Confocal endomicroscopy during brain surgery

Confocal endomicroscopy is a rapidly developing method, which has the potential to provide real-time in vivo histopathological information to neurosurgeons and pathologists.

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Fluorescence-based imaging techniques are emerging as valuable tools for enhancing intraoperative decision-making during surgical procedures. Particularly in the field of neurosurgery this method is becoming increasingly adopted in order to highlight vascular structures and tumors[i]. Typically, these methods are aimed at macroscopic visufalization of fluorescent areas and rely on surgical microscopes which are equipped with appropriate filters and light sources. For confocal endomicroscopy (CEM) this principle has been refined to visualize the microstructure of tissue at high magnification. It delivers images in real time and in vivo, i.e. without the need to extract tissue. In CEM a scanner probe (resembling a rigid endoscope) gently contacts the tissue surface in order to reveal cellular and architectural detail at the subsurface level based on a fluorescent agent. In addition, our latest CEM system is able to transfer images from the operating room via network, allowing a pathologist to read them from virtually anywhere. It therefore has the potential to complement the use of frozen sections in order to support neurosurgeons for intraoperative decisions.

Starting several years ago, ZEISS has pioneered CEM technology for neurosurgery together with a technology partner. The initial clinical and pre-clinical evaluation as a decision support tool for neurosurgery was performed with a system called FIVE 1 (in this guide referred to as Generation 1 (Gen1)). This experience was used as input to create the recently developed "Digital Biopsy Tool" for neurosurgical applications (referred to as Generation 2 (Gen2))[ii].

This article describes the technical principle of CEM, summarizes published studies as well as extensive unpublished experience, and describes basic principles of the interpretation of confocal images.

Technical principle of confocal endomicroscopy

Confocal laser scanning microscopy (CLSM) is an optical imaging technique which allows the selective visualization of a focal plane ("optical sectioning") in a sample. This is achieved by suppressing out-of-focus signals (i.e. background fluorescence) from above and below the selected focal plane using a pinhole. CLSM is widely employed in biomedical research for imaging of thick tissue. However, due to their size and shape bench-top confocal microscopes cannot be used for the visualization of living tissue in patients.

Therefore, in the Gen1 and Gen2 endomicroscopy systems miniaturization of the CLSM principle was successfully achieved in a hand-held probe, which can be inserted into the surgical cavity and put in direct contact with the patient's tissue. This makes it feasible to create a histology-like image ("digital biopsy") of a small area in front of the probe tip that is displayed at high magnification on a monitor (**figure 3**). The confocal image plane is parallel to the tissue surface and can be moved from the tissue surface down to deeper layers of the specimen via optics (**figure 1**).

Compared to Gen1, the Gen2 system contains technical improvements that facilitate the intraoperative application, e.g. regarding the shape of system and scanner probe, user interfaces, resolution, automated image acquisition modes, connectivity, etc. although the fundamental optical properties remain similar.

On a technical level the confocal imaging with the Gen1 and Gen2 systems works in the following way (**figure 2**):

A laser source (488 nm wavelength) transmits light through an optical fiber to the tip of the scanner probe. The fiber end is moved quickly by electromagnets in an XY scanning pattern. Via a lens system the light emanating from the fiber end is focused at an adjustable focus depth (Z-depth) into the patient's tissue. Due to the movement of the fiber end the position of the focal point in the tissue is moving, thereby scanning a target area in the focal plane in quick repetition. A fluorescent dye present in the tissue (see following chapter) is excited

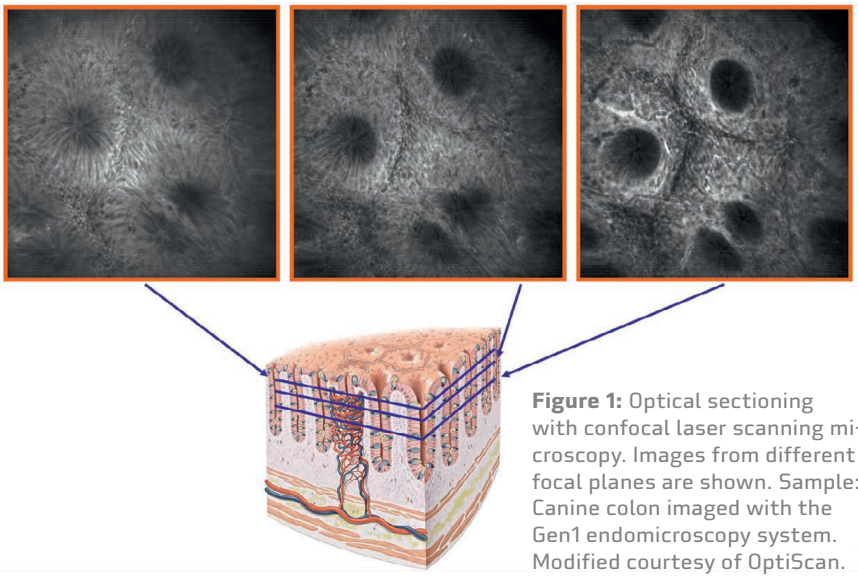

Figure 1: Optical sectioning with confocal laser scanning microscopy. Images from different focal planes are shown. Sample: Canine colon imaged with the Gen1 endomicroscopy system. Modified courtesy of OptiScan.

Image: ZEISS

by the laser light at the respective focal point and therefore emits fluorescence signals (**figure 4**). The lens system now serves to collect and focus the fluorescence light back into the tip of the light guide. The fluorescence light then passes an optical filter wheel and reaches a detector which converts the light intensity into a digital pattern. Since the XY position of the focal spot is known at any moment of the scanning process the digital pattern can be translated into a greyscale image of the field of view, which can be displayed on the monitor. Importantly, fluorescence light which does not arise from the current focal plane, is rejected since it is not focused on the fiber end. This means that the light guide acts like a confocal pinhole aperture known from conventional CLSM systems, thus improving the contrast of the image.

The main components of the Gen2 system are the mobile cart and the scanner unit (Figure 3). The cart houses the laser source, detector, PC, etc. and provides drawers for accessories as well as temporary storage of the scanner unit. A Full HD touchscreen monitor allows the control of all functions of the device, adjustment of the settings, and the display of confocal images during and after recording. In addition, a foot control panel permits the surgeon to control the main functions (focus depth, recording of images). In order to provide a sterile barrier during surgery the scanner probe and its cable need to be covered with a sterile sheath. This sterile sheath, at its tip, has a window of clear optical-grade plastic through which the illumination and signal light passes. As this window is part of the optical pathway, the sheath has to be used even if a sterile barrier is not mandatory (e.g. in ex vivo use).

The Gen2 system provides a field of view of 475 x 267 µm, which can be scanned with Full HD resolution. For live screening a higher refresh rate can be achieved by reducing the vertical resolution (i.e. number of horizontal lines). The focus depth can be adjusted in a range from the front window surface (~0 µm) down to ~200 µm below the tissue surface. Besides the acquisition of single images the system allows the recording of image series (i.e. continuous recording of images until stopped) and Z-stacks (automated recording of a user-defined range of images with selectable step size relative to the current focal plane).

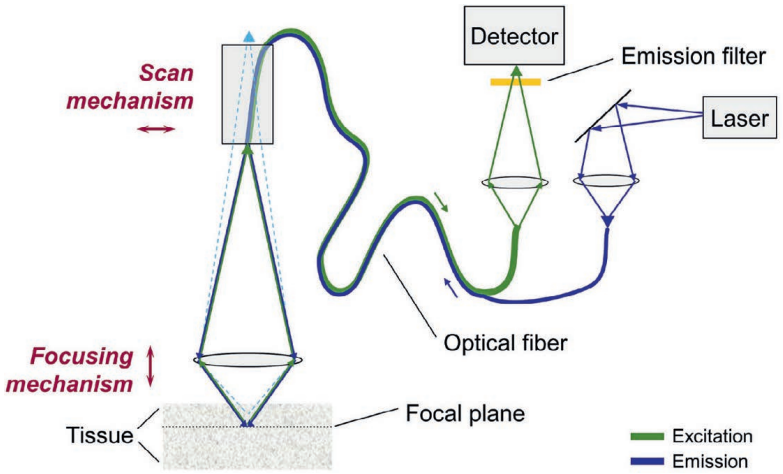

Figure 2: Functioning principle of the confocal endomicroscopy systems Gen1 and Gen2. Dashed lines depict out-of-focus fluorescence, which is not collected by the system.

The Gen1 and Gen2 systems are Class 3R laser products (not requiring personal protective equipment) with a nominal optical hazard distance (NOHD) of 71 mm extending from the probe tip. Since the maximum laser output at the scanner tip is only 1 mW there is no scientific indication of tissue damage whatsoever.

Fluorescent dye for confocal endomicroscopy

Visualization of tissue and cellular structures with the Gen1 and the Gen2 CEM system requires a fluorescent dye which stains cells and surrounding interstitial areas differently in order to create visible contrast. A variety of different fluorescent agents including fluorescein sodium (FNa), acridin orange, acriflavine and FITC- or Alexa Fluor 488-labeled antibodies have been successfully tested with the Gen1 system on brain tissue [1] -[3]. While their spectral characteristics fit well to the laser wavelength (488 nm) and emission filters used in the Gen1 and Gen2 systems, only one of them, FNa (figure 4), is cleared for use in humans, i.e. for retinal angiography. Two other dyes, which are nowadays used for the visualization of macroscopic structures in neurosurgery, namely indocyanine green (ICG) and 5-aminolevulinic acid (5-ALA), might also be interesting candidates but would require lasers of different wavelengths for optimal ex- >

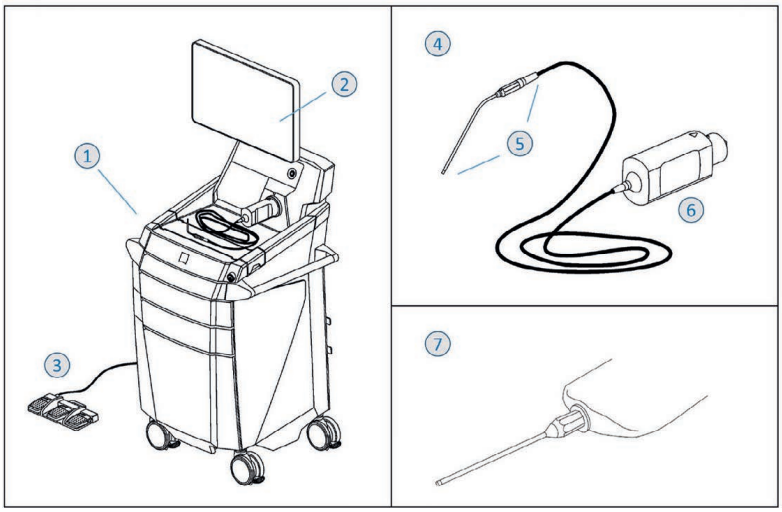

Figure 3: Main components of the Gen2 system including sterile sheath. (1) Cart, (2) touchscreen monitor, (3) foot control panel, (4) scanner unit, (5) scanner probe, (6) coupler unit, (7) sterile sheath

Images: Modified courtesy of OptiScan, ZEISS

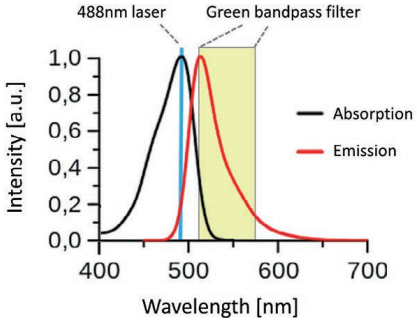

Figure 4: Fluorescence spectra of fluorescein. Excitation shown in black, emission shown in red.

citation. According to the current state of research, the Gen2 Digital Biopsy Tool should therefore best be used with FNa. Intravenous injection should be preferred compared to topical application since it allows more homogenous staining of the tissue.

FNa has been extensively and safely used for retinal angiography for more than fifty years. Similarly to its usage in ophthalmology, it can be used in the brain to visualize blood vessels. In contrast to most other tissues, where FNa penetrates quickly into the tissue surrounding the blood vessels, the blood-brain barrier (BBB) prevents this process under normal conditions. However, the dysfunction of the BBB in many intracranial tumors leads to an extravasation of the dye within in the tumor tissue. This fact was suggested as early as 1948 as a means for distinguishing tumors from normal brain intraoperatively. But only since the recent development of dedicated filters for surgical microscopes the number of clinical studies has increased.

The leakage of FNa into brain tissue at sites of a defective BBB is also the basis for visualizing the tissue microstructure with the Gen1 and Gen2 CEM systems, i.e. normal brain exhibits a very low fluorescence with the exception of blood vessels and some autofluorescent spots in cells [4]. However, the dye also appears to gradually leak into areas with intact BBB over time [1]. The dye is distributed in the interstitial fluid, i.e., it does not permeate the cytoplasmic membrane, and does not apparently interact with specific cell types. However, noted primarily from *ex vivo* and less so on *in vivo* imaging, some cells within the tumor are bright, which may represent FNa uptake following prolonged exposure to the dye, or influx of dye into damaged cells or cells undergoing deterioration.

The logical conclusion would be to use FNa for both macroscopic and microscopic fluorescence during the same operation, i.e. for visualization of tumor boundaries[i] as well as of cellular structures. However, there is still insufficient experience in this respect since the *in vivo* clinical studies (of the Gen1) in neurosurgery were performed without observing macroscopic fluorescence. Therefore, the dye was injected only 2-5 min before the imaging as opposed to a timespan of >30min, which was typically used in clinical studies on the macroscopic visualization of FNa.

Implementation during in vivo use

As pointed out above, fluorescein sodium (FNa) using intravenous injection might be the preferable fluorescent agent for use with the Gen2 system. In the following overview we shall focus on studies investigating the use of this dye in the context of neurosurgical oncology. After a summary of work published with the Gen1 system, we will shortly describe the first experience with the Gen2 system afterwards.

In a study from 2010 Sankar et al. [1] reported on their experience with Gen1 system for confocal imaging in a mouse model of glioma. They concluded that intravenous injection of FNa made it possible to distinguish between tumor and non-tumor tissue at tumor boundaries and to visualize features like hypercellularity and pleomorphism in the tumor. Moreover, CEM was reported to demonstrate the cortical vasculature effectively. In the following years several other studies of experimental meningiomas and gliomas in mice confirmed that FNa alone or in combination with other fluorophores can provide cytoarchitectural information for certain intracranial lesions [3], [5].

The first application of the Gen1 system in humans, using i.v. injection of FNa in 33 patients, was reported by Sanai et al. in 2011 [6]. For a variety of tumor histologies, including gliomas, meningiomas, hemangioblastomas, and central neurocytomas the system was shown to generate confocal images that are of a sufficient resolution for a neuropathologist to establish a preliminary diagnosis. Moreover, the authors suggested that CEM has the capacity to distinguish tumor margins from adjacent parenchyma. In a follow-up study this group systematically compared intraoperative confocal microscopy to corresponding H&E-stained sections from the same regions. They concluded that the results of both methods correlated surprisingly well and that many characteristic features of various brain tumors are reproduced by CEM [7]. The same group also showed on a

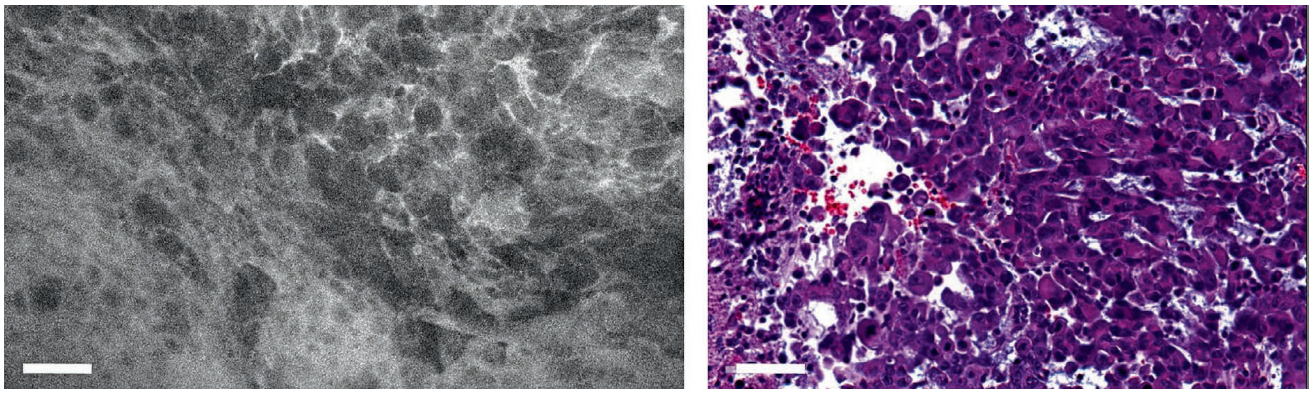

Figure 5: Confocal endomicroscopy of experimental glioma in mice (in vivo) using the Gen2 system and intravenously injected FNa. Matched confocal view (left) and H&E-stained sections (right). Scale bar, 50 µm.

large cohort of patients that CEM may make it feasible to distinguish different pathologies with high accuracy [7] and to diagnose gliomas and meningiomas with a specificity and sensitivity above 90% and comparable to those of frozen sections [8].

Based on promising intraoperative results of the Gen1 system, experimental gliomas in anesthetized mice were investigated using the Gen2 system and FNa injection. The results were very encouraging. [4] (**figure 5**). In addition, in a blinded observer study with the Gen2 system it was demonstrated that tumor and normal brain architecture could be distinguished well in mice [4]. Furthermore, preliminary experience gained *ex vivo* indicates that also the tissue microstructure of human samples can be observed very well with the Gen2 device [4] (**figure 6**).

Image interpretation

CEM enables the visualization of tissue architecture in real-time. Upon intravenous injection FNa is distributed in the tissue areas, where the BBB is defective. Due to the extracellular localization of the dye, cells generally appear as dark silhouettes, and intracellular components may be observed as shadows of varying grayscale intensities [2], [3]. However, observations made in mice and human *ex vivo* samples indicate that FNa may also accumulate in certain cell types following prolonged exposure.

CEM allows the visualization of cellular and architectural characteristics of many types of intracranial tumors in a way that strikingly matches H&E images from the same tumor [7]. These commonly identified features included, for example, cellular atypia, hypercellularity, infiltrating edges and necrosis in gliomas, deposition of collagen and psammoma bodies in meningiomas and fascicular growth pattern as well as cell morphology in schwannoma. For hemangioblastomas, lipid-laden stromal cells as well as the prominent vasculature could be demonstrated, whereas perivascular pseudorosettes were observed in the case of ependymoma [7]. Similarly, the Gen2 system has also been shown to deliver very promising results using experimental gliomas in mice and human biopsy specimens (Figure 5 and 6). While certain histological features (e.g. nuclear detail) may be less evident in confocal images compared to H&E images [7], certain other aspects may be even more visible (e.g. angioarchitecture and glomeruloid structures in high-grade gliomas) on CEM since living tissue can be analyzed [7].

As for conventional H&E-stained sections, the interpretation of CEM images requires a lot of experience. However, using blinded observers it has been shown that a blinded pathologist may be able to diagnose different tumor types with high accuracy [7], [8] and CEM might allow both neurosurgeons and neuropathologists to intraoperatively distinguish abnormal from normal tissue[6]. Recording of Z-stacks, which is now possible with the Gen2 system, may further support the interpretation of confocal data since the best focal plane can be selected from the stack, and artifacts by red blood cells may be easier to identify than on single images.

Importantly, the Gen2 system allows the pathologist to conveniently examine images via remote access virtually from anywhere. In the future, combined access to the 3D coordinates of the digital biopsies in the MR data and to the view seen with the surgical microscope at the time of CEM imaging might further facilitate to interpret images.

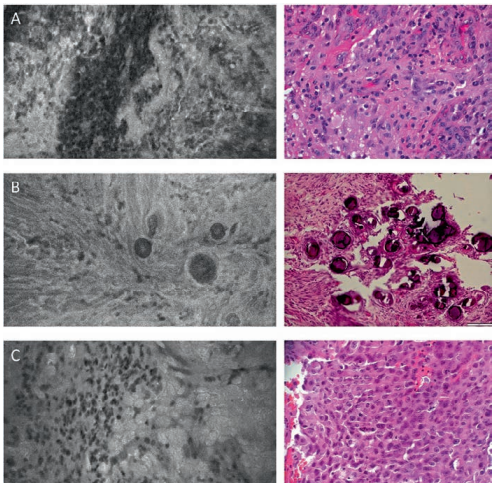

Figure 6: Confocal endomicroscopy of human brain tumors (ex vivo) using the Gen2 system and intravenously injected FNa. Matched confocal view (left) and H&E-stained sections (right). (A) glioblastoma, (B) meningioma, (C) metastasis of lung adenocarcinoma. Scale bar, 50 µm.

Conclusions

CEM is a powerful technology which has the potential to enter the neurosurgical operating room. It is expected that in future it will allow to perform digital biopsies and thus provide real-time in vivo histopathological information to neurosurgeons and pathologists, which may be used to support intraoperative decisions. ■

[i] Please use the fluorescent agent as per the approval status for the application in your country. Studies cited describe research work and may be based on off-label use of drugs.

[ii] Caution: The Generation 2 system is an investigational device, not for clinical use. Limited by law to investigational use. Not for sale. Future availability cannot be guaranteed. All referenced clinical tests with the generation 2 system were carried out under IRB and/or approved by an ethics committee.

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[8] N. L. Martirosyan *et al.*, "Prospective evaluation of the utility of intraoperative confocal laser endomicroscopy in patients with brain neoplasms using fluorescein sodium: experience with 74 cases.," *Neurosurg. Focus*, vol. 40, no. 3, p. E11, 2016.

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