1. Preamble

For enhancing intraoperative decision-making during surgical procedures, fluorescence-based imaging techniques are emerging as valuable tools. Particularly in the field of neurosurgery this method is becoming increasingly adopted in order to highlight vascular structures and tumors. Typically, these methods are aimed at macroscopic visualization 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.

ZEISS has pioneered CEM technology for neurosurgery together with a technology partner starting several years ago. The initial clinical and pre-clinical evaluation as a decision support tool for neurosurgery was performed with a system called FIVE 1 (manufactured by OptiScan). This experience was used as input to create a product for neurosurgical applications, the recently developed Digital Biopsy Tool CONVIVO.

This document addresses the following topics:

- Basics of confocal endomicroscopy
- Summary of published and unpublished experience
- Interpretation of confocal images

1 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.

An equivalent expression that is frequently used is confocal laser endomicroscopy (CLE)

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All referenced clinical tests with CONVIVO were carried out under IRB and/or approved by an ethics committee.

International edition: Not for use in US.
2. Technical fundamentals of confocal endomicroscopy

Confocal laser scanning microscopy (CLSM) 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.

For this reason, the miniaturization of the CLSM principle was successfully achieved in a hand-held probe of the endomicroscopy systems FIVE 1 and CONVIVO. The probe 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).

In comparison to FIVE 1, CONVIVO 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.

The confocal imaging with FIVE 1 and CONVIVO works on a technical level in the following way (Figure 2):

Light is produced by a laser source (488 nm wavelength) and is transmitted through an optical fiber to the tip of the scanner probe. The fiber end is moved quickly by electromagnets in an XY scanning pattern. The light emanating from the fiber end is focused via a lens system 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 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.
Figure 3. Main components of CONVIVO including sterile sheath. (1) Cart, (2) touchscreen monitor, (3) foot control panel, (4) scanner unit, (5) scanner probe, (6) coupler unit, (7) sterile sheath

The mobile cart and the scanner unit (Figure 3) are the main components of CONVIVO. 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).

CONVIVO 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).

It should be mentioned that FIVE 1 and CONVIVO are Class 3R laser products (not requiring personal protective equipment) with a nominal optical hazard distance (NOHD) of 32 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.

3. Use of fluorescent dye for confocal endomicroscopy

In order to create a visible contrast, a fluorescent dye which stains cells and surrounding interstitial areas differently, is required to visualize tissue and cellular structures with FIVE 1 and CONVIVO. A variety of different fluorescent agents including fluorescein sodium (FNa), acridine orange, acriflavine and FITC- or Alexa Fluor 488-labeled antibodies have been successfully tested with FIVE 1 on brain tissue\textsuperscript{1-3}. While their spectral characteristics fit well to the laser wavelength (488 nm) and emission filters used in FIVE 1 and CONVIVO, 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 excitation. According to the current state of research, the Digital Biopsy Tool CONVIVO 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.

For more than fifty years, FNa has been extensively and safely used for retinal angiography. 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 effusion of the dye within 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 basis for visualizing the tissue microstructure with the FIVE 1 and CONVIVO CEM systems is also the leakage of FNa into brain tissue at sites of a defective BBB, i.e. normal brain exhibits a very low fluorescence with the exception of blood vessels and some autofluorescent spots in cells$. However, the dye also appears to gradually leak into areas with intact BBB over time$. 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 usage of FNa for both macroscopic and microscopic fluorescence during the same operation, i.e. for visualization of tumor boundaries as well as of cellular structures, would be the logical conclusion. However, there is still insufficient experience in this respect since the observation of macroscopic fluorescence was not yet utilized in the in vivo clinical studies, which were performed with FIVE 1 in neurosurgery. 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.

$4$ 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.

4. Utilization during in vivo use
4.1. Experience from pre-clinical and patient studies
For use with CONVIVO, fluorescein sodium (FNa) using intravenous injection might be the preferable fluorescent agent, as pointed out above. 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 FIVE 1, we will shortly describe the first experience with the CONVIVO system afterwards.
The initial experience with the FIVE 1 system for confocal imaging in a mouse model of glioma was reported in a study from 2010 Sankar et al. 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.

In 2011, Sanai et al. reported the first application of FIVE 1 in humans, using i.v. injection of FNa in 33 patients. 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. The same group also showed on a large cohort of patients that CEM may make it feasible to distinguish different pathologies with high accuracy and to diagnose gliomas and meningiomas with a specificity and sensitivity above 90% and comparable to those of frozen sections.

Following up the promising intraoperative results of the FIVE 1 system, experimental gliomas in anesthetized mice were investigated using CONVIVO and FNa injection. The results were very encouraging (Figure 5). In addition, in a blinded observer study with CONVIVO it was demonstrated that tumor and normal brain architecture could be distinguished well in mice. Furthermore, preliminary experience gained ex vivo indicates that also the tissue microstructure of human samples can be observed very well with the CONVIVO device (Figure 6).
4.2. Anticipated workflow for the in vivo use of CONVIVO in human patients

The workflow outlined here is based on previous experience with FIVE 1 in human subjects as well as on the findings made with CONVIVO in anaesthetized mice and on human ex vivo samples.

We foresee several potential ways, how CONVIVO could support the surgery of brain tumors and complement frozen sections, although its efficacy for these purposes still needs to be proven in future clinical trials. First, CEM could be used to screen regions prior to performing biopsies. In this way the risk of collecting non-diagnostic tissue samples for frozen/permanent sections or biobanking might be reduced. Second, CEM could potentially be used to visualize tumor margins and may allow the interactive detection of small tumor areas and even disconnected islands of tumor cells in the infiltration zone. And third, CEM might be used to distinguish different tumor types intraoperatively thus allowing the neurosurgeon to refine the resection strategy at an early point.

The workflow for in vivo use during the surgery of brain tumors is expected to look like this:

1. System preparation: Before (or during) the surgery CONVIVO is positioned in the OR and prepared for operation by a circulator nurse using the touch screen, i.e. start-up, functioning test, entering patient name, connection to hospital network via Ethernet cable. Preferred default settings (laser power, gain, etc.), which are loaded during system start, can be set using an administrator account. Afterwards, the scanner probe is draped with the sterile sheath and the CEM system is now ready for use.

2. Administration of FNa. The dye is currently applied in accordance to the dosage used for established standard diagnostic procedures. Optimization of timing and dosage will be investigated within planned clinical investigations.

3. CEM Imaging: On one or several occasions during the resection of the tumor the neurosurgeon decides to perform digital biopsies with the CEM system. The scanner probe is handed to the surgeon and scanning is switched on.
   a. The scanner probe is placed gently on the brain surface in order to observe cellular structures in real time on the monitor.
   b. The position of the scanner probe in the situs is controlled by looking at the confocal images produced in the live view and by observing the scanner probe through the eyepiece of the surgical microscope or with the naked eye. If the Robotic Visualization System KINEVO 900 is used, both image modalities can also be displayed as a picture-in-picture mode on the KINEVO 900 screen or an external monitor. CONVIVO images can also be displayed directly on an external monitor. In addition, neuronavigation can be employed to localize the probe tip (see following chapter).
   c. Image recording: A foot control panel allows the surgeon to control the focus depth and to acquire images in 3 different modes: single images, image series and Z-stacks (all of which can also be accessed via the touch screen). Importantly, if using a KINEVO 900 or a PENTERO 900, a macroscopic photo of the surgical bed can be taken synchronously with the acquisition of CEM images.
   d. The circulator nurse can use the touch screen adjust settings for optimal image quality.

4. Reviewing and forwarding confocal images: The surgeon reviews the images acquired at one or multiple regions in situ. Selected images are exported to a shared drive in the hospital network or to the PACS server.

5. Feedback by a pathologist: The pathologist examines the confocal images via remote access and gives feedback to the neurosurgeon.
4.3. Further remarks regarding the clinical use of confocal endomicroscopy

First, the scanner probe can be operated in a free-hand fashion or mounted onto a retractor system\textsuperscript{6, 8}. Using a retractor which is tightened to a degree that allows both smooth movement and steady operation helps to diminish motion artifacts. Therefore, it has been reported that the images acquired in this way were initially of a higher quality compared to handheld operation. However, the latter is easier and the quality of the images improved with growing experience of the neurosurgeon\textsuperscript{8}.

Second, CEM allows digital biopsies to be performed at multiple spots in the situs, although the complete resection cavity is not covered due to the small field of view. However, the latter is not necessary in any case since CEM is designed to deliver high-value information from selected regions\textsuperscript{8}. Still, the number of spots that can be examined with CEM is almost unlimited since it does not require extraction of (potentially healthy brain) tissue or cumbersome sample processing - unlike frozen section analysis.

Third, intraoperative MR neuronavigation has been successfully employed to track the position of the probe tip during CEM imaging with the FIVE\textsuperscript{1} device. Results obtained with this system correlated well with neuronavigational imaging [6] (despite potential brain shift affecting the latter). Access to this information might also help the pathologist to interpret CEM images.

CONVIVO additionally provides besides technical improvements (design optimized for usage in the OR, completely new graphical user interface, improved resolution, etc.) some new features which are expected to constitute additional benefits for both neurosurgeons and pathologists: First, the possibility to acquire Z-stacks, i.e. the automated recording of a range of images from different focus depths, means the surgeon no longer needs to select the best focal plane for image interpretation. In contrast, the option of recording continuous image series may be useful for monitoring processes over time or simply storing all images during a session. Second, the video connection to external monitors and KINEVO\textsuperscript{900} (including picture-in picture display of the views of CEM and surgical microscope) allows the surgeon to adapt the data presentation to his/her needs. Third and probably most importantly, CEM data can be exported via network and reviewed by a pathologist via remote access (e.g. a VPN client). This could make the pathologist much more flexible since he would not need to be present in the pathology office in order to examine images.

A major advantage would be, if intraoperative CEM could be used in combination with macroscopic visualization of FNa fluorescence. In this case, a single drug could be employed for two different purposes during the same operation. However, further studies are needed regarding optimal dosage and timing during the procedure (see chapter 3).

4.4. Optimizing image quality

As discussed in the previous chapter, steady handling of the probe is important in order to avoid motion artifacts. This is expected to improve with increasing practice. In addition, it is advisable to change the probe position several times to acquire optimal images from a given area\textsuperscript{8}. Since red blood cells can confound the interpretation of CEM images it is recommended to irrigate the tissue field with saline and/or to clean the tip of the sterile sheath\textsuperscript{3}.

The optimal focus depth may be dependent on the type of tissue. However, the best images are usually obtained relatively close to the tissue surface since scattering and absorption lead to blurring and a reduction in fluorescence intensity when focusing deep into the tissue. Recording a Z-stack at each suspicious area makes it possible to cover a range of focal planes, from which the pathologist can then select the most suitable one.

Three parameters can be changed in the graphical user interface to optimize the image intensity/brightness (Figure 7):

1) Laser power: High laser power generally yields strong fluorescence intensity. However, prolonged exposure of a region to strong light can result in photobleaching of the dye. Therefore, the laser power should be reduced to the lowest level that still produces optimal results. 2) Brightness: The brightness of the image can be manually balanced using the “brightness” slider. If the auto brightness control is switched on, the software will automatically attempt to adjust the brightness in an optimal way. 3) Gain: This setting allows adjustment of the sensitivity of the detector. If the concentration of the dye in the tissue is very low, the gain can be increased, resulting not only in stronger signals but also in more noise.
Regarding optical filters, it is recommended to use the green bandpass filter for detecting FNa fluorescence as this filter provides images with the highest contrast.

The resolution should always be set to the highest available resolution when images are recorded. For screening tissue in live view, a lower resolution mode with a higher refresh rate can be chosen (Table 1).

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<td>1920 x 135</td>
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5. Interpretation of images

The visualization of tissue architecture is enabled by CEM 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\(^2\). However, observations made in mice and human ex vivo samples indicate that FNa may also accumulate in certain cell types following prolonged exposure.

The cellular and architectural characteristics of many types of intracranial tumors can be visualized by CEM 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, CONVIVO 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\(^1,6\).

Like for conventional H&E-stained sections, the interpretation of CEM images requires extensive 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 CONVIVO, 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.

Via remote access CONVIVO allows the pathologist to conveniently examine images 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.

6. Conclusion

CEM is a powerful technology with 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.
7. References


8. Abbreviations

FNa: Fluorescein sodium

NOHD: Nominal optical hazard distance

CEM: Confocal endomicroscopy (equivalent: CLE: Confocal laser endomicroscopy)

CLSM: Confocal laser scanning microscopy

ICG: indocyanine green

5-ALA: 5-aminolevulinic acid

H&E: Hematoxylin and eosin stain