The Utilization of Intraoperative Confocal Laser Endomicroscopy During the Fluorescence Guided Surgery for Brain Tumors

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Background
Intraoperative diagnosis in neurosurgery has traditionally relied on frozen and formalin-fixed paraffin-embedded section analysis of biopsied tissue samples. Although this technique is considered the "gold standard" for establishing a histopathologic diagnosis, it entails a number of significant limitations including the time required for transferring, processing, and interpreting the tissue, the presence of artifacts and sampling errors, as well as the differences present when comparing frozen and permanent sections that may lead to misdiagnosis. Rapid intraoperative diagnosis has become possible with refinement and miniaturization of the research-type confocal laser scanning microscope into a handheld confocal laser endomicroscope (CLE). We report the evolution of this technology and present analysis of recent use of the updated CLE tool on patients during fluorescein sodium-guided brain tumor surgeries.

Clinical Investigations
Our clinical experience with CLE includes 237 patients with gliomas, meningiomas and other CNS pathologies examined in vivo and in vivo using a Gen1 CLE and 48 patients using a Gen2 CLE (since 2016: 19 HGG, 3 LGG, 11 pituitary adenomas, 2 chondrocytomas, 2 metastases, 2 chiasmomas, 4 meningiomas, 2 treatment effects, 1 focal cortical dysplasia, 2 hemangioblastomas) examined ex vivo. The protocol was approved by the IRB of the Barrow Neurological Institute. St. Joseph’s Hospital and Medical Center, Phoenix, Arizona. Images were reviewed by a neuropathologist and 3 neurosurgeons. Since 2016 we optimized the protocol to work in combination with fluorescence-guided surgeries.

Confocal Laser Endomicroscope (CLE)

CLE Digital Biopsies with Fluorescein sodium (FNa): Image Features

Intraoperative Results

Diagnostic accuracy
Seventy-four consecutive adult patients (37 male and 43 female) were prospectively enrolled in the diagnostic accuracy study. There were 21 gliomas and 30 meningiomas. Multiple locations within the resection bed were imaged with CLE in vivo. The CLE specificity and sensitivity for gliomas were 94% and 91%, respectively. The frozen section specificity and sensitivity for gliomas were 94% and 95%, respectively. Both false negative and false positive values were evident in situations attempting to distinguish reactive changes and recurrent gliomas. FN and FP values for meningiomas are associated only with distinguishing between meningioma and schwannoma (Martirosyan et al., 2016)

References
BACKGROUND
Fluorescence guidance with 5-aminolevulinic acid (5-ALA) increases resection extent and may benefit surgical outcomes in high-grade gliomas. We investigated if a combination of various fluorescence guidance techniques can provide further advantage. We systematically assessed and compared the fluorescent patterns of 5-ALA-induced protoporphyrin IX (PpIX), fluorescein sodium (FNa) and indocyanine green (ICG) to identify GL261 gliomas in mice and RFP-US251 gliomas in rats.

METHODS
5-ALA (5mg), FNa (5 mg/kg) and ICG (20 mg/kg) were administered perioperatively. Fluorescence patterns were recorded with operative microscope, laser scanning confocal microscope, and confocal laser endomicroscope. Fluorescence was assessed quantitatively as a surface area of fluorescent positive tissue and qualitatively (false/true, positive/negative) as compared to HE-stained histology.

RESULTS
FNa highlighted a larger surface area (18.00 mm²) than white light (16.90 mm², p=0.016) or PpIX (16.00 mm², p=0.035). Both 5-ALA and FNa showed inhomogeneous staining patterns: multiple areas of equal staining, when PpIX was present and FNa was not, and vice versa. ICG was visible in 8/31 tumor samples, all immediately after injection, ICG did not reveal clear tumor margins, but stained non-tumor tissue and nearby vasculature. FNa signal was stronger (tumor to background ratio (TBR) 1.93±0.56) compared to 5-ALA (1.52±0.31; p<0.002). ICG TBR (2.75±0.87) was higher than FNa (p<0.006) or 5-ALA (p<0.001). Cell-level imaging revealed PpIX-positive, FNa-negative invading tumor cell groupings and areas. However, some tumor parts were PpIX-negative and FNa-positive, or PpIX/FNa-negative. Differences in FNa-negative (8/30, 27%) and PpIX-negative (12/30, 40%) tumor areas were insignificant, p=0.27. False positive FNa fluorescence (13/30, 43%) was more frequent than 5-ALA (4/30, 13%, p<0.01).

CONCLUSION
ICG highlighted tumor only within the first few minutes and stained mostly hypervascularized areas. Confocal and surgical imaging revealed inhomogeneous tumor border-staining with PpIX/FNa. Simultaneous administration of 5-ALA and FNa may provide additional benefit. Neither ICG, 5-ALA or FNa worked perfectly, emphasizing the need for more specific markers for fluorescence-guided brain tumor resection.

Methods

Animals
• 10-12 week old Female B6(Cg)-Tyrc-2J/J mice
• 5-ALA was injected intraperitoneally 2 hours before surgery (5 mg in 200 ul of 1% phosphate buffered saline)
• Glioma cells were injected intracranially 0.1 mm posterior to the bregma
• RFP was cloned into pLXSN (Clonetech, Mountain View, California, USA), plasmid (Dr J.B. Rubin, Washington University of St Louis, MO), and Lenti-X HT Packaging Mix (Clontech Laboratories, Inc.) and FUW-GL protein labeled tumor cells invading normal brain area, and absence of PpIX or FNa fluorescence in normal tissue.

Results

Figure 1. (A) Confocal laser endomicroscopy image of FNa stained background so tumor cells appear dark. (B) Confocal laser scanning microscope image illustrating tumor vs. normal brain tissue border. Top half of each image are tumor cells, and the bottom half is normal brain tissue. PpIX stains cytoplasmic inclusions in both tumor and normal brain, with lower brightness in tumor. FNa provides background stain for the tumor cells, which is not visible in normal brain. This is further illustrated in the merge panel.

Figure 2: Top Panel (A-C): Fluorescence imaging of mouse gliomas as seen sequentially on the operative microscope with filters. First column - white light (WL); second column – BLUE400 filter for 5-ALA-induced PpIX; third column – Yellow660 filter for FNa; forth column – overlay of PpIX and PpIX images. A. PpIX showed areas not highlighted by FNa (red arrows) B. FNa showed areas not highlighted by PpIX (yellow arrows) C. FNa and PpIX highlighted similar areas (green arrow). D. Difference in average surface of tumor area, as perceived under white light, FNa fluorescence, and PpIX fluorescence. N=35 images analyzed in each group. Images taken at similar area with different filters of operative microscope. E. Tumor-to-background ratio of PpIX, FNa and ICG as a measure of fluorescence signal. Ratio is a measure of fluorescence strength of the tumor compared to that of normal brain tissue, analyzed on ImageJ. N=48 images analyzed from operating microscope.

Figure 3. Laser scanning microscopy image. 5-ALA-induced PpIX highlighted brain tumor cells distant to the core, while FNa did not. Top row has highlighted blue and yellow squares, corresponding to similarly outlined blue (second row) and yellow (third row) images, which are enlarged. In the middle row, FNa successfully highlighted the sample taken from the tumor core (blue square), but did not do so in the bottom row, corresponding to the sample distant to the tumor core.

Figure 4. (A) Image illustrates individual tumor cells invading along vessels, as denoted by yellow arrows. 5-ALA-induced PpIX was able to visualize the tumor cells, which corresponded to the red fluorescent protein expression. (B) Laser scanning microscopy image illustrates tumor border showing red fluorescent protein labeled tumor invading normal brain area, and absence of PpIX or FNa fluorescence in normal tissue. (C) Laser scanning microscopy shows invasions of tumor into the normal brain along the vessels highlighted by PpIX. (D) H&E stain of representative brain showing invading tumor regions (yellow square) in the normal brain, similar to findings in (F).

Figure 5. Image acquired via Laser Scanning Microscopy. The two image panels show how PpIX accumulation in cells is visible under confocal microscopy as time progresses. At early time points (top row), protoporphyrin localizes diffusely to the cytoplasm. At a later time point (middle row), PpIX is trafficked into a more granular distribution, most likely representing cellular mitochondria. Bottom row: thick slice image acquired via Laser Scanning Microscopy. Images illustrate PpIX staining resulting in a heterogeneous degree of PpIX production on the cellular level, as noted in the second image with varying degrees of red intensity in various cells.

Figure 6. Image acquired via Laser Scanning Microscopy. Tumor border pictured above. With a weak 5-ALA signal, the tumor cells are visualized, but normal cells are visualized as well.

Figure 7. Biopsy-based comparison of the incidence and percentage of successful staining and over-staining in PpIX vs FNa in operating wide field microscope (Kinevo) and confocal microscopy, counted as “stain”, “misses”, “overstain”, “no overstain”. Total n=48 images analyzed under Kinevo operative microscope, n=33 for confocal microscope. P=0.48 for Stain, p=0.016 for overstain with operative microscope, P=0.046 for Stain, p=0.037 for overstain with confocal microscope.

Figure 8. Representative images of mouse gliomas in white light view and infrared 800 mode of the operating microscope. Upper row – brain was extracted within 3 minutes after intravenous ICG and FNa injection. Incongruent pattern of tumor labeling with ICG is observed, many vessels are highlighted and some tumor area is not highlighted with ICG, but was clearly fluorescent with FNa and 5-ALA. Middle row – retention of low ICG signal is visible 20 minutes after injection. Lower row – most of the brain slices exhibited no fluorescence when harvested after 30 minutes past ICG injection.

Figure 9. H&E confirmation of tumor cell mass (yellow arrow), distant to the main tumor mass above highlighted with PpIX but not with FNa (Similar to Fig. 3).

Figure 10. Image acquired via laser scanning microscopy. Images illustrate significant heterogeneity in tumor core with various markers visualized and non-visualized in neighboring cells. A. Labeled laser scanning microscopy images of Hoechst, PpIX, FNa, and red fluorescent protein. B. Merged overlays from panel A.