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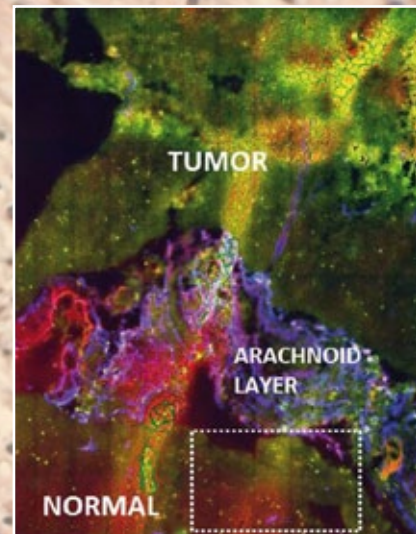
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Optical Biochemical Imaging: Potential New Applications in Neuro-Oncology

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Abstract: For the surgical treatment of malignant gliomas and adjustment of adjuvant therapies it is crucial to characterize the tumour as precisely as possible. This includes the determination of the exact tumour location as well as the analysis of its properties in order to define an accurate diagnosis as early as possible in the treatment process. New, purely optical, non-invasive techniques allow for the label-free analysis of tissue and are promising for neurosurgical applications. These techniques may be helpful for neuropathology and have the potential to be used for intraoperative *in situ* diagnosis of suspicious areas without

the need for removal biopsies. The ability of linear (Fourier-transform infrared, Raman [FT-IR], Raman), and non-linear (coherent anti-Stokes Raman scattering [CARS], surface-enhanced Raman scattering [SERS]), optical, vibrational spectroscopy, also in combination with multiphoton technologies (second harmonic generation [SHG], two-photon-excited fluorescence [TPEF]), to characterize brain tumours has already been shown in animal models of experimental glioma and on different human brain tumour entities. Based on biochemical composition, glioma tumours can be identified and characterized, the borders towards normal

brain tissue and infiltrative areas can be discerned with cellular resolution and morphological details help to identify functional fibre tracts. Label-free, optical biochemical imaging technologies can provide clinically relevant information and need to be further exploited to develop a safe and easy-to-use technology for intraoperative *in situ* diagnosis of malignant glioma. **Eur Assoc NeuroOncol Mag 2014; 4 (1): 20–6.**

Key words: brain tumours, intraoperative imaging, vibrational spectroscopy, coherent anti-Stokes Raman scattering (CARS)

■ Introduction

Malignant gliomas are a severe and life-threatening pathology because of their invading properties and limited intracranial space. To increase the probability of a positive outcome and to provide optimal, personalized therapy, it is crucial to precisely determine tumour localization and boundaries as well as the tumour's exact properties and characteristics. This information is required in the clinical time course as early as possible to adjust therapy accordingly.

Gliomas are typically resected by surgery followed by the application of adjuvant therapies, ie, irradiation and chemotherapy [1]. It is evident that especially high-grade malignant gliomas have to be removed as completely as possible while preserving the surrounding functional brain [2]. Preoperative MRI images provide information about the localization and size of the tumour. These images can be used for neuronavigation during surgery but they cannot compensate for intraoperative tissue changes and alterations, eg, shifts [1]. Intraoperative MRI requires profound constructional changes of the operating theatre, and special, expensive equipment [3]; it is time-consuming (15–30 min) and is used to optimize the extent of resection [4], ie, for resection control and not during ongoing surgery. Information about localization of certain tumour types can also be obtained by fluorescence monitoring: glioblastoma multiforme, the most malignant brain tumour in adults, accumulates 5-aminolevulinic acid and can be visualized intraoperatively by the fluorescence of the resulting me-

tabolites [5]. Other fluorescence dyes take advantage of tumour vessel depiction (indocyanine green) or of vascular leakage into the neoplastic tissue (fluorescein) and may be suitable for various brain tumour types, but none is commonly used for tumour resection [6]. The information obtained by intraoperative administration of fluorophores or contrast agents can be improved by the application of advanced optics like confocal or multiphoton technologies that permit to visualize fine structural details like cytoarchitecture [7]. Nevertheless, there is no method available that allows the localization of every type of brain tumour during surgery. The exact delineation between normal and tumorous tissue during surgery is still an unsolved problem. Additionally, it is not possible to detect small micrometastases before the breakdown of the blood-brain barrier with the technology clinically available [8, 9].

To optimize the resection strategy and decide upon radical resection of aggressive and highly malignant glioma, histopathological analyses are performed during surgery. Therefore, a biopsy sample of suspicious tissue is removed, tissue smears or sections are prepared and stained, and the tissue morphology is evaluated by a trained pathologist [10]. However, this method is time-consuming (approximately 30 min) and of a retrospective nature, ie only applicable after removal of the tissue, and does not allow for precise cancer recognition in those cases when representative specimens cannot be obtained. On the other hand, neurosurgery renders it possible to visually access the tumour and therefore theoretically opens the possibility to perform *in situ* diagnosis without tissue removal by applying non-invasive optical analysis of suspicious tissue.

Microscopy is a well-established technique to assess morphology of cells and tissue and to research and diagnose diseases of all kinds. Usually, special dyes are required to generate contrast in the substantially transparent tissue. There are a variety of dyes to visualize certain biochemical compounds or classes of compounds, ie classical stains discern basic or acidic compounds or antibodies are coupled to certain fluorophores or are visualized by chemical dye reactions

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and address very specifically markers on the cell surface or within the cells. Other dyes can be used to follow tracts and emphasize structural differences. However, most dyes are not approved for clinical use. New advanced optical methods allow the label-free investigation of tissues and have the potential for histopathological diagnosis [11] and even intravital application, a property that makes them useful in neurosurgery. They address biochemical properties (vibrational spectroscopy [12], coherent anti-Stokes Raman scattering [CARS], two photon-excited fluorescence [TPEF], second harmonic generation [SHG] [13]) and, to a certain extent, also morphological properties (CARS, TPEF, SHG) while other techniques focus on physical properties of the tissue (optical coherence tomography [14], intraoperative optical imaging [15]). Therefore, the present research focuses on the development of advanced, label-free imaging techniques for brain cancer pathology assessment and new tools for *in vivo* identification of glioma margins.

We present an overview of new biochemical imaging techniques that are already in use for experimental brain tumour research (vibrational spectroscopy, CARS, TPEF, SHG) and have the potential to be integrated in routine procedures during treatment and diagnosis of human malignant glioma [16].

Vibrational Spectroscopy

Vibrational optical spectroscopy comprises a series of different methods. In contrast to conventional staining methods they offer overall molecular specificity and utilize the interaction of electromagnetic radiation with the sample constituents to reveal chemical composition [17].

Specific vibrations of chemical bonds within the sample lead to characteristic alterations in electromagnetic radiation used for excitation and are visualized by a specific pattern of bands in the corresponding spectra (Figure 1). In case of biological samples and tissues, which consist of a variety of different biochemical compounds, vibrational spectra are the products of the complex overlap of multiple bands of the chemical bonds of all tissue constituents. Therefore, vibrational spectra comprise the entire information about cell or tissue biochemistry and are referred to as biochemical fingerprint.

Besides the identification of chemical compounds, also quantitative information is provided by vibrational spectroscopy. Changes in band amplitude are proportional to the concentration of a particular compound or functional group. Consequently, the spectra of biological specimens reflect both the structural complexity of the individual components and their relative abundances. Vibrational spectroscopy is regarded as the analytical method with the highest density of information.

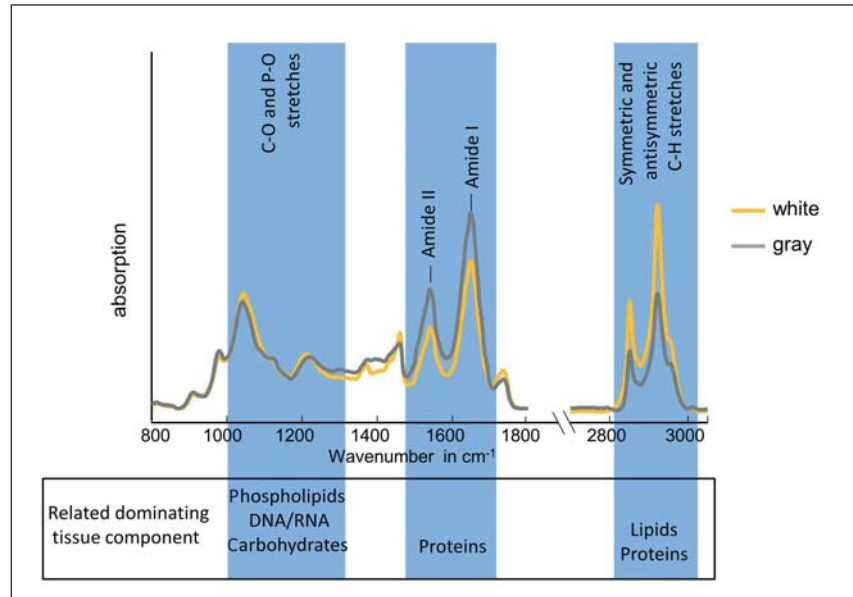


Figure 1. FT-IR spectra of grey and white matters of nervous tissue. The main bond vibrations and related dominating tissue components are indicated.

Spectroscopic information of several positions of a sample can be used to build images that visualize the underlying biochemical composition. Each point of the spatially resolved data set, the hyperspectral cube, contains the information of one spectrum. Usually, the application of advanced chemometric tools is required to extract clinically relevant features from the spectral datasets that can be displayed in different types of spectroscopic images [18]. Therefore, advances and research of new applications go hand in hand with advances in computer power and speed that are necessary to handle and analyze large datasets. Vibrational spectroscopic techniques therefore have been gaining increasing attention for biomedical applications, investigation of disease mechanisms, and diagnosis over the past years [17, 19].

Fourier-Transform Infrared Spectroscopy

Fourier-transform infrared spectroscopy (FT-IR) is based on the absorption of infrared radiation by the sample and its use in biomedical science has been studied for many decades. Starting with the investigation of single compounds, technical progress and advances in computational science permitted to analyze more complex tissue samples and to apply sophisticated analytical methods to large datasets. The entire compositional information about the biochemistry of nervous tissue contributes to FT-IR spectra. Figure 1 shows the typical FT-IR spectrum of grey and white matter. Bands in the region of 1000–1350 cm^{-1} are dominated by the vibrations of phosphate groups and carbohydrates and indicate mainly the presence of phospholipids, DNA/RNA, and carbohydrates in the context of cells and tissue [20]. Prominent bands of amide II and amide I bond vibrations of proteins are recognized at around 1550 and 1650 cm^{-1} , respectively. In the high-energy region from 2800–3000 cm^{-1} , bands related to C-H bond vibrations in lipids and proteins are found. Applied as an imaging technique FT-IR provides spatially resolved information about distribution of tissue constituents (Figure 2).

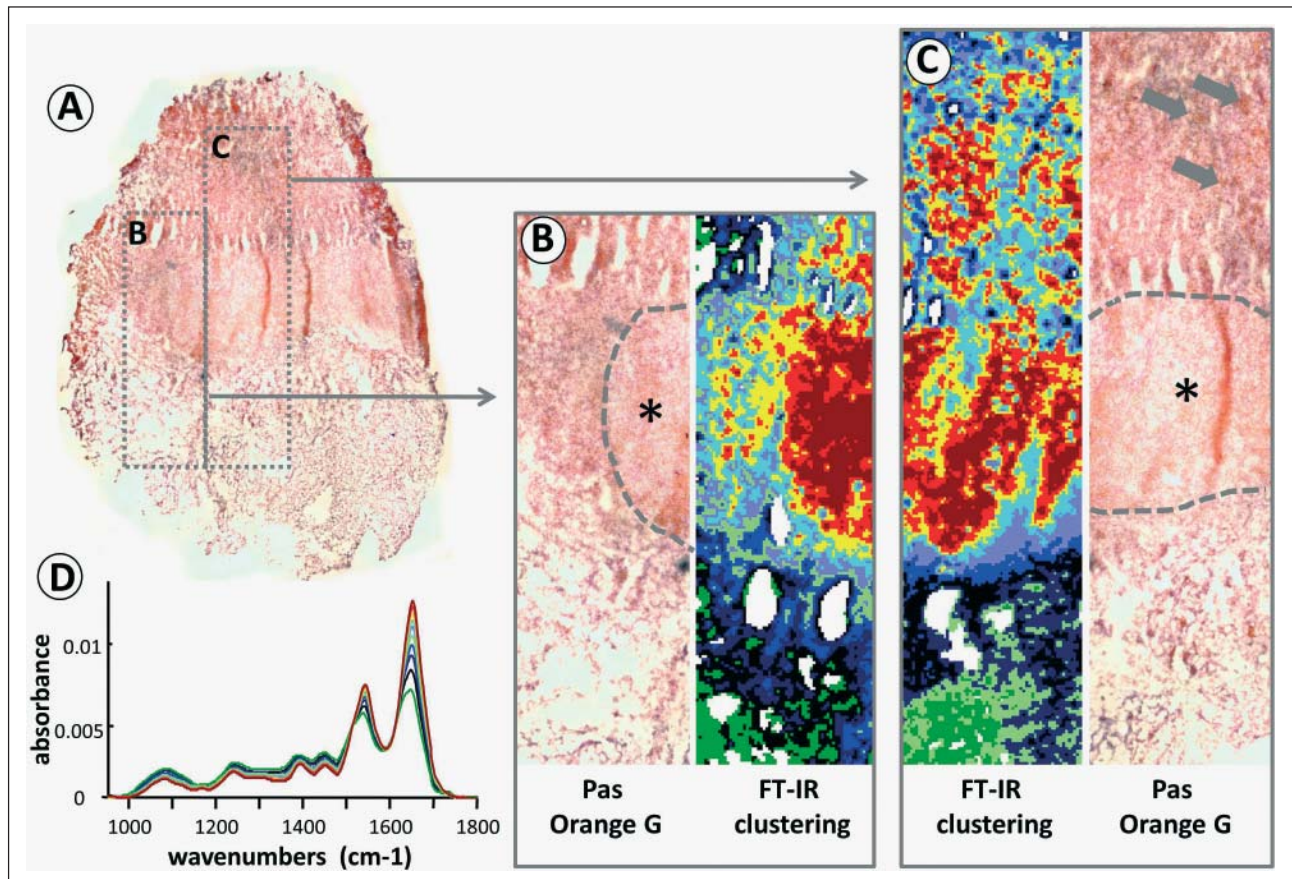


Figure 2. FT-IR imaging of human pituitary adenoma. (a) Overview of a cryosection of human pituitary adenoma stained using Pas-Orange-G and illustrating the production of human growth hormone (HGH). (b, c) High magnification of the areas indicated in (a) and corresponding FT-IR spectroscopic images obtained by cluster analysis. Large areas with increased HGH production are indicated by asterisks, local spots of hormone production are marked by arrows. (d) Representative spectra corresponding to area (b).

FT-IR spectroscopy has been intensively used to investigate brain tumours. Primary brain tumours as well as brain metastases of peripheral tumours can be localized and discerned from normal brain tissue with high specificity [21–23]. In case of brain metastases, the type of primary tumour can be identified [24]. The main components that allow differentiation of normal and tumour tissues and tumour-grading are the tissue lipid content and changes related to nucleic acids [25]. Additionally, collagen content and distribution of collagen subtypes are altered in brain neoplasms and can be analyzed by FT-IR spectroscopy [26].

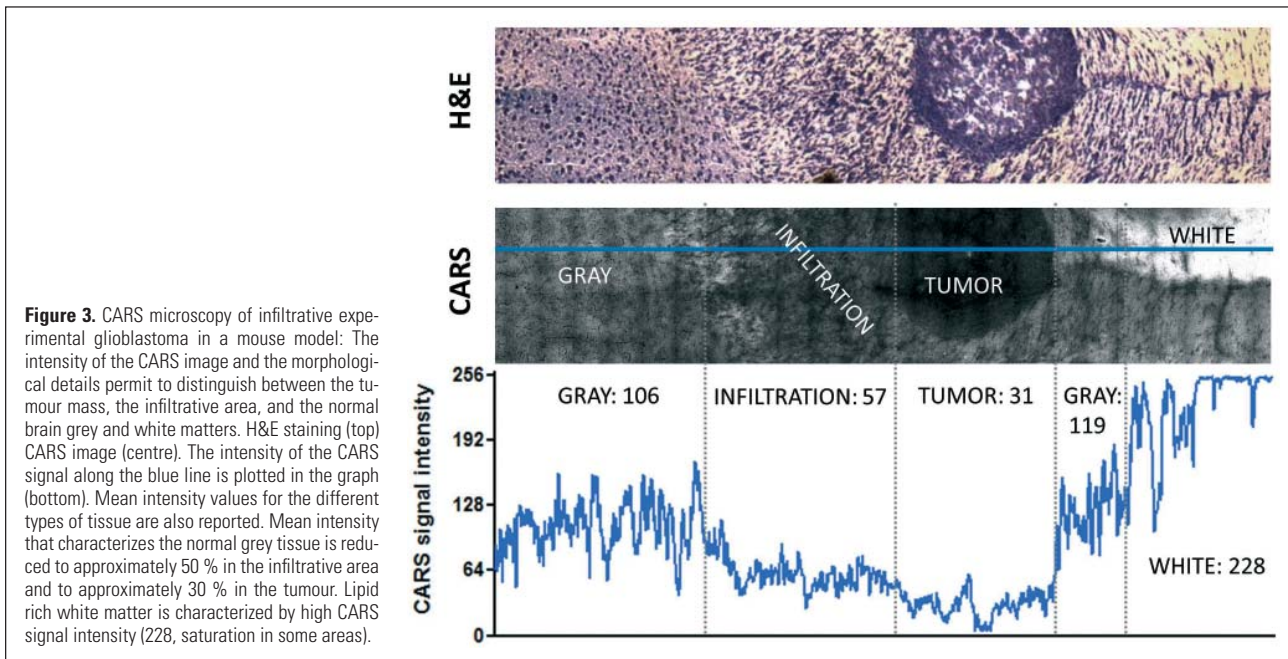
It was also possible to gain diagnostically relevant information, eg, the glioma grade, expression of hormones, or tumour vascularization can be assessed [27–29]. Figure 2 shows a tissue section of a human pituitary adenoma. Not only the tumour itself was identified but also the information about the pathologically and clinically highly relevant increased hormone production could be extracted from the spectral information using chemometrical analysis [28]. Cluster analysis sorts all spectra according to similarities and was used to build colour-coded maps of the tumorous tissue. Here, red pixels can be related to human growth hormone- (HGH-) producing tissue areas. In contrast, HGH-negative tissue areas are recognized in the blue clusters (Figure 2b,c). For cervical cancer, it has already been shown that infrared spectroscopy is able to make predictions at the molecular level [30].

ATR FT-IR

Attenuated total reflection FT-IR (ATR FT-IR) is a variant of infrared spectroscopy and offers the advantage of measuring non-transparent samples, eg, bulk tissue. This technique requires tight contact between the sample of interest and the core of the ATR device, the ATR crystal. Infrared radiation propagates in the crystal, generating an evanescent wave that penetrates a few micrometres of the sample. Spectral changes in the backscattered light are used to obtain information about the sample's biochemical properties. This is especially interesting for direct analysis of biopsy tissue [31]. Moreover, ATR FT-IR spectroscopy can be performed using a fibre optic probe [32] and can be implemented in an endoscopic setup. Therefore, it holds great potential for future clinical application and *in situ* diagnosis of malignant glioma. First results using optical ATR FT-IR spectroscopy for the analysis of native human brain tumour biopsies indicate the feasibility of this approach. It was possible to obtain high-quality spectra within minutes after tissue removal and to extract spectral differences in bands related to extracellular matrix components among different tumour types [31].

Raman Spectroscopy

Raman spectroscopy is based on the inelastic scattering of light and uses continuous wave lasers in the near-infrared for



excitation. In the Raman spectra, vibrational bands are better separated than in FT-IR spectra, therefore the spectra comprise a higher degree of information. Raman investigation of tissue permits to discriminate healthy from tumour and necrotic tissues in rat brain tissue samples [33] and was used to study brain functions in living mice and rats [34]. Brain injury caused by traumatic insults related to caspase-3-activated apoptosis can also be detected by Raman spectroscopy [35]. It has been demonstrated that Raman mapping can identify brain tumours in the living animal [36]. *Ex vivo* studies on human brain tumour samples have proven the ability of the technique to discern normal and tumorous tissues of adults [37, 38] and children [39]. Raman microspectroscopy of primary brain tumours can provide diagnostic information on the malignancy grade and cell density [40].

It has to be emphasized that the technique can be applied on native, non-dried tissue because the spectral contribution of water does not interfere with relevant bands of biological tissue as it does in FT-IR spectroscopy. Thus, artefacts due to drying and crystallization can be avoided [41] and, more importantly, this property makes Raman spectroscopy suitable for *in situ* diagnosis. First trials using fibre optic probes for *ex vivo* Raman spectroscopy of fresh human tumour samples proved the potential of the technique for grading of astrocytoma [42]. Current research focuses on the application of Raman spectroscopy to perform optical biopsies for tumour recognition [40], not only for brain tumours but also for other neoplasms eg, breast, skin, cervical, gastrointestinal, oral, and lung cancers [43].

Technical advances in the development and miniaturizing of Raman fibre probes may allow short acquisition times of approximately 10 s in concert with high-quality spectra acquisition [44, 45]. For gastric cancer diagnosis, Raman endoscopy has already been performed in a clinical context (> 300 patients) and provided diagnostic information [46].

Different techniques aim at enhancing Raman signal intensity in order to reduce acquisition time. Surface-enhanced Raman scattering (SERS) exploits the electrochemical interaction of molecules adsorbed by nanostructures. Applying the sample of interest onto a suitable surface enhances the Raman signal by as much as approximately 10^{10} . This technique is applicable for the analysis of chemical substances or single cells, but not for large tissue samples. Additionally, nanoparticles and compounds that exhibit strong SERS signals were employed as alternatives to fluorescent or colorimetric markers, and used for the detection and research of cancer and other diseases, eg, to visualize the distribution of known markers detected by classical immunohistochemistry [47]. In this context, spectroscopy is not used to reproduce tissue properties but to detect and reproduce the distribution of experimentally introduced compounds in a sample.

Resonance Raman spectroscopy exploits the amplification of the Raman signal that takes place when the energy of the exciting laser beam approaches the optical band gap of a tissue constituent, selected by appropriate tuning of the excitation wavelength. The Raman signal intensity is increased around 1000-fold and the resulting Raman spectrum is dominated by the bands of the resonance-enhanced molecule. Therefore, the main advantage over conventional Raman spectroscopy is mainly the ability to detect specific compounds at very low molecular concentrations, such as flavins, NADH, collagens, elastin, carotenoid, and the heme proteins [48].

The intrinsic characteristics of vibrational spectroscopic techniques, eg, being label-free and non-invasive, make them attractive for biomedical applications. However, these technologies generally require long exposure times and their lateral resolution is rather limited. Even if they offer great potential for detailed objective tissue classification in neuropathology (where time is not an issue) and are promising for *in situ* diagnosis of single suspect spots (lateral resolution/multiple acqui-

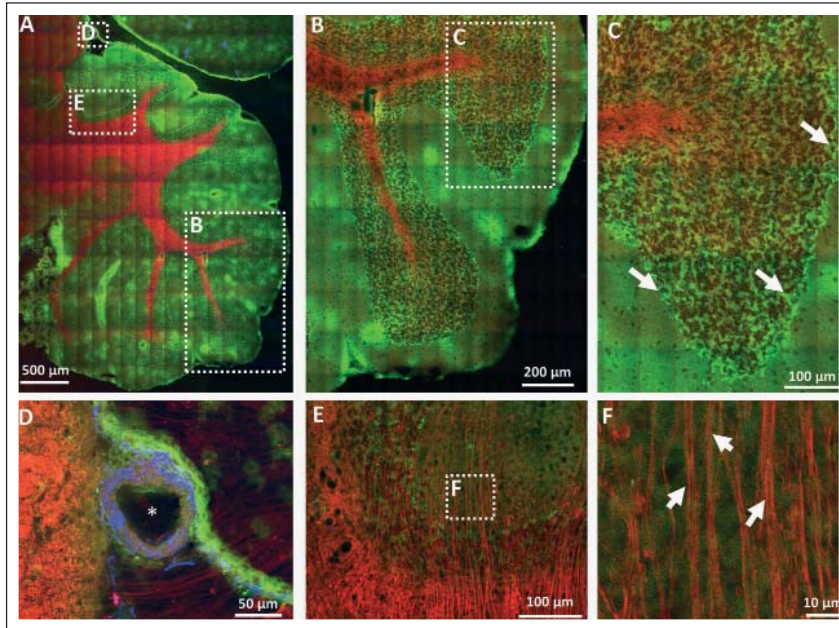


Figure 4. Multimodal NLO imaging of a mouse cerebellum (red: CARS, green: TPEF, blue: SHG). **(a)** Overview of the mouse cerebellum. **(b)** Magnification of the area indicated in **(a)**. **(c)** Magnification of the area indicated in **(b)**. Arrows indicate Purkinje cells characterized by punctuate fluorescence in the somata. **(d)** Magnification of the area indicated in **(a)**, a large blood vessel is shown (*). **(e)** Magnification of the area indicated in **(a)**, nerve fibres are indicated by intense CARS signal. **(f)** Magnification of the area indicated in **(e)**. Single axons can be discerned (arrows).

sitions are not required), their usefulness as an intraoperative imaging technology is limited.

■ Imaging Technologies

CARS Imaging

Nonlinear imaging techniques based on chemical contrast, such as coherent anti-Stokes Raman scattering (CARS), can overcome these limitations. CARS is a non-linear variant of Raman spectroscopy [49, 50]. It is based on resonant excitation of a single Raman band by using ultra-short tunable lasers and enables rapid acquisition of images. CARS images recorded at 2850 cm^{-1} mainly probe the spectral contributions of lipids [51, 52], which are important diagnostic markers of brain tumours [37], and enable visualization of tumour boundaries [51, 53]. In the CARS image, the tumour appears as a dark area surrounded by normal nervous tissue. This allows detection of primary brain tumours (gliomas), secondary brain tumours (metastasis) and of the tumour border with cellular resolution [54]. CARS imaging also permits to detect the infiltration of a tumour into normal, preserved brain areas. Figure 3 shows the CARS image and the corresponding H&E staining of a glioblastoma experimentally induced in a mouse brain. In the CARS image, the tumour can be recognized by its low CARS signal (dark region) and size and location matched the histological findings. The infiltrative zone was also characterized by a lower CARS signal intensity than in normal grey matter. CARS signal values along the blue line are shown in the graph and reveal the potential of CARS imaging for the development of an objective methodology for glioma analyses and characterization. Other tissue components may also be addressed by multiplex CARS and allow to address tumour-specific signals [55].

For possible intraoperative application, it is of ultimate importance to remember that CARS is a purely optical technique that requires no labelling but visualizes intrinsic tissue properties. So far, no phototoxic effects of CARS imaging have been shown to develop at the settings required for *in vivo* application and diagnosis [56].

Multimodal Nonlinear Optical Microscopy

CARS offers high-resolution imaging at video rate and can be combined with other nonlinear optical (NLO) microscopic methods on a single platform, allowing to retrieve a large amount of morphological and biochemical information from unstained, native tissue [53].

For multimodal NLO microscopy, the CARS signal (usually biochemical imaging of $-\text{CH}_2$ functional groups, ie, mainly lipids) can be acquired with other nonlinear processes that are simultaneously excited by the pulsed laser sources used to induce the CARS signal. Acquisition of endogenous TPEF provides a nonspecific signal that contributes to the visualization of tissue structure and morphology. In some cases, endogenous fluorescence can be directly correlated with a certain cell type like inflammatory microglia/macrophages [57] or subcellular structures, eg, mitochondria, or elastin fibrils. Sources of endogenous TPEF within nervous tissue are, among others, NADPH, FAD, lipofuscin, or Schiff's bases. Endogenous fluorescence has already been used to discern the structure of normal brain and glioblastoma [58]. Acquisition of SHG allows the selective visualization of fibrillar collagen, which constitutes an important marker for malignant glioma [26] and indicates the presence of fibrous structures or connective tissue, as well as of tissue vascularisation due to adventitial collagen.

Figure 4 shows multimodal NLO images of a mouse brain cerebellum at different magnifications to illustrate the technology's potential. Detailed information about tissue structures and morphology can be obtained without the application of any labels or dyes on unstained, native brain tissue. CARS imaging (red) permits to visualize myelin-rich fibre tracts, allowing to access overall cerebral layering (low resolution, Figure 4a), fibre alignment (Figure 4e), as well as single axons (high magnification, arrows in Figure 4f). Endogenous fluorescence of the tissue (TPEF, green) also contributes to the visualization of the layering of the cerebellum (Figure 4b). Cell nuclei are lacking fluorescent signals and appear as dark spots. In contrast, Purkinje cells of the cerebellum exhibit a marked, punctuated fluorescence pattern in cytosol (Figure 4c, arrows). Large blood vessels can be recognized by second harmonic generation signal (SHG, blue) caused by collagen fibres of the vessel wall (Figure 4d).

Multimodal NLO microscopy, integrating CARS, TPEF, and SHG, was used for pathological assessment of human brain tumours on tissue sections and provided exhaustive imaging of the tumour morphology [54]. In the case of glioblastoma, it was possible to detect the border of the tumour by analyzing CARS signal intensity as shown for the orthotopic mouse model (Figures 5a,b). Additionally, numerous small blood vessels inside the tumour mass were identified by means of the SHG signal. Finally, in the case of neuroma (Figure 5c), it was possible to detect profound differences between tumour and normal nerve tissue: in the tumour, CARS and TPEF revealed the morphology of the tissue showing that the cells were loosely arranged in an interwoven pattern and SHG imaging visualized the extracellular matrix alterations typical of the tumour. These NLO images illustrate that multimodal biochemical imaging technologies can provide detailed information about tissue components and structures and permit to detect pathological tissue alterations. Automatized analyses allow to access diagnostically relevant tissue parameters like nuclear density and size in NLO images [59].

There are already commercially available solutions to investigate SHG and TPEF for the diagnosis of skin cancer (Dermainspect). Small, portable platforms integrating CARS, TPEF, and SHG imaging [60] as well as multiphoton endoscopes [61] have been developed and represent the first step towards bedside application of these new technologies.

Perspectives

At the present developmental status, CARS and multimodal imaging as well as vibrational spectroscopy can be employed in histopathology to gain additional information about brain tumour micromorphology and structure. Also the value of these label-free noninvasive methods for research needs to be recognized by the biomedical research community that usually applies labels or dyes to achieve visualization of tissue composition or of specific structures. Further research will then succeed in finding diagnostic and predictive markers using vibrational spectroscopy that is (compared to immunohistochemistry or molecular biology) cheap and fast.

The usefulness of biochemical imaging methods for neurosurgery has been widely demonstrated in proof-of-principle experiments with small sample sizes. Experiments have been performed *in vivo* in animal models [34, 36] and first studies have been performed on native *ex vivo* human brain tumour biopsies [31, 58]. Retrospective analyses indicate vibrational spectroscopy as a useful intraoperative tool for tumour border definition and detection of high-grade tumour residues [62]. To transfer these techniques into a clinical environment a large

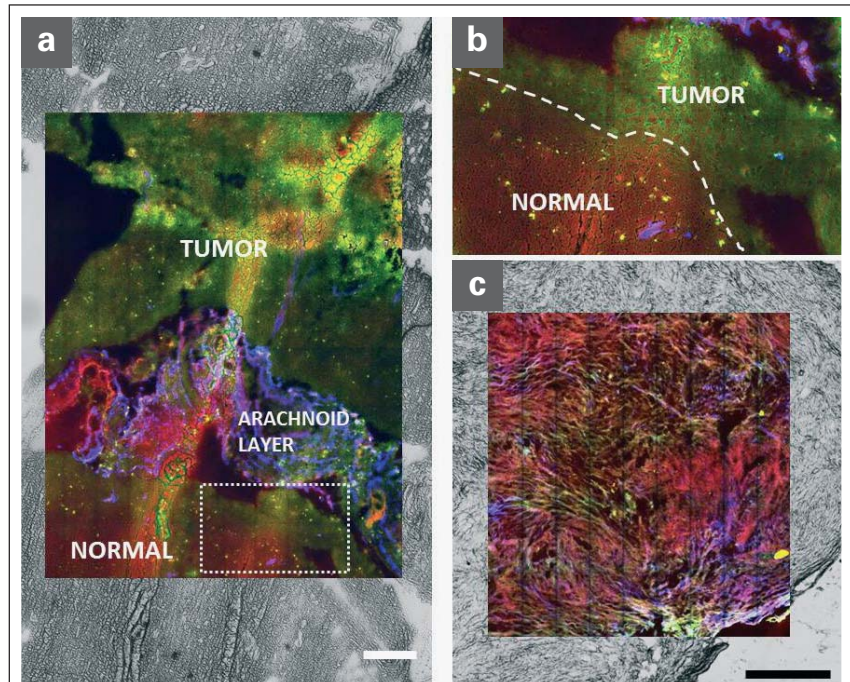


Figure 5. Multimodal NLO imaging of human tumours (red: CARS, green: TPEF, blue: SHG), overlaid with the bright field images of the unstained sample. The technique allows to retrieve detailed morphochemical information about tissue structure and properties on unstained samples. **(a)** Cryosection of human glioblastoma (scale bar: 200 μ m). **(b)** High magnification of the border between tumour and normal tissue as indicated in **(a)**. **(c)** Cryosection of human neuroma (scale bar: 0.5 mm).

set of glioma tumour samples is needed to match spectroscopic and clinical data of an extended set of patients.

In addition, technical engineering needs to improve miniaturisation for, eg, implementation in endoscopes, to improve scanning rates for usable imaging speed, and to verify the bioinformatics algorithms for data processing to extract and visualise relevant information in an adequate form to the neurosurgeon or neuropathologist. For routine intraoperative application, the surgeon needs an easy-to-handle device that delivers biochemical information about tissue status to be integrated in the established datasets, thereby facilitating and improving glioma resection. The compatibility with existing systems like surgical microscopes and endoscopes as well as with neuronavigation is essential for implementation in clinical routine.

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Conflict of Interest

The authors state that no conflict of interest exists.

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