Linköping University Medical Dissertation
No. 1581

Fluorescence Guided Resection of Brain Tumors

Evaluation of a Hand-held Spectroscopic Probe

Johan Richter

Department of Biomedical Engineering
Division of Neurology, Department of Neurosurgery

Linköping University, SE-581 85 Linköping, Sweden
Linköping 2017

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- Evaluation of a Hand-held Spectroscopic Probe

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Cover photo: Flashlight effect of the hand-held probe under blue light from the microscope for fluorescence guided surgery (2012)

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Mathilda
und
Max und Niklas
och
Malva

*”Sometimes, the greatest problems
are the ones we do not see”*

(Jonna Bornemark; Swedish philosopher)

Abstract

Malignant gliomas grow infiltrative in the brain and can therefore not be completely removed by neurosurgical means. However, for an optimized oncological treatment it has proven useful to resect as much as possible of the tumor tissue. The identification of the tumor in the marginal zone is difficult but crucial. Studies have shown that visualization of the specific enhancement of 5-aminolevulinic acid (5-ALA) in the tumor can help to maximize the resection. The Department of Biomedical Engineering, Linköping University, has developed an optical hand-held probe (HHP) to identify tumor tissue with a high sensitivity by means of fluorescence spectroscopy.

The technical design and the optical properties of the probe were gradually developed in a standard neurosurgical setting during resection of malignant gliomas. The device could easily be implemented in the operating room, meeting all requirements in terms of sterile handling and without interference of any kind with other equipment. The integration of the device in a navigation system and its use in combination with a blue light surgical microscope were simple. Measurements in 27 operations during resection of malignant gliomas were compared to results from biopsies from the same tumor locations. The equipment was tested as a stand-alone device ($n = 180$), integrated in a navigation system or in combination with the blue light microscope ($n = 190$). A ratio calculated from the measurements enabled objective and comparable values for different tissue types, in correspondence with the findings from the histopathological examinations and in accordance with the navigation system as well as with the surgical microscope.

The marginal zone was explored and tumor fluorescence could be identified beyond the fluorescence as seen through the microscope. A higher sensitivity of the HHP was confirmed; the specificity was lower.

The combined use of the HHP with a navigation system and with a surgical microscope was beneficial.

Sammanfattning

Maligna hjärntumörer växer infiltrerande i hjärnan och kan därför inte helt avlägsnas genom kirurgiska operationer. För en optimerad behandling har det emellertid visat sig vara av värde att avlägsna så mycket som möjligt av tumörvävnaden. Identifiering av tumören i gränsszonen är mycket svårt, men avgörande. Studier har visat att visualisering av den specifika laddningen av 5-aminolevulinsyra (5-ALA) i tumören kan bidra till att maximera resektionen. Institutionen för Medicinsk Teknik (IMT) på Linköpings universitet, har utvecklat en liten handhållen optisk prob (HHP) för att identifiera tumörvävnad med hög känslighet med hjälp av fluorescens-spektroskopi.

Den tekniska konstruktionen och de optiska egenskaperna hos proben utvecklades stegvis genom testning i flera neurokirurgiska operationer för resektion av maligna gliom. Utrustningen uppfyllde alla krav när det gällde steril hantering i operationssalen och kunde användas utan störningar av något slag med annan operationsutrustning. Integreringen i ett navigerings-system och användningen i kombination med ett kirurgiskt mikroskop för fluorescens-styrd kirurgi var oproblematiska. Mätningar under 27 operationer vid resektion av maligna gliom jämfördes med resultat från biopsier från samma tumörtagningsställen. Utrustningen testades såväl som en fristående enhet ($n = 180$) och som integrerad i ett navigationssystem eller i kombination med mikroskopet ($n = 190$). En särskild kvot beräknad ur mätningarna möjliggjorde objektiva och jämförbara värden för olika vävnader, i överensstämmelse med resultaten från de vävnadspatologiska undersökningarna och i överensstämmelse med navigationssystemet såväl som med det kirurgiska mikroskopet.

Tumörernas gränsszon undersöktes och tumörfluorescens kunde identifieras bortom fluorescensen som mikroskopet visade. En högre känslighet hos HHP bekräftades; specificiteten var lägre.

Den kombinerade användningen av HHP med ett navigationssystem och med ett kirurgiskt mikroskop visade sig vara fördelaktig.

List of Publications

Paper I:

Ilias Michael, Richter Johan, Westermarck Frida, Brantmark Martin, Andersson-Engel Stefan, Wårdell Karin
Evaluation of a Fiber-Optic Fluorescence Spectroscopy System to Assist Neurosurgical Tumor Resections
Novel Optical Instrumentation for Biomedical Applications III / SPIE – International Society for Optical Engineering, Vol. 6631, 1-8, (2007)

Paper II:

Haj-Hosseini Neda, Richter Johan, Andersson-Engel Stefan, Wårdell Karin
Optical Touch Pointer for Fluorescence Guided Glioblastoma Resection Using 5-Aminolevulinic Acid
Lasers in Surgery and Medicine, 42:1, 9-14 (2010)

Paper III:

Richter Johan, Haj-Hosseini Neda, Andersson-Engel Stefan, Wårdell Karin
Fluorescence Spectroscopy Measurements in Ultrasonic Navigated Resection of Malignant Brain Tumors
Lasers in Surgery and Medicine 43:8–14 (2011)

Paper IV:

Richter Johan, Haj-Hosseini Neda, Hallbeck Martin, Wårdell Karin
Combination of Hand-Held Probe and Microscopy for Fluorescence Guided Surgery in the Brain Tumor Marginal Zone
Photodiagnosis and Photodynamic Therapy 18:185–192 (2017)

Related Publications

Wårdell Karin, Blomstedt Patric, Richter Johan, Antonsson Johan, Eriksson Ola, Zsigmond Peter, Bergenheim Tommy, Hariz Marwan

Intracerebral microvascular measurements during deep brain stimulation implantation using laser doppler perfusion monitoring
Stereotactic and Functional Neurosurgery, 85(6), 279-286 (2007)

Richter Johan, Haj-Hosseini Neda, Andersson-Engels Stefan, Wårdell Karin

Tumor resection with fiber-optic fluorescence spectroscopy system, ultrasound based neuronavigation and peroperative CT-scan
XVIII Congress of the European Society for Stereotactic and Functional Neurosurgery (2008)

Haj-Hosseini Neda, Richter Johan, Andersson-Engels Stefan, Wårdell Karin

Photobleaching behavior of protoporphyrin IX during 5-aminolevulinic acid marked glioblastoma detection
Journal of Photonic Therapeutics and Diagnostics V; SPIE, 716131/1-8 (2009)

Richter Johan, Haj-Hosseini Neda, Wårdell Karin

Fluorescence Spectroscopy based identification of Glioblastoma multiforme
American Association of Neurological Surgeons, Denver (2011)

Haj-Hosseini Neda, Lowndes Shannely, Richter Johan, Wårdell Karin

Blood interference in fiber-optical based fluorescence guided resection of glioma using 5-aminolevulinic acid
Proc. of SPIE, vol 7883 (2011)

Wårdell Karin, Zsigmond Peter, Richter Johan, Simone Hemm
Relationship between laser Doppler signals and anatomy during deep brain stimulation electrode implantation toward the ventral intermediate nucleus and subthalamic nucleus
Neurosurgery, (72), 2, 127-140

Haj-Hosseini Neda, Richter Johan, Hallbeck Martin, Wårdell Karin
Low dose 5-aminolevulinic acid: Implications in spectroscopic measurements during brain tumor surgery
Photodiagnosis and Photodynamic Therapy, 12/2, 209-214 (2015)

Richter Johan, Haj-Hosseini Neda, Hallbeck Martin, Wårdell Karin
“Fluorescence Guided Resection of Brain Tumors: Combination of Hand-held Spectroscopic Probe and Microscope”
Meeting of the Scandinavian Neurosurgical Society, Lund (2015)

Haj-Hosseini Neda, Milos Peter, Richter Johan, Hallbeck Martin, Wårdell Karin
A Multipurpose Guidance Probe for Stereotactic Biopsy Procedures
XXIIInd Congress of the European Society for Stereotactic and Functional Neurosurgery Madrid, Spain (2016)

Haj-Hosseini Neda, Milos Peter, Richter Johan, Hallbeck Martin, Wårdell Karin
Optical guidance for stereotactic brain tumor biopsy procedures- preliminary clinical evaluation
SPIE Photonics West (2017)

List of Acronyms

5-ALA	5-AminoLevulinic Acid
ALS	Amyotrophic Lateral Sclerosis
BBB	Blood Brain Barrier
CBTRUS	Central Brain Tumor Registry US
CNS	Central Nervous System
CSF	Cerebro Spinal Fluid
DTI	Diffusion Tensor Imaging
DWI	Diffusion Weighted Imaging
FGR	Fluorescence Guided Resection
FGS	Fluorescence Guided Surgery
GBM	Glioblastoma (multiforme)
Gy	Grey (unit of radiation)
HHP	Hand Held Probe
KPS	Karnofsky Performance Score
LED	Light Emitting Diode
MRS	Magnetic Resonance Spectroscopy
MEP	Motor Evoked Potentials
MS	Multiple Sclerosis
MGMT	O6-methylguanine–DNA methyltransferase
OR	Operating Room
OTP	Optical Touch Pointer
PNS	Peripheral Nervous System
PET	Positron Emission Tomography
PD	Proton Density Weighted Imaging
Pp IX	Protoporphyrin IX
QOL	Quality Of Life
SEP	Somatosensory Evoked Potentials
VEP	Visual Evoked Potentials
WHO	World Health Organization

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Introduction

During the course of this project the neuro-engineering group at the Department of Biomedical Engineering (IMT) and the Department of Neurosurgery at the University Hospital, Linköping, have evaluated a small optical probe, developed as a hand-held device (HHP) connected to an all-in-one combined light source with registration equipment including processing software. It was designed to identify specifically fluorescing brain tumor tissues with high sensitivity in order to optimize resection of malignant gliomas. The tool should enable objective grading of the signals registered in order to identify different tissue types and varying degrees of tumor infiltration in the marginal zone towards the normal brain tissue. The equipment was tested as a stand-alone system and together with other surgical systems. This thesis presents first measurements with the tool under real intraoperative conditions during brain tumor surgery. Gradual modification of the system was based on the early experiences from application of the system in patients. The subsequent implementation of the tool in combination with a navigation system and with a surgical microscope for fluorescence-guided neurosurgery was evaluated. The results of the research are presented in four papers published in international journals.

This thesis elucidates the clinical focus in the development of the hand-held probe system, as previously described in all technical aspects in the thesis ‘Fluorescence Spectroscopy for Quantitative Demarcation of Glioblastoma Using 5-Aminolevulinic Acid’, Linköping Studies in Science and Technology Dissertations, No. 1463 (Haj-Hosseini, 2012).

1 Anatomy

1.1 Central Nervous System

The central nervous system (CNS) consists of the brain and the spinal cord, both in turn consist of grey and white matter.

The grey matter consists of the cell bodies of neurons, the actual nerve cells and the actual processing of neural impulses has been presumed to be generated here. The white matter, formed by the bundles of axons that constitute the actual nerve fibers connecting the brain, has in the past been accredited only to facilitate the transport of the signals from the neurons and furthermore to bind the cerebral tissue together. In this sense the white matter has in the past been considered as merely supporting the neurons, but not otherwise influencing the neural activity (Allen and Barres, 2009, Petré, 1972).

In the brain the grey matter is on the outer surface and in different deep-seated nuclei or networks (Fig. 1). The volume ratio of grey to white matter varies with age between 1.3 to 1 and 1 to 1.5, reaching the highest relative amount of white matter around the age of 50 without any difference between the sexes (Miller et al., 1980).

Figure 1: Grey and white matters of the CNS are differently organized in the brain and in the spinal cord. The neurons are in the grey matter, as are the dendrites, while the longer axons are in the white matter.

1.2 Glia

Recent reports count $80-90 \times 10^9$ nerve cells in the adult human brain. The glia system has been estimated to account for 2/3 of the brain's weight and a relation of 10:1 glia cells for each neuron has been colported through textbooks for many years; both statements without clear evidence. Some recent authors claim to have found ratios up to 50-80:1 glial cells per neuron although others suggest ratios close to 1. The ratios seem to vary at different locations of the brain and at different ages and stages of development (Azevedo et al., 2009).

Both grey and white matter is built up from different types of glial cells in addition to the neurons and the axons. The white matter consists of the longer axons from the neurons while the shorter axons and the dendrites are located in the grey matter. The greater part of the mass of the white matter consists of myelin sheaths formed by glia cells that surround the axons. The glial cell systems of the CNS have different shapes and functions (Fig. 2).

Figure 2: Different glia cells have different functions in the CNS. Astrocytes seem to have the most complex relation to the neurons, axons and blood vessels. The most common and also the most malignant gliomas are associated with astrocytic origin.

The astrocytes ($\approx 40\%$) and oligodendroglia ($\approx 45\%$) form the category of what has been called the supportive tissue. The microglia ($\approx 10\%$) have phagocytic abilities and constitute the brains macrophages as a part of the immune-defensive system. The ependymal cells ($\approx 5\%$) shape the walls of the ventricular system including the central canal of the spinal cord.

Astrocytes are of two types, protoplasmatic and fibrillary. The first kind forms sheaths around synapses and enwraps blood vessels, creating the neuropil, i.e. non-myelinated glial areas in the grey matter. The second kind reaches axons in the white matter through the nodes of Ranvier and also enwraps blood vessels.

Oligodendrocytes build up the myelin sheaths around axons in the white matter forming interneuronal connections and tracts between different cortical areas and nuclei in the brain and spinal cord. In the white matter glia cells outnumber the neuronal tissue at a ratio of 10:1 (von Bartheld et al., 2016, Herculano-Houzel, 2014). The optical properties of the axonal sheaths giving the white color tone are related to the relatively high partition of lipids in the myelin cells (O'Brien and Sampson, 1965).

Astrocytes are active in forming synapses. They can ensheath a large number of synapses and by modulating the chemical conditions in the synaptic gap the electric signals between neurons and along the dendrites and axons may be altered (Barres, 2008). Both astrocytes and oligodendrocytes communicate with other glial cells as well as with neurons. It seems that these processes are active during the whole development of the CNS also at the embryonic level and glia precursor cells are known to be active in directing neurons in their migration to the right locations. Furthermore, the different glia cells are involved in various processes occurring in response to different lesions to the brain.

The overall rate of brain energy metabolism is strongly linked to the generation of neuronal electrical activity and hence the cerebral cortex, harboring the neurons, has much higher metabolism than white matter. To maintain the rate of energy production the brain is

totally dependent on a continuous supply of glucose and oxygen from the blood stream. Hence, regional cerebral blood flow varies greatly with tissue type and gray matter has 3-4 times higher perfusion compared to white matter. Such differences in flow rate hold a potential for tissue differentiation through spectroscopic measurements.

1.2.1 Blood Brain Barrier

The glia system has one of its important roles in forming the blood brain barrier (BBB) and the blood-CSF-barrier (BCSFB), which is built up similarly in the entire CNS. Since Ehrlich in 1885 showed that not all substances added to the blood circulation pass over to the brain or the CSF, a BBB was postulated by Lewandowsky in 1900 and in 1913 Goldman could describe the existence of a CSF-blood-barrier (Hawkins and Davis, 2005). It was not until the late 1960's, with the help of electron microscopes, that the morphological correlate was found in the tight junctions of the endothelium in the capillary system of the brain. These membranous obstacles are penetrable for lipophilic, but not for hydrophilic substances, yet several parallel mechanisms of active transport between and through the cells are known for selective passage of molecules (Banks, 2016). This is of general significance for pharmacological treatment of diseases of the CNS and in particular for substances that are intended to reach brain tumors, including the uptake of the fluorophore five-aminolevulinic acid (5-ALA), which is the actual core of this thesis. The BBB may become disrupted in pathological conditions leading to edema formation in the brain. Not only can abnormal passage of substances play a pathophysiological role in certain conditions but it can also be utilized in radiological diagnosis with the passage and accumulation of contrast in e.g. tumors or areas of inflammation and, further, it is the basis for treatment with cytotoxic agents in the CNS. The BBB may also be opened artificially as part of treatments protocols.

2 Pathology

2.1 Central Nervous System Tumors

CNS tumors occur in approximately 22.3 cases per 100 000 inhabitants, with varying incidence rates in different age groups. Approximately 1/3 (32%; CBTRUS) of CNS tumors are malignant. Not all intracranial tumors are primary brain tumors.

Tumors of the CNS comprise an astounding variety of neoplasms and the WHO classification counts in all 154 different CNS tumor forms (Tab.1). They can emerge from a variety of intracranial tissues such as the meninges, nerve sheaths, blood vessels, from glandules, etc. (Louis et al., 2016) thus forming a large group of “extra-axial”, mostly benign tumors, of high neurosurgical significance. In contrast, true brain tumors originating from the glial cells are mostly malignant and only glial cell tumors will be further considered here.

WHO classification of tumours of the central nervous system			
Diffuse astrocytic and oligodendroglial tumours			
Diffuse astrocytoma, IDH-mutant	94003	Neuronal and mixed neuronal-glial tumours	
Diffuse astrocytoma, IDH-wildtype	94004	Dysembryoplastic neuroepithelial tumour	94130
Diffuse astrocytoma, NOS	94005	Ganglioglioma	94131
Anaplastic astrocytoma, IDH-mutant	94013	Neuronal	
Anaplastic astrocytoma, IDH-wildtype	94014	Neuronal	
Anaplastic astrocytoma, NOS	94015	Neuronal	
Glioblastoma, IDH-wildtype	94021	Neuronal	
Glioblastoma, IDH-mutant	94022	Neuronal	
Glioblastoma, NOS	94023	Neuronal	
Anaplastic oligodendroglioma, IDH-mutant and 1p/19q-codeleted	94031	Neuronal	
Anaplastic oligodendroglioma, IDH-wildtype	94032	Neuronal	
Anaplastic oligodendroglioma, NOS	94033	Neuronal	
Other astrocytic tumours			
Pilocytic astrocytoma	94041	Neuronal	
Piloastrocytic astrocytoma	94042	Neuronal	
Subependymal giant cell astrocytoma	94043	Neuronal	
Atypical pilocytic astrocytoma	94044	Neuronal	
Anaplastic astrocytoma	94045	Neuronal	
Ependymal tumours			
Ependymoma	94051	Neuronal	
Piloependymoma	94052	Neuronal	
Myxopapillary ependymoma	94053	Neuronal	
Ependymoma, NOS	94054	Neuronal	
Anaplastic ependymoma	94055	Neuronal	
Other glomas			
Choroid plexus papilloma	94061	Neuronal	
Anaplastic choroid plexus papilloma	94062	Neuronal	
Choroid plexus carcinoma	94063	Neuronal	
Meningeal tumours			
Meningeal schwannoma	95001	Meningeal	
Meningeal schwannoma	95002	Meningeal	
Meningeal schwannoma	95003	Meningeal	
Meningeal schwannoma	95004	Meningeal	
Meningeal schwannoma	95005	Meningeal	
Meningeal schwannoma	95006	Meningeal	
Meningeal schwannoma	95007	Meningeal	
Meningeal schwannoma	95008	Meningeal	
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Meningeal schwannoma	95061	Meningeal	
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Meningeal schwannoma	95067	Meningeal	
Meningeal schwannoma	95068	Meningeal	
Meningeal schwannoma	95069	Meningeal	
Meningeal schwannoma	95070	Meningeal	
Meningeal schwannoma	95071	Meningeal	</

Table 1: The WHO Revision of 2016 counts more than 50 different tumors of glial origin (overview only).

True brain tumors were first described in 1863 by R Virchow, who also first reported on the glia cells already in 1856 (Kettenmann and Verkhratsky, 2013). Golgi introduced the term glioma in 1884, after having found astrocytic cells in some tumors of the brain. In fact, as known today, nearly all of the primary brain tumors originate from the glial cells. This large group of the gliomas accounts for 25% of all primary CNS tumors and 75% of all malignant CNS tumors (Fig. 3).

In the WHO classification there are 51 types of gliomas, among them astrocytomas, oligodendrogliomas and ependymomas, but also ‘other astrocytic gliomas’ and ‘other gliomas’, as well as choroid plexus tumors, neuronal and mixed neuronal-glial tumors of several subsets.

Figure 3: The group of Gliomas has several subsets of tumors with different biological characteristics and of divergent pathological significance, The largest entity among the Gliomas is the most malignant form, the Glioblastoma, which can be primary or secondary due to malignification of other gliomas.

2.2 Glial tumors

It has been suggested that gliomas are derived from neural stem cells or glial progenitor cells rather than from derailed mature glial cells. Some of the embryonic and fetal mechanisms of proliferation and

migration of stem cells and progenitor cells may to some extent still be present in the adult, especially in the subventricular zone and in the white matter (Claes et al., 2007). The growth pattern is infiltrative in a unique manner for these tumors even in comparison to other forms of cancerous diseases and the behavior of these tumors has been compared to “a guerilla war”, hence the “impossibility to treat with classical methods without collateral damage”. With higher WHO-grade the heterogeneity of the tumors increases and the most malignant gliomas incorporate different characteristic features of all other grades on different locations within the tumor; the reason why the glioblastoma used to be suffixed ‘multiforme’. Glioblastomas may rarely be multicentric. The entity “gliomatosis cerebri” is a widespread form in its own category engaging multiple lobes of the brain.

Gliomas are graded I-IV in the WHO/CNS-scale classification scale of CNS tumors with increasing degree of malignancy foremost based on histological and immunohistochemical features of the tumors (Louis et al., 2016). The latest revision of the WHO list from 2016 also includes molecular criteria. Examples of tumors corresponding to the listed grades are pilocytic astrocytoma of grade I; diffuse astrocytoma, oligodendroglioma and ependymoma of grade II; anaplastic astrocytoma, anaplastic oligodendroglioma and anaplastic ependymoma of grade III; glioblastoma of grade IV. The grades I and II are also summarized as low-grade tumors, of which grade I often displays more circumscript growth and sometimes can be classified as benign. Gliomas WHO-grade II-III tend to progress and transmute into malignancy. Tumors of grades III and IV are lumped to form a common group of high-grade, primary malignant gliomas, and grade IV is also the common endpoint of progressively malignifying gliomas, regardless of origin. There are to some extent differences between the grades III (anaplastic glioma) and IV (glioblastoma) in histological appearance and in prognosis, but not in the surgical approach.

2.3 Glioblastoma

The glioblastoma represents more than 50% of all gliomas and corresponds to grade IV in the WHO-classification (high-grade, fast growing). Being the most malignant and also the most common form of the primary brain tumors, the glioblastoma (GBM) confronts neurosurgeons with a severe challenge. It is a well-known fact that surgery alone cannot cure the illness, due to the infiltrative growth pattern of this tumor and it is a difficult task to actually define and offer optimal help to patients.

FIG. 86.—Gross appearance of a spongioblastoma multiforme (Surg. No. 15808) in left hemisphere of a patient aged 43 brought to hospital in coma after three months' symptoms mistaken for encephalitis lethargica. After a hypertonic saline infusion a right subtemporal decompression was performed without avail. Death four days after admission. (Four-fifths natural size.)

Figure 4. Bailey and Cushing described the most malignant glioma in 1927; at that time still named Spongioblastoma uniforme and multiforme, today differentiated as primary and secondary Glioblastoma.

Since the thorough description and classification of tumors of the glioma group including a correlated study on the prognosis by P Bailey and H Cushing in 1927, there has until quite recently been but little progress in treatment and survival from glioblastoma. Initially the authors called the tumor spongioblastoma (Fig. 4), but later adopted the term glioblastoma, as already suggested by Mallory in 1914 and published in 1925 (Mallory, 1924). The prognosis has

remained dismal, although survival rates have improved, especially in the last decade (deSouza et al., 2016).

The incidence is generally 3-6/100 000 inhabitants (Ostrom et al., 2016) although some reports show higher incidences in e.g. Germany and Sweden (Bahemuka, 1988), which may suggest a link to high socioeconomic levels and to individuals of European origin. It generally affects males more than females (1.3-1.6:1) and occurs with a peak incidence in the 5th-6th decade of life (Vårdprogram, 2016).

Median overall survival after combined surgical and oncological treatment for confirmed glioblastoma WHO-grade IV is less than 1.5 years. Yet 30% of the patients may live 2 years or more and some investigations have reported on up to 10% of 5-year survival (Oertel et al., 2005), although long-term survival is more frequently reported to be 2-5% (Kelly, 2010) (Ostrom et al., 2016).

In up to 75% of the cases the cause of death is due to direct effects of the recurrent tumor, while in the remaining cases complications of the disease including adverse effects of the treatment modalities were identified as causes of death (Sizoo et al., 2010). Tumor recurrence occurs in up to 90% within the immediate vicinity of the original location (Roy et al., 2015) and only in rare cases on remote locations.

Histological examination of GBM presents a multitude of malignant features typical for neoplasms. Cells of fibrillary astrocytic appearance are arranged in the shapes of palisades and show varying size, reduced cytoplasm, necrotic and sometimes cystic compartments, vascular proliferation and thrombotic veins (Louis et al., 2016). The morphological elements of the tumor alone do not sufficiently characterize the diversity of its biological propensities. Molecular genetic and immunohistochemical investigations have become routine in many neuropathological centers throughout the world and are today also part of the WHO definitions of the glial tumors. GBM can be primary (90%) or secondary (10%). Both forms are of astrocytic origin and with similar histological build-up, but

with quite different genetic profiles. The secondary GBM develop from WHO grade II or III astrocytoma and seems have a better prognosis than the primary form (sometimes referred to as 'wild type'), given the appropriate choice of therapy based on molecular analysis (Ohgaki and Kleihues, 2013).

The GBM is almost always located subcortically in the white matter of the supratentorial cerebral hemispheres. They are uncommon in the posterior fossa and very rare in the spinal cord. GBM rarely spread outside the original location although perivascular growth, although disseminations through the CSF to the spinal canal as drop metastasis or generalized dissemination after shunting of CSF do occur (Buhl et al., 1998).

3 Diagnosis

3.1 Clinical presentation

The clinical presentation of intracranial tumors depends on their topographic location in the brain and dysfunction can be caused both by destruction of tissue and by compression of structures in the vicinity of the tumor location or even beyond. Focal impairment such as aphasia, alteration of the sensorium and motor dysfunction may result in 25-40% of the patients. Diffuse headache can precede more distinct symptoms in 50-70% of the cases, sometimes for many months (Krauseneck and Mertens, 1987). Altered behavior with changed personality and character may indicate an intracranial process in 30-50% of the cases and also epileptic seizures occur as presenting symptoms in 30-50 %.

3.2 Radiology

Often, the first diagnostic measure in cases of a suspected cerebral lesion will be a CT-scan, which in emergency cases tend to be performed without contrast enhancement. The next necessary step is magnetic resonance tomography, without and with contrast media (Gadolinium). T1-weighted images with contrast media, the corresponding T2-weighted and FLAIR images (Fig. 5), diffusion weighted imaging (DWI) and diffusion tensor imaging (DTI), proton density (PD) weighted images and also magnetic resonance spectroscopy (MRS) give a correct tumor diagnosis in most cases although primary brain tumors can be mimicked by other pathologies. The indication for tumor treatment or abstention from treatment can therefore not rest on imaging alone. (Law et al.).

Figure 5: Typical presentation of a Glioblastoma in the right temporal lobe as visualized in different MR-sequences (T1 without and with Gadolinium contrast media, T2, FLAIR) and planes (axial, coronal, sagittal).

3.3 Biopsy

For any kind of further specialized oncological treatment a biopsy will be necessary, to yield a reliable histopathological diagnosis, usually extended with immunohistochemical and molecular examinations.

Biopsies from brain tumors can be performed as an open approach procedure, although in the majority of the cases the operation will be carried out stereotactically or navigated by frameless systems through a burr-hole trepanation. A Sedan type biopsy needle (Backlund, 1971) is pushed along a calculated trajectory into the tumor and several samples are withdrawn, which then are sent for intraoperative cytological examination. This is time consuming since the patient must wait in the OR until it is confirmed that relevant tissue actually has been collected, which may, at times, include several sampling attempts..

It is clear that the oncological treatment will be more effective if a gross total cytorreductive operation has been successful. Nonetheless, there is also data to indicate that in cases where only partial resection ($< 90\%$) of tumor is possible, because of unacceptable risks for neurological injury in critical areas, instead a biopsy followed by oncological treatment may be more profitable to the patients, given the lesser surgical risk.

4 Treatment

4.1 Radiation

The basic concept for treatment of malignant gliomas consists of the combination of surgery, radiation and chemotherapy, which has been shown to be able to provide the best overall survival and the longest progression-free intervals (Stummer et al., 2000, Stummer et al., 2008). The combination of surgery and radiation has proved to double the survival rates compared to surgery alone and the addition of chemotherapy, especially since the introduction of temozolomide, has augmented the effect of treatment even further (Stupp et al., 2005). Conventional radiation therapy interferes with cell function through destruction of the replication systems (DNA). In contrast, the principles of radiosurgical methods (Gamma-Knife, LINAC, X-Knife, Cyber-Knife) are based on controlled and precisely targeted high-energy bundles of ionizing radiation focused with the help of a stereotactic system in order to directly destroy the tumor. This treatment modality has so far not contributed substantially to improve the therapy of malignant gliomas (Binello et al., 2012).

Radiation as a sole treatment modality is a rare option reserved for cases not eligible for resective surgery and not responsive to chemotherapy. External beam whole brain treatment has a maximum dosage at around 60 Gy due to tissue toxicity, which to some extent can be reduced by fractionated stereotactic irradiation (Malmström et al., 2012, Combs et al., 2005). Reirradiation can be tried, as nowadays better results can be expected, due to more precise methods (Combs et al., 2007).

4.2 Chemotherapy

Before the development of temozolomide, malignant brain tumors were considered more or less resistant to other kinds of chemotherapy, with some exception for the nitrosourea substances ACNU (nimustine), BCNU (carmustine) and CCNU (lomustine). These lipophilic substances pass the BBB, but the patients often

suffered severe side effects, while only 1/5 of all tumors responded at all (Grisold and R., 2013). Chemotherapy is rarely carried out as a stand-alone therapy except in cases of recurrent glioblastoma.

4.3 Neurosurgery

4.3.1 *Criteria for surgery*

The since long established concept of combined treatment for malignant brain tumor is based on maximum cytoreductive debulking, so-called gross total resection (GTR), as a precondition for oncological treatment on the cellular level (Young et al., 2015). Depending both on the extent and the location of the tumor as well as the clinical condition of the patient, it must be decided whether an operation can be performed without causing harm and reduced quality of life. For this reason the main strategy is based on a defensive approach, meaning that tumor tissue preferably should be left behind, rather than to risk destruction of functional neural tissue and cause severe impairment. The impact of many medical, psychological and social factors increases with age and more than 50% of the patients with GBM are over 65 years of age when first diagnosed. Age and clinical performance status, i.e. Karnofsky Performance Index (KPI), are the independent prognostic factors above all, but can never be exclusion criteria alone. The response to corticosteroid medication, with or without remission of neurological symptoms, may help to estimate what can be achieved by a surgical resection of the tumor. By stabilizing the BBB, corticosteroids can help to indicate what is caused indirectly by compression of the brain or by the perifocal edema as opposed to direct destruction of the engaged area.

The decision to operate for resection will ultimately comprise evaluation of the tumor's extent and localization on an anatomical and functional basis. If basal ganglia or eloquent cortical areas are affected, permanent and severely disabling impairment is at risk and will contradict the resection of the tumor. In these cases an operation 'for resection' may be rejected and an oncological treatment

recommended. This, however, does require an adequate histological and biochemical diagnosis, which thus necessitates another kind of operation, ‘for biopsy’. A biopsy usually still can be taken, as an operation through a simple burr-hole generally constitutes a minor endeavor, to obtain a histopathological and biochemical diagnosis as a prerequisite for planning of oncological treatment in such cases.

4.3.2 Local routine neurosurgical set-up

Unlike low-grade gliomas, which can be considered for awake surgery, malignant gliomas are operated only under general anesthesia at the Neurosurgical Department, Linköping University Hospital. In similarity to operations on low-grade tumors, functional control in malignant tumor surgery is accomplished by neurophysiological mapping of the motor strip and monitoring of sensory and motor evoked potentials (SEP, MEP). A navigation system for topographic orientation, frameless or with a stereotactic frame is usually applied. The routine always includes an operating microscope for meticulous dissection and since the microscopes (M720 OH5, Leica GmbH, Wetzlar, Germany) were equipped with specific luminescent filters (FL400), the operations on suspected malignant gliomas are generally performed as fluorescence guided surgery (Hefti, 2010), using the fluorophore 5-ALA (Gliolan[®], Medac GmbH, Wedel, Germany).

4.3.3 Operative strategy

The procedure is carried out through an open bone flap approach under direct vision and the microscope is usually not applied before preparation in the subcortical tissues. The white light from the microscope serves the stereoscopic magnification within the surgeon’s visual field. The blue light filter can be activated at any time for detection of possible fluorescence, but usually not until the preparation has reached subcortical structures. After completing the resection of the tumor tissue as far as possible along the defensive line of strategy, the site is closed and the patient observed postoperatively up to 24 hours.

4.3.4 Postoperative follow-up

In this center an early MRI is routinely carried out within 72 hours postoperatively, to confirm or reject GTR (Forsting et al., 1993). When remaining parts of the tumor are found and GTR is aimed for, most cases are offered a second procedure within days to remove the residual. Weekly multidisciplinary conferences with neurosurgeons, neuropathologists, neuroradiologists and neurooncologists (including video-links to remote referring hospitals) decide how to proceed. From that point on the patients are generally referred to oncological treatment. The clinical follow-up as well as radiological control exams are performed by neurooncologists along standardized schedules and the patients are regularly discussed in the team along the course. Reoperation is performed in approximately 10-30% of all cases, although no fixed rationale for the decision can be outlined. Aggressive treatment of recurrent GBM has been shown to be able to maintain quality of life (QOL) and to prolong survival even in elderly patients (Stark et al., 2007).

At this center we have treated two patients with a 7-year-survival during the last decade but in general our results match current international statistics based on 50-60 cases newly diagnosed and operated each year.

4.4 Standard surgical procedure

When the dura mater is opened, the surface of the brain only rarely reveals the location of the tumor, but sometimes a slight edema of the cortical structures can be discerned. Both hyperemia as well as hypovascularized fields on the cortex can be suspected of a deep-laying tumor. Palpation of the cortical surface can reveal an area of induration. The use of intraoperative ultrasound or navigation systems has improved the precision to find the optimal site for cortical incision tremendously. In the preoperative planning the access route should be selected to avoid eloquent structures. Neurophysiological cortical mapping including both evoked potentials and stimulation of functional areas can be applied for

further precision. Thus having located and approached the tumor the surgeon can usually debulk much of the tumor center without risk. The surroundings of the tumor may appear discolored, deviate in texture from white matter, cysts may open and collapse and some parts may be well circumscribed and easily removed but inevitably the surgeon will encounter the well-known gradual transition zone from apparently pathological tissue into a zone of brain-resembling tissue.

That is the situation when the decision of how to limit the resection has to be made.

The actual debulking is carried out with various manual dissecting instruments, with or without the use of an ultrasound aspirator. Many instruments can be integrated into the navigation system and navigation data can be upgraded based on intraoperative imaging systems such as ultrasound or intraoperative MRI. At the beginning of the dissection the approach is naturally directed inwards from the margin of the tumor, but thereafter it usually proceeds from the inside towards the margins, which provides better access and vision and safer identification of the transition zone. Meticulous preparation based on all visual and palpable aspects of the tissues at the tip of the instruments is crucial to preserve function.

4.5 Navigation

The clinic introduced frameless navigation in 2001; at that time the Sonowand system combined an ultrasound- and a navigation device (MISON, Trondheim, Norway). In 2015 the Stealth system (S7; Medtronic, Minneapolis, MN, USA) was implemented. The Sonowand device enabled real-time adjustment of the navigation following brain shift (due to mass reduction during tumor resection) by an *in situ* ultrasound uptake, and recalculation of the registered coordinates. The Stealth system does not include this feature, but has increased precision of the registration for navigation. The Sonowand proved to be a reliable system and was predominantly used during

the project presented here; it allowed for easy to handle integration of the probe by simple registration and recalibration.

4.6 Fluorescence Guided Surgery

The true extension of the malignant glioma tissue infiltrating the normal brain tissue is not known. Until this day, therefore, no clear criteria have been defined to support the surgeon's decision where to limit the resection of malignant gliomas. Generally GTR is pursued, since it has been shown that significantly longer progression free interval and longer overall survival is possible if GTR approach > 98% of the tumor compared to the often used >90% definition of GTR (Lacroix et al., 2001). In the quest to achieve maximal radicality possible discrepancy between the surgeon's ambition and safe dissection in the marginal zone may jeopardize neural functions, although surgeons often tend to overestimate the extension of their resections (Orringer et al., 2012) (Fig. 6).

Figure 6: The example resembles a spherical tumor with a homogenous density of tumor cells. In reality the tumor is inhomogeneous. Yet the model demonstrates the risk of possibly leaving substantial masses of tumor by an incomplete resection.

Increased surgical safety during dissection in this marginal tumor zone exactly what has been improved by the development of fluorescence-guided surgery (Stummer et al., 1998) with modern microscopes equipped with filters for detection of the fluorescence (Figs 7-8). The fluorescence is emitted from metabolized 5-ALA, i.e.

protoporphyrin IX (PpIX) accumulated in the malignant glioma cells and is perceived as red (“strong”) or pink (“weak”) luminescence.

Figure 7: The surgical microscope for fluorescence-guided resection has contributed substantially to maximize the rate of gross total resection. The surgeon has to separate “no fluorescence”, “strong” or “weak” fluorescence.

Figure 8: a: blue light mode; b: white light mode

The perception of that fluorescence, however, is still a subjective matter of the surgeon; the task is to resect areas of “strong” fluorescence and to rather void areas of “weak” fluorescence. It is not known, however, what “strong” respectively “weak” fluorescence means in terms of tumorous content in the tissue. There is some evidence, that 5-ALA enhances in tumor tissue beyond the extent of the contrast media enhancing parts (Fig. 9).

Figure 9: Problems of the Glioblastoma; dismal prognosis, infiltrative growth with a variety of tissue types without membranous limitation.
Green – resectable; red: not resectable?

5 Biomedical Optics in Brain Tumor Surgery

5.1 Light Interaction with Tissue

Laser light is to some part reflected from different tissues, but to some part it propagates into them. There it is absorbed and also scattered, depending on the different structures of different tissues (Fig. 10). The light is mainly absorbed by so-called chromophores; molecules that define the color of the tissue due to different absorption (and non-absorption) properties, such as content of water, lipids or proteins. Among the latter is e.g. hemoglobin. The absorbed light carries energy that is transferred to the atoms and molecules of the cells and is partly transformed into heat or partly re-emitted as fluorescence.

Figure 10: Different light interactions in tissue

Fluorescence occurs when the incident light excites electrons in the tissue molecules to reach a higher energy level. This is only temporary (usually within milliseconds), as opposed to the process of phosphorescence (minutes or hours), which is slower due to another behavior of the electrons in their excited state. The excited electrons will return from the higher level to their ground state and then release the energy as light, now at a longer wavelength (corresponding to lower energy) than the originally exciting light (Masters, 2010)]. Substances capable of this are called fluorophores. Tissues have specific absorption and emission spectra and at some light wavelengths the absorption is reduced, i.e. the excitation

intensity is higher. Fluorophores can be excited at different wavelengths, but the fluorescence is always re-emitted at a specific wavelength. Substances can thus be identified by their fluorescence.

5.2 Photosensitizers

Fluorophores can be endogenous or exogenous. Exogenously delivered substances may enhance fluorescent properties of endogenous fluorophores. Porphyrins can be both endogenous and exogenous and have been found to be cytotoxic when exposed to light in the presence of oxygen, i.e. some of them are photosensitizing agents. The discovery of the substance class around 1900 first led to the development of photodynamic therapy, since the 1980's used in various cancer treatments, but later also to the development of applications for the fluorescent properties of these substances. A variety of photosensitizers of different origin have since been developed and are still experimented with, e.g. fluorescein or porphyrin-based Photofrin and Foscan[®]. Another precursor of porphyrin is 5-ALA.

5.3 Five-Aminolevulinic Acid

Porphyrins and their derivatives are ubiquitous in biological systems, in different cells, in enzymes or in mitochondria's. The compound 5-ALA is a natural substance, which in plants leads to synthesis of chlorophyll or in animals e.g. to cobalamin, but in humans almost solely to synthesis of heme, a process that takes place in every cell of the body. Externally administered, depending on the dosage, it can interfere in the heme cycle of the tumor cells by overload of the enzyme ferrochelatase with its photosensitizing metabolite protoporphyrin IX (PpIX) (Heyerdahl et al., 1997). It has peak fluorescence at 635 nm when excited at wavelengths around 400 nm. The process in combination with oxygen in the tissue is also called photobleaching, since the fluorescence diminishes. As much as it may be of advantage in PDT, during intraoperative diagnosis prior to resection it is not desired.

In doses of maximum 20mg/kg 5-ALA the photobleaching and the cytotoxic effects can be avoided by protection from intensive light sources, e.g. by keeping the patient in a dimmed room or directing the operating room lamps aside from exposed parts of the patient's skin.

The 5-ALA enhances specifically in brain tumors, due to the fact that the fluorescing metabolite PpIX is synthesized at a much higher rate in the tumor cells than in the normal brain tissue (Guyotat et al., 2016). Other fluorophores, e.g. dyes such as fluorescein, are synthesized in the liver and thus have to pass the BBB. It has not yet been explained how external application of 5-ALA can influence the rate of PpIX synthesis in brain tumors. A combination of active transport mechanisms through the cells and a disrupted BBB around malignant gliomas may be involved. Leakage through the BBB can allow for some of the fluorescing substance to migrate further into the edema and the surroundings, e.g. along white matter tracts and thus may sensitize distant tumor cells outside areas of ruptured BBB for laser excitation. However, PpIX does not accumulate in normal brain tissue to the amount that fluorescence will be seen under the microscope. Whether higher dosage of ALA, beyond the recommended microscopy dose of 20mg/kg, may cause higher amounts of PpIX outside of the tumor, however, has not been demonstrated.

5.4 Surgical Application

The use of visualizing agents in neurosurgery, e.g. the employment of dyes, infused into the targeted tissue, dates back more than half a century. In 1948 the use of fluorescein for localization of brain tumors was first reported (G and Peyton, 1948). In 1957 Kurze introduced the surgical microscope into neurosurgery and today microsurgery is established as a routine technique in neurosurgery worldwide (Uluc et al., 2009). The combined use of fluorescence and surgical microscopes, however, was not established until half a century later.

Studies on endogenous fluorophores, i.e. the autofluorescence of brain and tumor tissues have been performed. The autofluorescence, however, results from many different fluorophores. The variation of the contents of fluorophores depends on many factors, which has made the interpretation of the signals from measurements difficult, especially in the brain. Stummer was the first to publish a report on the use of modified surgical microscopes equipped with filters for detection of fluorescence from metabolized 5-ALA.

Exogenously increased enhancement of PpIX in the malignant glioma by metabolism of 5-ALA is accomplished by administering 5-ALA dissolved in water to the patient. The patient should receive the medication approximately 2-3 hours prior to the planned resection of the tumor, for maximum enhancement and fluorescent response during the resection. The dose is adjusted to 20mg/kg bodyweight, following the recommendations from the manufacturer Medac for use with operating microscopes (EMA, 2007) (Fig. 11).

Figure 11: The fluorophore 5-ALA is given to the patient dissolved in water 2-3 hours prior to the operation.

The optical measurements may be started from the very beginning of the tumor preparation. Under the blue light filter in the operating microscope a red luminescence can be seen in the tumor. Normal brain tissue will be perceived as blue. The surgeon has to differ between “solid” and “vague” red fluorescence, meaning “strong” and “weak” respectively. Strong fluorescence can be safely resected outside eloquent areas, whereas weak fluorescence seems to coincide

with the tumor marginal zone, thus potentially leading the surgeon into functional tissue domains. In 2008 Stummer could demonstrate that the rate of GTR in 243 cases of malignant gliomas could be substantially increased. It was found that the method was highly specific, but the sensitivity was low. Knowing that the tumor growth often extends beyond the contrast media enhanced areas as seen in MR exams, there is a need for a method to detect tumor with a higher sensitivity (Guyotat et al., 2016).

5.5 Early Hand-held Probe Systems

Early attempts in the development of the hand-held probe system used in this project date back to the 1990's. Andersson-Engels and Wilson investigated the possibilities of using autofluorescence or exogenous fluorophores to induce tissue specific fluorescence and whether such fluorescence could be visualized by a registration system, either in form of a curve or an image, to identify specific tissues including tumors (Andersson-Engels, 1992). The basic design of such a system is outlined in Fig 12.

Backlund envisioned that a device for 'optical biopsy' could be combined with a resection instrument, allowing the surgeon to identify pathological tissue in real-time while dissecting and to remove it immediately (Backlund, 2004)(Fig 13).

Figure 12: General principle of a fluorescence detection system for point measurement (upper image).

Figure 13: The vision of Professor Backlund was very obvious; the combined instrument would give the surgeon real-time information about the character of the tissue dissected.

It was already then recognized that hematoporphyrine derivatives would be eligible as exogenous fluorophores, that artifacts caused by ambient light sources could be reduced by using a pulsed light source and that the interpretation of the signals could be improved by calculating a ratio between the autofluorescence and the specific fluorescence recorded (Sternborg et al., 1996). Based on these concepts an apparatus for fluorescence spectroscopy was assembled (Fig. 14).

Figure 14: One of the first systems, developed in Lund; it was heavy and vulnerable, therefore difficult to transport and to implement.

A minor study was carried out in the Neurosurgical Department of the Linköping University in 2000; focused on biopsy of malignant brain tumors (Backlund et al., 2002) (Fig. 15). It became part of a thesis on different optical methods of tissue identification (Pålsson, 2003). One important achievement was the observation that several light sources and spectra were not necessary; emission at 405nm was sufficient to detect PpIX-fluorescence, with lesser influence from the autofluorescence than at 337 nm, which had shown some advantage in demarcation for identification of autofluorescence. This, however, was not specific for malignant gliomas.

Figure 15: Fluorescence spectroscopy measurements were carried out as “optical biopsies” at the Neurosurgical Department, Linköping University Hospital. The regular biopsies confirmed the findings of malignant glioma. The device in the foreground of the image is the same as in Figure 14.

The study on the malignant brain tumor biopsies was only published as a conference abstract, but it ignited the idea of developing a new system in the Department of Biomedical Engineering at Linköping University.

The idea of the new project was to identify malignant brain tumor tissue *in situ* during resection, hence extending the project into clinical neurosurgery. The maturation of the technique involved much work on adaption and design suitable for the operation room setting. The examinations performed with the new system are presented in the following chapters.

6 Aim of the Thesis

The overall aim of the thesis was to introduce into a clinical setting a hand-held probe for fluorescence detection for identification of tumor tissue and to evaluate the performance during brain surgery.

The following specific aims were of special interest:

- Reliable measurement of specific fluorescence
- Integration of the device into the operating room
- Detection and elimination of sources of error
- Evaluation of combination with an ultrasound and navigation system
- Evaluation of combination with a fluorescence microscope

and the aim above all:

- Identification of the marginal zone.

7 Material and Methods

7.1 Portable System with Light Emitting Diode (Paper I)

The older system had been quite large, heavy and vulnerable, which meant difficult to transport and to deploy. Main requirements for a new device were that it should be more easy and robust to handle, but it should also deliver more precise results by a better light source and new processing software, yet also be cost effective. Recent developments at that time had let the use of a light emitting diode (LED) seem advantageous in a prototype of a new system, although not allowing pulsed light emission. The excitation source and the registration unit were fitted into a small box of 25x30x14 cm at a weight of 3 kg, which was substantially less than the previously used boxes. A schematic view of the system is shown in Figure 16. The high power LED (200mW) constituted a considerable financial gain compared to the laser beams previously used. It delivered the necessary energy with sufficient precision by adapting the filters and the special optical fiber used. The high energy was necessary considering the loss of energy in the transmission into the fiber cable. At the tip of the fiber the energy was 1mW. The equipment was sterilized with the Sterrad[®] sterilization system (low temperature, peroxide). The actual hand-held probe, the size of a narrow pen, was provided with a plastic funnel, non-translucent, to shield off ambient light from the overhead lights and the surgical lamps as well as from the surgical microscope at the tip of the probe and in the measurement area, i.e. the actual resection area in the surgical field (Fig. 17, 25).

Figure 16: The prototype had an LED light source (A), i.e. a continuous wave. The probe, i.e. the light transmission unit consisted of an emitting fiber with collecting fibers arranged around it (B), with exciter and emitter filters (I, II). The signals were passed to a spectrometer (C). A funnel was attached to the probe for suppression of ambient light by covering the tip during measurements.

Figure 17: The plastic funnel reduced the influence of ambient light; the autofluorescence without (left) and with (right) shielding funnel.

Before the system was implemented in the OR it was tested in measurements on skin that had been prepared with cream containing 5-ALA (16%, Metvix, Photocure ASA, Norway). Measurements were carried out after 4 hours (Fig. 18a-b).

Figure 18a: Testing the system on skin, prepared with 5-ALA-cream

Once the system was calibrated and found to reliably register fluorescence it was possible to measure on human brain and brain tumor tissue in accordance with the ethical approval obtained for the purpose.

Figure 18b: The system showed a typical response at 635nm over the cream-treated areas, also a minor peak at 704nm – the typical pattern known from studies with the older device.

The patients were given 5mg/kg of 5-ALA dissolved in orange juice 2-3 hours prior to the operation. The set-up was designed not to interfere with the standard procedure for resection of malignant brain tumors. During the measurements the surgeon had to state what kind of tissue was suspected at the tip of the probe; normal brain or tumor (Fig. 19).

Figure 19: After measurement on one spot a biopsy was taken from the same site. In this image the funnel is retracted to show the placement of the probe.

This and any other event occurring were noted in the protocol. The measurement was then carried out, the duration 1000 ms. The registrations were made under white light conditions and also with the overhead lights dimmed and the surgical microscope turned away from the measurement site. Finally, biopsies were taken on the sites of measurement and separately noted.

This examination protocol introduced for this first survey was later adapted for all further measurements with the modified system.

7.2 Portable System with Pulsed Laser (Paper II)

The experiences with the LED-/continuous wave-system and the evaluation of the results made improvements of the system necessary, in order to get signals of higher purity and at the same time ensure practical applicability in terms of avoiding disturbances of the surgical routine.

The concept of using a pulsed light source was adapted (Andersson-Engels, 1992). A new laser with 50 mW, capable of both pulsed and continuous mode, was installed (Fig. 20). The higher output energy would be expected to increase the photobleaching process, but a new electronic control of the registration unit with gated detection of the signals made it possible to catch the stronger signal by synchronization, fast enough to give a more precise measurement of the captured spectra. Of the two different probe constructions tested with the first system, the one with the registering fibers placed

around the light source in the center was chosen. The funnel on the tip of the probe was no longer used, since ambient light was reduced by faster measurements, now 400ms (repeated twice, i.e. 0,8s), exciting energy at 5mW. Safety calculations ruled out any hazards, as the tissue temperature increased only 1°C after as long as 1,5 minutes.

Figure 20: The improved system featured the basic design and used the original probe with the circular arrangement of the emitting respectively collecting fibers, but had new equipment inside the box with electronic control and a pulsed laser.

A: Light source B: Spectrometer. C: Probe, flexible fiber optic.

For quantification of the signals registered a formula for calculation of the measurements was introduced (Haj-Hosseini et al., 2009), compensating for the stronger bleaching effect and for variations of the exciting energy due to different angles of the probe during the measurements (Fig. 21). The baseline was the peak of the autofluorescence at 635nm (I_{base}). It was subtracted from the peak value at the same wavelength (I_{635}) and then divided by the peak value of the autofluorescence (I_{autofl}). As the ratio would give equal results after repeated measurements, the bleaching effect could be neglected. Since the bleaching also would be only very localized, measurements could be repeated in the immediate vicinity. The resolution of the registrations resembled a tissue area of $\leq 2 \text{ mm}^2$.

Figure 21: The ratio ‘K’ facilitated objective quantification of the signals, i.e. comparability of the amount of fluorescing tissue.

The registrations of the signals obtained were evaluated on accuracy and the recalculated measurements were compared with the histological examinations of the biopsies from the resection cavity and also related to the surgeon's estimation of the tissue type. All measurements in this study were based on a dosis of 5mg/kg, like in the previous study.

Several patients were examined during the development and adjustments and one example of a case with a high-grade glioma was published (Paper II) to demonstrate the effectiveness and the advantages of the new system. The system was ready for a clinical application in a clinical routine setting.

7.3 Combination with a Navigation System (Paper III)

The next step was to perform measurements on a series of patients in order to identify different tissue types (and ultimately to detect the marginal zone) by systematic implementation of the calculated ratio (mentioned above). The following studies were performed on several consecutive cases of malignant gliomas. The new system was integrated into a navigation system for more precise localization to compare with the bioptic samples; biopsy being the gold standard as a measure for the precision of the fluorescence registration as well as for the navigation (Fig. 22).

Figure 22: The new system was integrated into a navigation system. During the measurements the feedback was withheld in order not to influence the surgeon's judgment of the tissue types.

The navigation system used at that time was the SonoWand (Mison A/S, Trondheim, Norway), which included an ultrasound-based system for recalculation of the topographic parameters to account for the shifting of the cerebral structures during the on-going resection.

The HHP was calibrated into the navigation system. Measurements were registered on different spots, e.g. "solid tumor" or "tumor margin" in accordance with the images from the navigation system and biopsies were taken from these spots. The procedure was reported in Paper III. Again the 5-ALA-dosis was 5mg/kg.

7.4 Combination with a Surgical Microscope (Paper IV)

The first three studies were all performed on patients that had received a preoperative 5-ALA-dosis of 5 mg/kg. The next study was performed in combination with the meanwhile established surgical microscope for FGS. The general requirement for the use of these microscopes is a 5-ALA- (Gliolan®) -dosis of 20 mg/kg. For comparability of the measurements this dosis was adapted also for the HHP. Measurements were performed on spots where the microscope visualized fluorescence, "strong" and "weak" and intentionally also beyond in order to seek the marginal zone of the tumor (Fig. 23).

Figure 23: The operations were performed under normal conditions, in both white light and blue light mode. The HHP measurements were carried out under blue light.

The system had been further developed and could now take advantage of the laser's capability to operate in both pulsed and continuous mode, allowing for single spot measurements as well as for registrations with a moving probe along the suspected marginal zone of the tumor (Fig. 24). The output energy was raised to 10mW for higher precision; the measurement time remained 800 ms in the pulsed mode, capturing 3 spectra. In the continuous mode the measurement time could be elongated to 160s, then capturing up to 200 spectra. The precision of the continuous mode was necessarily lower due to the movements of the probe.

Figure 24: The computer screen of the registration unit was occasionally turned to the surgeon for immediate feedback, especially useful when measuring in the continuous mode.

Focus was on performing measurements of fluorescence with the HHP and retrieving biopsies from localizations where the microscopic view of the fluorescence was considered “weak” by the surgeon, as opposed to “strong”. The latter is known to correspond well to core tumor tissue, but the former has not been defined, although thought to correspond to the “outskirts” of the tumor, i.e. the marginal zone. The surgeon stated the judgment on which tissue type to suspect, including ‘healthy brain’, like in the previous studies. In the evaluation of the histopathological diagnoses different categories were formed: ‘normal brain’, ‘necrosis’, ‘core tumor’ and ‘intermediate inflammatory zone’. The ‘intermediate zone’ was subdivided into ‘gliosis with evidence of tumor’ and ‘gliosis without evidence of tumor’.

7.5 Patients

The patients examined had been referred under the diagnosis of an intracranial tumor and were selected if a malignant glioma was suspected. The gender distribution corresponded to general statistics on epidemiology. All in all 25 patients operated on with the HHP were included; aged 18-82 years, 14 males and 11 women. Informed consent was obtained from every patient. Ethical approval from the local board was granted for all studies within the project (Project No.: M139-07, 2012/333-32).

7.6 Data analysis

For the study with the ultrasound navigation system (Paper III) 180 fluorescence samples were captured together with 20 biopsies and 30 ultrasound images. In the comparison with the blue light microscope (Paper IV) the HHP was used at 193 sites and in combination with the microscope in 70 positions. A total of 79 biopsies were harvested. The ratio was calculated for each spectrum, and compared statistically with the naked eye inspection, ultrasound data and microscopy images respectively. For the data analysis and statistical tests MATLAB® (The MathWorks, Inc., USA) was used. In Paper III the fluorescence data were divided in two groups; spectra with a

PpIX peak and without PpIX peak. Spectra with clearly identified artifacts, for example, through light interference from the microscope or from blood staining of the probe, were excluded for further analysis. In Paper IV signals affected by blood were included in the comparison of the HHF-probe and the FGR-microscope, but signals totally blocked by blood were removed from the comparison with histopathology.

The number of true positives (TP), true negatives (TN), false positives (FP) and false negatives (FN) were defined based on the histopathology diagnosis to calculate the diagnostic performance parameters. True positives included the low and high-grade tumor tissue, intermediate inflammatory zone with tumor cells and necrotic tissue with tumor cells. True negatives included intermediate inflammatory zone without tumor cells; the reverse was valid for the false positives and false negatives. Two diagnostic performance parameters were calculated as defined by sensitivity, $TP/(TP + FN)$ and specificity, $TN/(TN + FP)$. Within the FGR-microscopy data, any visible fluorescence, i.e. “weak” and “strong”, was grouped as tumor positive. For the statistical analysis, values of 0, 1 and 2 were assigned to “none”, “weak” and “strong” fluorescence.

Each spectrum was evaluated by forming a ratio (Fig. 21). Statistical comparisons between the groups in Paper III were done using Fisher’s exact test with double-sided 95% confidence interval and the nonparametric Mann–Whitney U-test for grouped samples was used for statistical comparison between the calculated fluorescence ratios according to the tissue type groupings. In the data analysis for Paper IV the Linear Pearson correlation was implemented to investigate goodness of fit (R^2) and p-value between the calculated fluorescence ratios measured by the HHF- probe and the grades of fluorescence seen by the surgeon through the FGR-microscope. Also a receiver operating characteristics curve (ROC) was plotted to compare the classification efficiencies of the FGR-microscopy vs. spectroscopy.

Approximately 1-2 mm large biopsies were taken in the operating room. In the pathological examination routine the samples were paraffin embedded and formalin fixed. Using a microtome, 4 μ m sections were cut and put on objective slides. The samples were subsequently examined under a microscope to determine the presence of the type of malignancy and cancer grade. The biopsy samples were categorized depending on the neuropathology diagnosis into one of the groups of non-malignant brain tissue, low grade tumor (LGT, corresponding to WHO grade II; some samples were found in the mixture of grades within tumors of higher malignancy), high-grade tumor (HGT, corresponding to WHO grades III and IV), necrosis containing tumor and the intermediate inflammatory zone which was further divided in two categories, namely 'gliosis with evidence of tumor cells' and 'gliosis without evidence of tumor cells'. The neuropathologist was blinded to the fluorescence measurements. Each biopsy was separately graded as first described by the surgeon and then correlated to the fluorescence.

8 Results

8.1 First Experiences; the LED System (Paper I)

The general handling of the tool and the registration units was easily adapted by all of the staff as well as the surgeon and the biomedical engineers, who registered the measurements. The implementation caused no difficulties in the OR (Fig. 25). The flexible fiber cable was at a length of 3m long enough to keep the necessary distance to the sterile field. No infections were caused by any unusual circumstances in cases where the HHP had been used.

The system thus proved robust and easy to transport and to install on site, as it consisted only of the small box described above, the fiber cable with the probe and a laptop computer.

Figure 25: The handling in the OR caused no difficulties, neither any sterility hazards nor any interference with other instruments.

Covering the probe with the non-translucent plastic funnel did reduce ambient light artifacts and actually helped to provide cleaner signals, but it turned out to be impractical clinically. Two versions were tested: one of soft plastic material, which did keep the position along the rigid metal tube of the HHP, but which was difficult to move along the probe; the other one of rigid plastic material, which was easily moved along the probe, but which did not stay in place (Fig. 26). Unless the tip of the probe was fully covered and protected from ambient light the signals were imprecise and not useful.

The funnel made it difficult for the surgeon to see where the probe was actually positioned. Also the microscope and the surgical lamps had to be turned away from the surgical field and the overhead lights were to be dimmed. This prolonged the measurements, as special care must be taken to avoid lesioning of the tissue or displacement of the tip of the probe.

Figure 26: The HHP was initially held with the shielding funnel, while using an LED as the light source. The funnel reduced artifacts from surrounding light sources, but it obstructed the surgeon's view.

Some of the measurements had to be repeated several times due to minimal blood interference at the site of measurement, although it was rinsed with saline and seemed clean to the naked eye, even under vision through the microscope, before placing the probe.

In spite of these practical circumstances, the main finding was that the system could identify malignant brain tumor tissue and differentiate it from healthy brain, as shown in Figure 27.

Figure 27: In accordance to the expected signals from the skin measurements, the HHP showed the typical fluorescence peak at 635 nm when measuring on tumor tissue, whereas surrounding healthy brain tissue did not show the peak, only autofluorescence. The two measurements were made in close proximity.

The basic design of the system was confirmed as useful for the purpose, but it needed further refinement, i.e. more power and precision.

8.2 Seminal Improvement; the Pulsed Laser System (Paper II)

With the pulsed laser system ambient light suppression was effective, although the signals recorded still improved with shading of the tip of the probe, e.g. by holding a hand between the microscope and the measurement site for the duration of the 3-4 seconds measurement. The surgeon was still able to control the position of the probe in the resection cavity (Fig. 28).

Figure 28: With the pulsed system the measurements could be performed under white light conditions with a high degree of artifact suppression. However, the registrations were more precise, if the measurement site was shaded, e.g. with a hand under the microscope for a few seconds.

The measurements with the pulsed laser could clearly differentiate healthy brain, grey or white respectively, from tumor tissue or e.g. blood obstruction of the tip of the probe (Fig's. 29a-d).

Using the ratio between the measured specific fluorescence and the autofluorescence (presented in chapter 7.2 above), it was possible to obtain reproducible and objective values corresponding to the different tissues examined, i.e. “normal brain” and “tumor”. In a number of spots within areas macroscopically considered as “normal brain” by the surgeon, a fluorescence signal was detected and tumor was histologically confirmed (Fig. 30).

Figure 29 a) Brain tissue was safely identified, signaling a curve without a fluorescence peak at 635 nm; b) White matter could be differentiated from grey matter due to different optical properties. A smaller portion of tumor tissue, i.e. of a lower level of infiltration, could be seen in this case; c) Artifacts could be identified and differentiated from other findings of cerebral or tumoral tissues. Blood often covered the tip of the HHP; saline cleansing easily cleared it; d) The HHP could be used for measurements on any surface in the operational situs, but it could also measure in depth. The surgeon was able to estimate what lay ahead of the instruments in the direction of the resection.

Figure 30: These measurements show the situation in one location, where the surgeon initially was reluctant to resect the tissue, in the vicinity of a blood vessel. The surgeon had stated the suspected diagnosis as “not tumor” before measuring with the HHP. When the HHP displayed the typical curve for malignant tumor tissue, it was decided to resect. Afterwards the control measurement on the location did not show any more tumorous peak. The vessel remained unharmed. Histology confirmed tumor growth in the specimen.

Initially the results of the measurements were not disclosed to the surgeon during the operation, in order not to influence the surgeon's decisions. Later in the course of the project verbal feedback was given, as the accuracy of the measurements was increasingly evident. The new system with the pulsed laser light source proved easier to use; the ambient light was no longer a relevant factor of disturbance, the measurements and the registrations were faster and the signals were more precise. The identification of malignant tumor tissue and the differentiation from healthy brain was swift and safe.

8.3 Hand-held Probe and Navigation (Paper III)

Intraoperative navigation was useful when performing fluorescence measurements and it proved easy to calibrate the hand-held probe into the navigation system. Not only could the HHP be used as a pointer in the resection cavity, it could also be used to penetrate the depth of the tumor. The signals thus retrieved gave the surgeon an idea of the tissue type beyond the surface of the resection cavity and simultaneously the navigation could show the distance of the probe tip to normal brain (Fig. 31).

Figure 31: The HHP was easily integrated into the navigation system. The probe could be used as a pointer and the measurements of the fluorescence could be compared to the radiographic localization. The shielding funnel was still used in some of the measurements with the navigation system, but was removed after the evaluation of the pulsed system indicated that it was redundant.

Like in the previous studies, the protocol demanded that the surgeon should state which type of tissue was being measured before the actual measurements were made. The fluorescence peaks were then noted and a snapshot from the screen of the navigation system was taken (Fig. 32).

Figure 32: Examples of snapshots from the navigation system screen, displaying the position of the tip of the probe in the ultrasound image. The arrows point at the different measurement spots with matching fluorescence curves. The corresponding biopsies confirmed the categorization based on the fluorescence ratio. A: “tumor margin”, B: “solid tumor”, C: “necrosis”.

The surgeon could base the pre-measurement statement of the diagnosis not only on the visual impression from the operation site but also from the navigated position. The categories for the statements were labeled “not tumor”, “gliosis”, “tumor margins”, “solid tumor”, “necrosis”, and “unclassified tissue” (Fig. 33).

Figure 33: The median values of the ratios calculated were compared to the surgeon's visual diagnosis. Both the general tendency of the fluorescence measured and the ratios calculated did confirm the surgeon's statements on the tissue type, although the correlation was not as high as with the histological findings.

The histological examinations of the specimen taken from measurement spots were compared to the fluorescence ratios calculated. Zero value was calculated for "necrosis" and also for "not tumor", which matched the histological diagnosis. Higher values correlated with "solid tumor" and lower values were found for "gliosis" and the "tumor margin" categories, also in accordance with the histology (Fig. 34).

Figure 34: Increasing fluorescence ratio values were well related with the histological findings in the tissue samples measured.

When comparing the accuracy of ultrasound based navigation system and of fluorescence measurements to detect tumor as verified in the histological specimens, it was found that despite HHP being slightly superior to navigation, the combination of the systems resulted in the highest count of true positive observations (Fig. 35).

Figure 35: “True positive” diagnosis was higher with the HHP (here referred to as OTP, as in Papers II and III) than with the ultrasound based navigation system. The combination of the systems gave even better results. TP = True Positives, TN = True Negatives, FN = False Negatives, FP = False Positives.

The calculated ratio made it possible to identify and differentiate various tissue types in correlation to the radiographically visualized extension of the tumor by the strength of the recorded signals, i.e. the height of the peak in the curve on the computer monitor. It was noted, however, that the ratio values were not comparable from case to case, but only within the series of measurements from one patient at a time; a ratio of ‘2’ or ‘0.5’ would not necessarily correspond to “solid tumor” or “gliosis” in every patient. It was understood, that the degree of PpIX enhancement would depend on many different factors between different patients and also that the measurements were carried out in different manners in each case. Yet, the relation of the ratios between the different tissue types in each case was retained.

A further minor modification of the new system now allowed for two types of measurement method: single spot measurement, with the HHP held on one spot while repeating the measurements 3-5 times within 2.4-4 s; and continuous line measurement, with a repetition of the spectra up to 200 times while moving the probe along an imaginary line, where the tumor extent was suspected to be limited towards the normal brain (Figs. 36a-b, 37). This latter mode was tested in some cases and seemed promising, but will need refinement of the interface, which is planned as a separate project. In the project presented here, all evaluations were based on single spot measurements.

Figure 36a: In the continuous mode measurement the number of spectra captured was increased to a maximum of 200. The figure shows several overlapping spectra of different peak height, as the tumor content of the tissue varied.

Figure 36b: In the continuous mode measurement several signals were obtained by moving the HHP along a line across the tissue. This was useful to delineate the tumor extent. The figure shows excerpts from a recorded film of such a measurement, where the surgeon could see the changes in the curve in real-time.

Figure 37: In the continuous mode the probe was moved in zigzag-manner across the anticipated limitation of the tumor extent, in order to see the variations of the fluorescence peaks and where the fluorescence diminished.

8.4 Hand-held Probe and Microscope (Paper IV)

When using the microscope for fluorescence-guided surgery it was necessary to keep lights dimmed in the OR. As the blue light mode was switched on from white light, a second of blinding occurred before the surgeon's eyes were adapted to the darker surgical field and structural details could be discerned. The microscope then provided an overview of the resection site to the degree of magnification desired. The HHP could be introduced into the setting without any difficulties.

Two incidental observations were made during the measurements under blue light conditions. First, at a distance of 2-3 mm from the tissue surface the HHP induced fluorescence visible through the microscope in areas were biopsy confirmed finding of tumor, but where no fluorescence had been seen through the microscope before using the HHP there, thus acting in similarity to a flashlight. Second, it was noted that the signal registration was undisturbed by ambient light sources in this set-up, thus eliminating the need for pulsed mode of the laser. Both observations were reserved for future studies. Examples of typical blue light visualizations are shown in Fig. 38.

Figure 38: Typical aspects under the microscope during blue light vision; a) no fluorescence, except at the tip of the HHP (flashlight effect) b) diffuse “weak” fluorescence

The median HHP fluorescence ratios calculated for the classes of fluorescence used with the microscope i.e. “no fluorescence”, “weak” and “strong” fluorescence were calculated to 0.3 ($n = 131$), 1.6 ($n = 34$) and 5.4 ($n = 28$) respectively (Fig. 39). The magnitude of these ratios are similar to ratios calculated for spots based on histopathological diagnosis, thus indicating that the microscopical observations were made in areas of “no tumor”, “tumor margin” or “core tumor”. These values corresponded to the values calculated for biopsy specimen from measurement spots, thus confirming e.g. “core tumor” or “tumor margin”. Of the 131 measurements in areas where no fluorescence had been seen through the microscope, the HHP detected fluorescence in 88 sites (67%).

Of the biopsy specimen graded “high grade tumor” 91% had shown fluorescence with the HHP, but only 25% through the microscope. Histopathologically, the category “gliosis” or “marginal zone” was subdivided during the histopathological examinations, since gliotic tissue was found “with evidence of tumor cells” and also “gliosis without evidence of tumor content”. The two subsets of “gliosis” had shown fluorescence in 77% and 66% respectively using the HHP and had given different ratios of fluorescence for “gliosis with evidence of tumor” versus “gliosis without evidence of tumor” respectively.

Figure 39: a) The ratios calculated from the HHP fluorescence measurements in correspondence with the fluorescence observations as seen through the microscope, when using the categories “none”, “weak” and “strong” fluorescence. b) The ratio differences between the histopathological categories were significant even between the subcategories from the marginal zone.

For these categories the sensitivity of the HHP was higher than that of the microscope, but the specificity was lower, as the HHP had detected gliotic tissue without evidence of tumor as well. Setting the threshold for resection to the microscope category “strong”, would correspond to HHP ratios >12 in order to reach the same specificity as the microscope. The sensitivity, however, equaled that of the microscope at a ratio of 7.6 (Fig. 40). These ratio values corresponded to “strong” fluorescence under the microscope and to “core tumor” in the histopathological exams throughout the series of measurements in most patients. They are significantly higher than the ratio values found in “weak” fluorescent areas, where in many cases “core tumor” was found in the biopsies taken there.

Figure 40: ROC/AUC curves; comparing threshold levels for the sensitivity and specificity of the HHP and the FGS microscope. The HHP can detect fluorescence with a higher sensitivity in areas that display “weak” fluorescence under the microscope, but the specificity is lower.

Some of the spots where the HHP detected fluorescence while the microscope did not, were at angles not accessible for direct vision with the microscope, e.g. immediately after the entrance zone under the cerebral cortex.

The HHP signaled negative fluorescence in 11% of spots that were seen as tumor positive with both “weak” and “strong” fluorescence. These negative measurements could be explained by external factors like blood covering the tip of the probe or the measurement being carried out inaccurately, e.g. by wrong angulation of the probe towards the tissue.

The use of the HHP in combination with the microscope caused no difficulties in the practical handling. The measurements with the HHP caused no disturbances of the viewing of the fluorescence through the microscope, e.g. increased bleaching at the measurement spots; this would in any case not be detectable for the surgeon, since the measurement areas were negligibly small in relation to the tumor extent and the bleaching effect of the HHP on the tissue was minor.

9 Discussion

This thesis presents a small hand-held tool that was developed to detect malignant brain tumor tissue by means of fluorescence spectroscopy. It was found that reliable measurements of specific fluorescence could be obtained with high sensitivity for tumor and that various tissue types including normal brain could be objectively differentiated. The device could easily be integrated in the OR without any negative interference with this very special environment. During the early stages of development several technical modifications were needed to improve the detection performance and sources of error were eliminated. The new tool was easy to integrate with other surgical instruments and it proved an important adjunct to standard surgical techniques. Integration with the neuronavigation system was found to improve the accuracy of either system in defining the tumor margin. When extending the application to the surgical microscope and the microsurgical technique the new fluorescence detection probe proved to augment the surgeons' performance beyond visible tumor limits.

The main advantage of the tool is its high sensitivity for tumor fluorescence and the calculation of objective values for the degree of fluorescence registered. There are many possibilities as to how the tool can be used and improve the surgical treatment of GBM.

There is no cure for GBM and the survival rates and quality of life have until recently been constant for many decades. It is known that the amount of residual tumor after resection has a decisive bearing on survival since the effects of oncological treatment depends on the actual tumor cell burden. It is clear from many studies that tumor remnants in excess of 10% of the tumor volume carry a very dismal prognosis. The actual amount of residual tumor tissue depends on the tumor size as illustrated in Fig. 6 and also a few percent of a large tumor remaining after surgery represents a significant tumor cell burden. On the other hand, even lobectomies incorporating vast margins of normal tissue surrounding a GBM, as practiced in the

early history of neurosurgery (Gardner, 1933), did not significantly influence the recurrence rate. Therefore, surgical removal of the tumor tissue can at best be the prerequisite for the oncological therapy.

The modern surgical approach for maximum resection of malignant brain tumor is counterbalanced against preservation of function and quality of life, along the principle of a defensive strategy. To achieve very high levels of cytoreduction, dissection must extend into the outer edge of the marginal zone of the GBM, where obvious tumor gradually evades into macroscopically normal appearing brain. It is crucial to find technologies that identify discrete tumor infiltration at this level and simultaneously apply techniques that can identify functional tissue of high significance, both cortical and deep fiber tracts, to prevent dissection at the edge of the marginal zone taking place in normal and eloquent brain tissue.

In some anatomical locations such as the brain stem or basal ganglia GBM cannot be safely resected at all and in such cases biopsy followed by oncological therapy is the strategy of choice for optimal preservation of life quality. Other GBM may be located in regions permitting radical excision without causing severe neurological deficits. However, many GBM involve or grow close to critical structures. The pursuit of GTR carries a significant risk of functional injury especially for the deep connective fiber tracts in the hemispheres. To protect such structures a number of techniques can be used to increase the dissection safety, thus extending the possibilities to resect the outer marginal zone of such tumors. To balance methods with high sensitivity for tumor infiltration, neurophysiological monitoring with MEP, SEP and VEP or direct stimulation of the motor fibers in the internal capsule is routinely applied. Tumors affecting the speech areas can be operated with the patient awake in local anesthesia. This technique is rather widely used for low-grade tumors but for GBM it is less frequently used, although we are currently expanding this possibility. Intraoperative imaging can be used to update the reference information in the

navigation system and both ultrasound and especially intraoperative MRI can be used. Intraoperative MRI allows for detailed updating on tumor remnants and also e.g. on the location of white matter tracts in relation to resection.

The optimal monitoring method depends on the nervous function at risk. For motor and sensory system mapping, electrophysiological methods offer good opportunities for safe mapping whereas for the visual system is less suited for this kind of monitoring. Although VEP can be used, the visual tract is best localized using intraoperative navigation. To protect language function, both cortical and connective fibers such as the arcuate fasciculus, awake surgery is necessary for monitoring. The intraoperative navigation technique suffers from a major drawback due to “brain shift” caused by resection of tissue, which leads to incongruence between the actual positions of structures in the cranial cavity in relation to the original position displayed in the preoperative images on which the navigation is based. Therefore, imaging in intraoperative navigation must be upgraded during surgery. In this project, ultrasound was used for this purpose in a specially designed system, the Sonowand. Today, the interest for intraoperative MRI is expanding and many institutions embark on this technically challenging method.

It has been reported in several international publications that the surgeons’ generally overestimate the degree of the resection during the procedure, in comparison to postoperative radiological control exams (Albert et al., 1994, Forsting et al., 1993, Eljamel et al., 2013). The advent of FGS has to some degree helped the neurosurgeons to overcome this discrepancy and improve the extent of resection. With this technique the surgeon is, during the operation, enabled to discriminate “solid red” from “vague red” fluorescence, of which the former can be resected, whereas the latter should not, according to general recommendations (Stummer et al., 2000). The “solid red”, i.e. “strong” fluorescence has been demonstrated to exhibit tumor tissue. The “vague red”, i.e. “weak” fluorescence is assumed to correspond to what has been called the tumor margin or

marginal zone (instead of a border). But the “weakly” fluorescing areas contain tumor tissue, although to an uncertain degree due to inhomogeneous infiltration of normal brain tissue. Using the established FGS microscope, there are no objective parameters that differentiate “strong” fluorescent tissue from “weak” fluorescence other than the surgeon’s eyesight in combination with experience and acquired skills, which are subjective qualities. Here, the use of the HHP can fulfill an important task, as ratio values can be compared and a decline of fluorescence, i.e. lesser tumor content in normal brain resembling areas, can be recognized and clearly differentiated.

The HHP, with its high sensitivity, should find a useful position within a frame of several different tools used in neurosurgical tumor resection. The original vision of Prof. Backlund consisted of a combined light source and a suction device and this idea is still valid (Backlund et al., 2002). Of special interest is the combination with the ultrasound tissue aspirator and with electrodes for intraoperative neurophysiological stimulation. Such electrodes could in fact be shaped as a dissector, suitable for classical manual tumor removal. Creating such a multipurpose tool with dual functions, i.e. high sensitivity for tumor cell detection through fluorescence and balanced by protection of eloquent tissue through electrical stimulation, is a close perpetuation from Prof. Backlund’s original vision.

One finding yet to be further explored in this context, is a “flashlight effect”, noted when using the HHP in conjunction with the surgical microscope. This phenomenon would allow the tip of the light source to be mounted a few millimeters proximal to the tip of the surgical tool, thus avoiding interference with the function of the tool. It is of interest to combine the HHP with other kinds of continuous data that can be used for delineation of the outer edge of the tumor marginal zone. Other research groups have reported on the use of highly sensitive spectroscopic probes for tumor detection, e.g. in combination with intraoperative MRI (Eljamel et al., 2013, Roberts

et al., 2011), with encouraging results, although not further specified. Recently Fluorescein, which has been used in the past, has seen a revival and is integrated in the latest generations of microscopes as fluorescence at 560 nm (Chen et al., 2012, C. Lane and A. Cohen-Gadol, 2013). Theoretically detection of Fluorescein should be possible using a different light source in the HHP system and it is likely, that switching between the two should be technically possible. It remains to be explored, whether Fluorescein could be detected in such an objective manner, as demonstrated for 5-ALA in the here presented project. So far, the Fluorescein560 technique is as much a matter of the surgeon's subjective judgment as is the established FGS with 5-ALA. One possible advantage of combining these two fluorescence methods could theoretically be that Fluorescein 560, which simply seems to pass the disrupted BBB, will accumulate in areas corresponding to contrast enhancement on MRI; thus helping to alert the surgeon that the tumor marginal zone is entered. The gradual transition in ALA/PpIX fluorescence from "strong" to "weak" seems less precise to define this line of demarcation, since it is enhanced beyond it.

Further, the rate and pattern of regional blood flow varies with anatomical location in the brain and also with tissue type. The HHP can be combined with a small Doppler probe (Wårdell et al., 2013) thus allowing for integration of fluorescence and specific flow patterns which could open up for more detailed tumor margin zone identification. Such an extension of the HHP's capability with the detection of blood flow, i.e. blood vessels, is currently being explored for use in navigated biopsies of suspected brain tumors.

One major difference between the HHP and microscope FGS is the possibility to explore the depth of the tumor resection field by inserting the HHP into the tissue. In combination with the navigation system this option facilitates the planning of the surgeon's strategy. This can overcome the relatively short measurement depth, approximately a millimeter, when measuring on the surface.

More data is needed on the ALA/PpIX metabolism and fluorescence of tumors other than GBM, although it is known that several other forms of malignant glioma such as anaplastic oligodendroglioma and ependymoma or some cancer metastases show PpIX fluorescence (Hefti, 2013, Ewelt et al., 2015). This implies that a variety of lesions suspected for malignant CNS tumor are within the category of possible detection by means of fluorescence spectroscopy. The biopsy procedure can be shortened very significantly also, thus avoiding repeated sampling along different trajectories, which carries an increased risk for bleeding. As soon as fluorescence is detected, a relevant biopsy can be taken and the procedure can be closed. First results from our on-going study seem to confirm such significant advantages.

Also, in open biopsy procedures 5-ALA/PpIX-fluorescence can be used to verify suitable spots for tumor sampling.

It is an important finding that tumor fluorescence levels vary between different patients, although the distinct differences between normal tissue, tumor margin and tumor core are retained in all individuals. Consequently, it is not possible to construct an automatic algorithm to identify tumor and base resection on figures or derived maps alone. Rather, handling of the marginal zone still depends on extensive experience of the surgeon in order to recognize different tissues, not only visually, but also and importantly, tactile differentiation of tissue texture; the high sensitivity of the HHP constitutes no shortcut to the limit of resection, but could instead lead to uncritical resection, if relied on inappropriately.

Known problems with FGS microscopy, such as e.g. diffuse appearance of fluorescence in the ventricular walls without macroscopic evidence of tumor growth, are not resolved by use of the probe. It may be assumed that the fluorescent tissue in the ventricular wall contains tumor cells. Although the HHP also detects fluorescence there, there is no clear evidence in the literature to support the decision whether to resect or not, which so far cannot rely on any clear evidence in the literature; there is no explanation

for the phenomenon known and there are only a few studies performed on a comparison of postoperative courses in cases after resection or non-resection of the fluorescing ventricular wall (Tejada-Solis et al., 2012) (Moon et al., 2016). It may be assumed that the fluorescent tissue in the ventricular wall contains tumor cells, yet it is suggested not to resect diffuse fluorescing areas in the ventricular wall, since complications may be incalculable. Engagement of the ventricular wall by malignant gliomas is an independent bad prognostic factor, but it is suggested not to resect diffuse fluorescing areas in the ventricular wall, since complications may be incalculable.

Also, an optimal dosis of the 5-ALA medication is uncertain. For the blue light microscope, a dosis of 20mg/kg is recommended. In the development of Gliolan[®] (Medac, Germany) doses of 0.2mg/kg and 2mg/kg were tested before 20mg/kg was found to meet the requirements of the FGS, as facilitated by the commercially available microscopes from various manufacturers. Apparently other doses, e.g. 10mg/kg, were not systematically tested (or not published). The HHP proved to be able to detect fluorescence in accordance with histological findings in brain tumor tissue at 5mg/kg, even after up to 10 hours. Although adverse effects of Gliolan[®] are not a major problem, a lower dose would seem to enhance a reduced risk of such events. Additionally, a lower dose would be more cost effective, given equal detection rates by adequate equipment. Our microscopes were not able to detect fluorescence when the patient had received 5mg/kg of Gliolan[®]. In another on-going project (EudraCT 2013-005565-40) at the Depts. of Neurosurgery and Biomedical Engineering, in which children with malignant gliomas are operated by means of FGS, observations imply a different metabolism of 5-ALA compared to adults; postoperative skin measurements (Haj-Hosseini et al., 2015) have shown slight fluorescence in adults, but not in the children examined so far. Obviously, we need to understand more about the effects of 5-ALA in the human brain and in malignant brain tumors.

Further research regarding these implicit questions will have to be carried out. Also, the HHP will be further developed for practical use, e.g. the interface, as the computer screen has its shortcomings from the surgeon's position; an external display could become part of an interface development, but acoustic feedback may be favored over further visual distraction.

In conclusion, the hand-held probe system can reliably and in an easy-to-handle manner detect malignant primary brain tumor tissue with a high sensitivity. The system is robust, mobile and cost effective.

Both single spot and continuous line measurement techniques can be applied to detect and delineate tumor tissue, where the navigation systems or the surgical microscopes do not reveal it. The combination of the systems is beneficial.

The HHP can contribute to higher precision in the intraoperative observations during surgery of malignant gliomas and ultimately support the surgeon's decision on resection.

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12 Review of papers

During the course of this project a small optical probe was developed as a hand-held device. It was designed to register 5-ALA/PpIX fluorescence in brain and tumor tissues with a high sensitivity and it should enable objective grading of the signals in order to identify different tissue types with varying degrees of tumor infiltration.

Within the frame of this thesis, the system was implemented and evaluated during brain tumor resection in four steps:

- 1) A fluorescence spectroscopy system using an LED-diode was assessed during a few surgical sessions. The system build up was presented and the feasibility of tumor tissue detection with the hand-held probe during brain tumor surgery was demonstrated. Due to weak signals further development of the system was necessary.
- 2) The second setup resolved the problems with ambient light in the operating field by introducing a pulsed spectroscopy system. The measurements proved to be very precise and allowed the quantification of the signals in correlation to the different identified tissue types as given by the pathological diagnoses from biopsies.
- 3) In the third phase evaluation was done on a series of nine patients. The system was used together with ultrasound-guided navigation. The lower dose of 5 mg/kg 5-ALA was still used, as in the previous trials, and the expected results were reproducible as for reliable tumor identification and discrimination from brain tissue. The faster measurements were less susceptible for artifacts arising from ambient light. The equipment was implemented in an easy-to-handle fashion for the surgeon as well as for the staff.
- 4) In the fourth trial a combination with the established fluorescence microscopy technique was investigated (Paper IV). For this purpose the recommended ALA-dose for use with the microscope of 20 mg/kg was administered. The hand-held probe proved to be able to detect tumor in areas beyond detection by the microscope.

Papers

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