact studies in this area. Through improved molecular labeling and confocal endoscope technology, as well as thorough and effective translation of this technology into the operating room, LSCE will provide a powerful tool for achieving better and safer patient outcomes in the neurological operating room in the future.

Disclosure

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