CHAPTER 6

TIME GATED FLUORESCENCE SPECTROSCOPY AND STEADY STATE FLUORESCENCE IMAGING OF BRAIN TUMOR TISSUES

6.1 INTRODUCTION

In the previous chapters, it has been observed that steady state native fluorescence spectroscopy of blood plasma may be used as a potential technique in the characterization of normal and diseased subjects. In addition to these well established applications of steady state fluorescence spectroscopy for \textit{in vitro} and \textit{in vivo} characterization of biological materials, time gated fluorescence spectroscopy and imaging of tissues also plays a vital role in the current research on optical detection of tumors. It is based on the specific localization of administered fluorescent molecules (fluorophores) in tissue or cell structures or the characteristic fluorescence signatures of endogenous fluorophores present in cells and tissues. In this context, the present chapter describes the results of a pilot study carried out on \textit{in vitro} time gated fluorescence spectroscopic characterization and steady state fluorescence intensity imaging of tissues from solid brain tumors.

The major cause of morbidity and death in malignant brain tumor cases is recurrence of tumor at the original site (Wagnieres et al 1998). Surgical removal of brain tumor is the most common initial treatment received by brain tumor patients. Surgical resection can benefit the patients in several ways: for example, it relieves the mass effect of tumor on neurological tissue and allows histological diagnosis of the tumor, which directly affects the direction of follow-up therapeutic strategy (Toms et al 1999). Many studies have demonstrated that aggressive surgical resection enhances the survival length and quality of life for brain tumor patients (Wisoff et al 1998 and Hess 1999). Therefore, the goal of brain tumor resection procedures is to maximize tumor removal with minimal neurological damage. To achieve this goal, accurate intraoperative identification of brain tumor margins during craniotomy is required.
Intraoperatively, brain tumor margins are currently determined by neurosurgeons by visual inspection and information provided by surgical navigation systems that are based on computerized tomography (CT)/magnetic resonance images (MRI) and/or intraoperative ultrasound (IOUS) (Auer and Velthoven 1990). However, these techniques have several limitations that prevent surgeons from achieving a complete tumor resection. First, the true infiltrating margins of primary tumors may not be visible on CT/MR images, even T2 weighted MR images, unless the tumor cells are sufficiently dense. Secondly, registration errors and intraoperative brain shift can degrade the spatial accuracy of the surgical navigation system by as much as 1 cm (Hill et al 1998 and Dorward et al 1998). Moreover, gliomas, unlike most metastatic tumors, do not typically possess clear boundaries, i.e. the margins appear blurred in the IOUS images. Further, it is often difficult, even for experienced neurosurgeons, to visually differentiate low-grade gliomas and associated tumor margins from normal brain tissues.

Although on-site pathology provides accurate diagnosis, it is time consuming and expensive and as such it is not used routinely for tumor-margin detection. Hence, there is a clear need for the development of a real-time, guidance tool that allows intraoperative detection of brain tumor margins with high sensitivity to facilitate complete or high-degree brain tumor resection. In this context, several studies have been reported on the potentiality of optical spectroscopy to fulfill this need (Stummer et al 1998a, Lin et al 2000 and 2001). These studies have been based on the measurement of steady state fluorescence spectra of tissues arising from either intrinsic fluorophores (Wagnieres et al 1998) or administered exogenous fluorophores such as HpD (Andersson-Engels et al 1989) or precursors such as Aminolevulinic acid (ALA) (Stummer et al 1998a). However, limited studies have been reported on the applications of time-gated fluorescence spectroscopic characterization and imaging of brain tumor tissues (Wagnieres et al 1998). In this regard, the present chapter reports on the measurement of time gated fluorescence spectral characteristics and images of normal and solid tumor tissues from human glioblastoma patients treated with 5-ALA.

6.2 MEASUREMENTS ON STANDARD FLUOROPHORES

The fluorescence lifetime values of standard fluorophores such as NADH, flavin, collagen and elastin were measured using time correlated single photon counting (TCSPC)
method. Time gated fluorescence spectra were also measured for these fluorophores for five
variable time gates of constant width viz., 0-5 ns, 5-10 ns, 10-15 ns, 15-20 ns & 20-25 ns and at
365 nm excitation using a mercury lamp. The results of these studies are presented in this
section.

6.2.1 Measurement of Fluorescence Lifetime

In order to optimize the time gates used for fluorescence spectral measurements, the
fluorescence lifetimes of some common fluorophores present in tissues such as NADH, flavin,
collagen and elastin were measured by TCSPC method, using the experimental arrangement
shown in Figure 2.5. The details of the experimental procedure are given in Chapter 2. To be
brief, a frequency doubled laser diode at 390 nm was used and all the photon events were
accumulated in a multichannel analyzer to obtain the fluorescence decay curves. The
fluorescence decay curves were analyzed using a nonlinear least-square reconvolution and two
exponential fitting algorithm as mentioned in Chapter 2.

For this study, 10 µM solutions of NADH and flavin in Phosphate buffered saline
(PBS) of pH 7.4 were used whereas the fluorophores collagen and elastin were used in powder
form. The fluorescence decay curves of NADH and flavins are shown in Figures 6.1a and b
respectively. Figure 6.1a gives a rapidly decaying component with a short lifetime of
0.6 ± 0.09 ns and a second component with a longer lifetime of 2.3 ± 0.3 ns. The relative
amplitude of the rapidly decaying component is found to be more than that with a longer
lifetime. In the case of flavin, a rapidly decaying component with a lifetime value of 0.74 ± 0.28
ns and a second component with a longer lifetime of 4.8 ± 0.15 ns were found (Figure 6.1b).
However, in this case, it is found that the relative amplitude of the second component with a
longer lifetime is more than that of the rapidly decaying component.

The fluorescence decay curves of collagen and elastin are shown in Figures 6.2a and
b respectively. In the case of collagen, two components were found one with a shorter lifetime
of 0.88 ± 0.07 ns and the other with a longer lifetime of 6.26 ± 0.17 ns. Similar results are
obtained in the case of elastin in which a component with a shorter lifetime of 1.46 ± 0.07 ns
Figure 6.1 The fluorescence decay curve of (a) NADH and (b) Flavins measured by TCSPC technique.
Figure 6.2 The fluorescence decay curve of (a) Collagen and (b) Elastin measured by TCSPC technique
and a second component with a relatively longer lifetime of $6.62 \pm 0.11$ ns were found. In the case of both collagen as well as elastin, the relative amplitude of the longer lifetime component is more than that with a shorter lifetime. The nanosecond lifetimes of the fluorophores calculated from the decay measurements, the relative amplitudes of the two species and the Chi square values of the exponential fit are tabulated in Table 6.1. From the table, it can be seen that both collagen and elastin have longer lifetime values when compared to NADH and flavin.

<table>
<thead>
<tr>
<th>Fluorophore</th>
<th>$T_1$ (ns)</th>
<th>$T_2$ (ns)</th>
<th>$I_1$ (%)</th>
<th>$I_2$ (%)</th>
<th>Chisq.</th>
</tr>
</thead>
<tbody>
<tr>
<td>NADH (10 μM)</td>
<td>0.60 ± 0.09</td>
<td>2.30 ± 0.30</td>
<td>70.43</td>
<td>29.57</td>
<td>1.2</td>
</tr>
<tr>
<td>Flavin (10 μM)</td>
<td>0.74 ± 0.09</td>
<td>4.8 ± 0.15</td>
<td>6.85</td>
<td>93.15</td>
<td>1.2</td>
</tr>
<tr>
<td>Collagen (solid)</td>
<td>0.88 ± 0.07</td>
<td>6.26 ± 0.17</td>
<td>30.63</td>
<td>69.37</td>
<td>1.2</td>
</tr>
<tr>
<td>Elastin</td>
<td>1.46 ± 0.07</td>
<td>6.62 ± 0.11</td>
<td>33.59</td>
<td>66.41</td>
<td>1.1</td>
</tr>
</tbody>
</table>

### 6.2.2 Measurement of Time Gated Fluorescence Spectra

In order to study the time resolved spectral characteristics of the fluorophores, time gated emission spectra of NADH, flavin, collagen and elastin were measured as mentioned in Chapter 2. All the fluorophores were used in solid form for this study. A Q-switched Nd:YAG laser with the third harmonic at 355 nm and a fluorescence microscope coupled to the laser were used for the time gated fluorescence spectral measurements. An optical multichannel analyzer combined with a self-constructed monochromator was used to detect the fluorescence signals. Five time gates, viz. 0-5 ns, 5-10 ns, 10-15 ns, 15-20 ns and 20-25 ns were used for the spectral measurements. Fluorescence emission spectra were also measured at 365 nm excitation.
using a high pressure mercury lamp as a continuous wave (cw) source, for comparison with the time gated spectra.

Figure 6.3a shows the time gated as well as 365 nm cw excited emission spectra of NADH. It is found that the fluorescence spectra show a prominent emission peak around 470 nm. The intensity of this emission peak is found to be maximum at 365 nm cw excitation and it varied for different time gates. In the time gate of 5-10 ns, the intensity of the emission peak is found to be more than that at 0-5 ns. Further, it is found that the emission peak intensity decreased with increasing time gates. Figure 6.3b shows the time gated as well as 365 nm cw excited emission spectra of flavin. The fluorescence spectra of flavin show a primary emission peak around 565 nm and the intensity of this emission peak is found to be maximum at 365 nm cw excitation. In the time gated spectra, the emission peak intensity around 565 nm is found to be maximum at 5-10 ns and it decreased for increasing time gates.

Figure 6.4a shows the time gated and 365 nm cw excited fluorescence spectra of collagen. It can be seen that the fluorescence spectrum of collagen at 365 nm cw excitation shows two emission peaks around 440 and 470 nm, with the intensity at 470 nm being more than that at 440 nm. Similar spectral characteristics are observed in the time gated spectra at 0-5 ns and 5-10 ns. However, beyond 10 ns, the emission peak around 440 nm is found to be more dominant than that around 470 nm. Figure 6.4b shows the time gated as well as 365 nm cw excited fluorescence spectra of elastin. In this case, the fluorescence spectrum at 365 nm excitation shows a prominent peak around 475 nm and a small hump around 440 nm. In the case of time gated emission spectra, at 0-5 ns, and 5-10 ns, the emission peak around 475 nm is found to be prominent whereas beyond 10 ns, the emission peak around 440 nm appears to be prominent.

6.3 IN VITRO TIME GATED FLUORESCENCE SPECTRA OF NORMAL AND TUMOR BRAIN TISSUES

Time gated fluorescence spectra of were measured in vitro on 15 normal sites and 16 tumor sites from cryosections of tissues collected from five patients with malignant
Figure 6.3  Time gated and 365 nm excited steady state (cw) fluorescence emission spectra of (a) NADH and (b) Flavin
Figure 6.4  Time gated and 365 nm excited steady state (cw) fluorescence emission spectra of (a) Collagen and (b) Elastin
glioblastoma following oral delivery of 5-ALA in a dosage of 20 mg/Kg body weight. The spectra were measured from the tumor center, tumor border and normal tissues as judged under white light and later confirmed with histopathological examination. The time gated emission spectra of the cryosections were measured under a fluorescence microscope adapted for fluorescence excitation, as mentioned earlier. The details of the materials and methods are given in Chapter 2. In addition to the time gated spectra, the fluorescence spectra of the cryosections were also measured at 365 nm and 395-440 nm excitations using a Mercury lamp, with appropriate filters, as mentioned in Chapter 2. All the spectra were corrected for background signals of the glass slides on which the cryosections were mounted.

Figure 6.5 shows the representative time gated fluorescence spectra of a normal brain tissue. As it is found that beyond 20 ns, the fluorescence emission from the standard fluorophores are weak, for the measurements on tissue cryosections, only four time gates, viz. 0-5 ns, 5-10 ns, 10-15 ns and 15-20 ns were used. It can be seen from the Figure 6.5 that the fluorescence spectra of normal brain tissue exhibits a primary emission peak around 440 nm and a hump around 470 nm which may be assigned to the enzyme bound NAD(P)H present in tissues and the structural proteins, collagen and elastin (Wolfbeis and Leiner 1985 and Ramanujam 2000). It is also found that the intensity of this emission peak around 440 nm is maximum within the time gate 0-5 ns and it decreased with increasing time gates. The time gated fluorescence spectrum of normal brain tissue is found to be least intense in the time gate 15-20 ns. Also it is observed that the hump around 470 nm is less distinct in the time gated spectra, beyond 15 ns.

Figure 6.6a shows the representative time gated emission spectra measured from the periphery of a solid tumor of the brain. In this case, it is found that the emission spectrum exhibits a primary emission peak around 440 nm and a distinct secondary emission peak around 475 nm, which may be assigned to the enzyme bound NAD(P)H, collagen and elastin present in tissues. It should be noted that the primary emission peak around 440 nm is more intense within the time gate 0-5 as well as 5-10 ns, whereas beyond 10 ns, the intensity of the primary emission peak decreases with increasing time gates. It is also observed that the secondary emission peak around 475 nm which may arise from the intrinsic fluorophores, NAD(P)H.
collagen and/or elastin is more intense in the time gate, 0-5 ns and it becomes less prominent with increasing time gates.

Figure 6.5  Time gated fluorescence emission spectra of a normal site on a brain tissue cryosection

Similarly, Figure 6.6b shows the representative time gated fluorescence spectra of the center of a solid tumor cryosection. It can be seen that as in the case of the tumor boundary, the emission spectra of tumor center also shows a primary emission peak around 440 and a secondary emission peak around 475 nm which may be assigned to enzyme bound NAD(P)H, collagen and/or elastin. It should be noted that in the case of tumor center, the primary emission peak is more intense within the time gate 10-15 ns, than at 0-5 ns. Also, the fluorescence intensity of the emission peaks around 440 and 475 nm is found to decrease beyond 10 ns, with increasing time gates.
Figure 6.6  Time gated fluorescence emission spectra of a brain tissue cryosection at the site of (a) tumor periphery and (b) tumor center
Figure 6.7a shows the representative steady state fluorescence spectra of normal brain tissue, periphery of brain tumor and the tumor center, measured at 365 nm cw excitation of a mercury lamp. From this figure, it can be seen that the fluorescence spectrum of the tumor boundary exhibits two well defined emission peaks around 440 and 475 nm as observed in the case of the corresponding time gated emission spectra. Normal brain tissue is also found to exhibit similar spectral characteristics at 365 nm excitation. However, the fluorescence emission from normal brain tissue is found to be less than that of the tumor boundary. In the case of tumor center, the fluorescence emission is found to be least intense when compare to normal and tumor periphery. In this case, the emission peak around 475 nm is not so distinct as the tumor boundary.

In order to check the possible emission from ALA-induced Protoporphyrin IX (PpIX) in normal and tumor tissues obtained from patients administered with 5-ALA, fluorescence emission spectra were also measured at 395-440 nm excitation using a mercury lamp. Figure 6.7b shows the representative fluorescence spectra of normal brain tissues, tumor boundary and tumor center, measured at 395-440 nm excitation using mercury lamp. It is found that the normal brain tissues show enhanced fluorescence in the green region and emission maxima were observed in the range 520-545 nm. These native fluorescence emission characteristics may possibly be assigned to flavins. In the red region, the normal brain tissues exhibit weak fluorescence peaks around 635 nm. In the case of tumor boundary, it can be seen that the native fluorescence in the green region is significantly less when compared to normal tissues. However, in this case, two well defined emission peaks are observed in the red region around 635 and 672 nm. Similar peaks are also observed in the fluorescence spectrum measured from the center of a solid tumor. However, in this case, the fluorescence intensities of the emission peaks around 635 and 672 nm are found to be more than that of tumor boundary. The weak fluorescence emission peak observed in normal brain tissues around 635 nm and the well defined emission peaks observed in the case of tumor boundary and center of the brain tumor around 635 and 672 nm may be assigned to ALA induced endogenous PpIX in tissues (Stummer et al 1998a).
Figure 6.7  Steady state fluorescence emission spectra of normal and tumor sites on a brain tissue cryosection at a) 365 and b) 395-440 nm excitations
6.4 IN VITRO FLUORESCENCE IMAGING OF NORMAL AND TUMOR BRAIN TISSUES

In addition to the measurement of time gated and continuous wave excited fluorescence emission spectra, transillumination video images and fluorescence images were also recorded for normal and tumor brain tissues at each site studied. Transillumination and fluorescence images of the cryosections were recorded under the fluorescence microscope adapted for the measurement of time gated spectra. The fluorescence was excited by a high pressure mercury lamp at 365 nm and 395-440 nm and the corresponding fluorescence images were grabbed by a highly sensitive NCA camera. For each site, transillumination images under white light were recorded as an average of 2 images and fluorescence images were recorded as an average of 50 images, both using 10x microscope objective. The details of the experimental set up are given in Chapter 2.

Figure 6.8a-b show the representative fluorescence images of a normal brain tissue at 365 and 395-440 nm excitation. From the figures, it can be seen that normal brain tissues exhibit prominent native fluorescence in the blue and green regions. The fluorescence in the blue region may be attributed to the emission from NAD(P)H, collagen and / or elastin whereas the intense green fluorescence may have originated from flavins. These observations are in agreement with the emission peaks observed in the corresponding fluorescence spectra of the normal tissues (Figures 6.7a-b). Figure 6.8c shows the corresponding transillumination image of the normal brain tissue. Similarly, the representative fluorescence images of periphery of a solid tumor at 365 and 395-440 nm excitation are shown in Figures 6.9a-b respectively. It can be seen that the tumor periphery shows bright autofluorescence in the blue region (indicated by arrow heads) as shown by Figure 6.9a. Figure 6.9b shows that at 395-440 nm excitation, the fluorescence image of the tumor periphery shows bright red fluorescence indicating the accumulation of ALA-induced Pp IX in the tumor tissues. This is in agreement with the observations of fluorescence spectral measurements and this bright red fluorescence may be considered as the origin of the well defined red emission peaks observed in the fluorescence spectrum of tumor periphery at 395-440 nm excitation. The corresponding transillumination image is shown in Figure 6.9c which indicates the highly densed cell structure of the tumor tissues.
Figure 6.8  Steady state fluorescence images of a normal site on a brain tissue cryosection at (a) 365 nm excitation, (b) 395-440 nm excitation and (c) the corresponding transillumination image under halogen lamp.
Figure 6. Steady state fluorescence images of a tumor periphery site on a brain tissue cryosection at (a) 365 nm excitation, (b) 395-440 nm excitation and (c) the corresponding transillumination image under halogen lamp.
Figures 6.10a-b show the representative fluorescence images of a solid tumor site at 365 and 395-440 nm excitation. The corresponding transillumination image is shown in Figure 6.10c. It is found that in the case of tumor center, the autofluorescence in the blue region is not so bright as in the case of tumor periphery and normal tissues. However, at 395-440 nm excitation, it is observed that the tumor tissues at the central site of the solid tumor exhibit bright reddish fluorescence as observed in the case of tumor periphery. This agrees with the results of the fluorescence spectral measurements and it is possible to assume that this bright red fluorescence observed in the fluorescence images may be the origin of the red emission peaks observed in the fluorescence spectra of tumor center measured at 395-440 nm excitation. This bright red fluorescence observed in the case of tumor center indicates the possible accumulation of ALA-induced endogenous porphyrins in tumor tissues.

6.5 DISCUSSION

Among the many factors determining the prognosis in patients suffering from malignant gliomas, the extent of surgical resection remains controversial (Stummer et al 1998a). Therefore, methods that improve intraoperative identification of malignant tissue, enabling more selective and thorough tumor resection, may be of value. Previous approaches to contrasting malignant gliomas using intravenous administration of fluorescent markers have not gained wide acceptance. Possible contamination of the surgical cavity by blood containing the fluorescent marker may have limited the usefulness of this approach. Also, the accuracy of tumor detection may have been limited, because intravenously administered fluorescent markers spread from edema-producing lesion with accompanying edema into surrounding non tumor tissue (Stummer et al 1998a).

Recently, a novel method for visualization and photodynamic therapy of skin, bladder and gastrointestinal tumors has been investigated. This method uses the putative capacity of malignant tissue to preferentially synthesize or accumulate fluorescent and photosensitizing endogenous porphyrins after excess administration of 5-ALA, a naturally occurring precursor in the heme biosynthetic pathway. If this technique can be applied to
Figure 6.10 Steady state fluorescence images of a tumor site on a brain tissue cryosection at (a) 365 nm excitation, (b) 395-440 nm excitation and (c) the corresponding transillumination image under halogen lamp
human malignant gliomas, porphyrin accumulation would allow fluorescence detection within the brain. The obvious advantage would be that fluorescence would be restricted to malignant cells, without contamination of the tumor cavity by blood borne marker or spreading of the tumor with tumor edema (Stummer et al 1998a).

In this context, several studies were carried on animal tumor models and human subjects with malignant gliomas to demonstrate the significant accumulation of porphyrin fluorescence in experimental brain tumors after intravenous administration of 5-ALA (Stummer et al 1998a,b and 2000). These studies have focused mainly on the characteristic red fluorescence exhibited by malignant gliomas due to preferential accumulation of ALA-induced PpIX in tumor tissues, in the steady state spectral measurements and video & fluorescence images. However, as mentioned earlier limited studies have been carried out on the time resolved fluorescence characterization of brain tumors (Wagnieres et al 1998) and the fluorescence characteristics exhibited by the intrinsic fluorophores such as NADH, flavins in brain tumor tissues.

Under these circumstances, a pilot study was carried out on 15 normal and 16 tumor sites in cryosections of tissue biopsies obtained from five patients with malignant glioblastomas. Time gated emission spectra of the cryosections were measured at 355 nm using a Nd:YAG laser. The time gates were optimized by an initial study performed to estimate the fluorescence lifetime values of standard fluorophores. Steady state fluorescence spectra were also measured at 365 and 395-440 nm excitation using a high pressure mercury lamp. For all the normal and tumor sites studied, transillumination images and fluorescence images at 365 and 395-440 nm excitation were also recorded. The results of this preliminary study showed that normal sites exhibited characteristic fluorescence emission peaks around 440 & 470 nm at 365 nm excitation and around 520-545 nm at 395-440 nm excitation. The intrinsic fluorophores possibly contributing to the emission peaks observed around 440 and 470 nm are enzyme bound NAD(P)H, collagen and/or elastin.

The steady state fluorescence spectra of normal and tumor sites on the cryosections of brain tumor biopsies measured at 365 nm excitation show that normal brain tissues and the
tumor peripheries exhibit the emission peaks around 440 and 470 nm. However, in the case of tumor center, the emission peaks around 440 nm are more pronounced than those around 470 nm. The emission peaks observed in the steady state fluorescence spectra of tissue biopsies at 365 nm excitation may be attributed to the intrinsic fluorophores mentioned above. Also, it is observed that the fluorescence intensity of tumor periphery is more than that of normal tissues as well as tumor center. This may suggest increased concentration of NAD(P)H in tumor periphery when compared to normal tissues. This is in agreement with the reported results in the case of ovarian tumors that NADH is postulated to increase in tumors as a result of alterations in blood flow, decreased pH of the tissue and, abnormal mitochondria as well as abnormal transport of electron carrier molecules into the mitochondria where the electron transport takes place (Brewer et al 2002). It should also be noted that the fluorescence spectrum of the tumor center is less intense when compared to the tumor periphery and normal tissues.

Despite these observations, the steady state fluorescence measurements did not provide any further information about the absolute contribution of those fluorophores emitting prominently in normal or tumor tissues. In this regard, time gated emission spectra provide sufficient information. For instance, in the case of normal tissues, the emission spectra within the time gate of 0-5 ns are more intense when compared to the other time gates. However, in the case of tumor center, the emission spectra within the time gate of 5-10 ns are more prominent when compared to other time gates. At this juncture, it should be noted that though the emission peak observed around 440 nm is found in both normal brain tissues as well as tumor center, the more intense spectra of normal tissues and tumor center respectively within 0-5 ns and 5-10 ns time gates indicate the following: the emission peak around 440 nm observed in the case of normal tissues may be dominated by enzyme bound NAD(P)H whereas in the case of tumor center, the structural proteins may be the promising fluorophores. This is evident from a comparison of the time gated emission spectra of the standard fluorophores with that of tissue biopsies. The time gated spectra of collagen and elastin show that the emission peak around 440 nm are prominent at longer time gates, even beyond 15 ns. In the case of tumor periphery, however, it can be seen that the emission spectra within the time gates 0-5 ns as well as 5-10 ns are equally dominant and the peak around 440 nm is not so prominent as in the case of tumor center.
This may be inferred as indicating that both NAD(P)H as well as collagen may be contributing equally to the fluorescence emission observed in tumor peripheries.

However, in the case of steady state fluorescence spectroscopy, several researchers have reported decrease in the overall fluorescence intensity in tumor tissues which may be caused by morphological changes resulting from cellular proliferation and mucosal thickening (Dhingra et al. 1996 and Zonios et al. 1996) when compared to normal tissues. This results in decreased contribution from the highly fluorescent collagen present in the extracellular matrix, basement membrane and submucosa in tumor tissues. It is also reported that any alterations in tissue architecture that inhibit the ability of excitation photons to reach the natural fluorophores or of the fluorescence emission photon to escape from the tissue and be detected by the spectroscopic system would affect the fluorescent signature. In the case of cervical tissues, it has been reported that contribution of collagen fluorescence decreases and the contributions of NADH fluorescence increase as the tissue progresses from normal to dysplastic (Brookner et al. 2000). However, attempts to correlate fluorescence intensity to epithelial thickness have thus far been unsuccessful (Schomacker et al. 1992) and further detailed histologic studies are required to confirm this hypothesis. It must be pointed out that, although epithelial thickening occurs in some dysplasias, it is not a universal finding (Ramzy 1983, Robbins et al. 1984 and del Regato et al. 1985) and that thickening of the epithelium may be associated with a variety of chronic irritative conditions as well (Ramanujam et al. 1993).

Further, as a supporting evidence of the observations in the present study, at this juncture, it is worth to mention the following: Interstitial collagens are located in the leptomeninges and the fibromuscular layer of large blood vessels in the brain. Type IV collagen, mainly present in capillaries and large blood vessels, is the principal collagenous constituent of most basement membranes (Chintala and Rao 1996). The most common molecular form of type IV collagen is a heteropolymeric molecule, [α1(IV)]2 α2 (IV); other types of homopolymeric forms, [α1(IV)]3 and [α2(IV)]3, occur as well, and three additional type IV collagen chains are known to exist in kidney (Gunwar et al. 1990). Type IV collagen is secreted and assembled as a procollagen molecule, in which, each chain has an apparent molecular weight of 160,000-180,000. Recent studies have shown that the glioblastoma cells
are also capable of synthesizing type IV collagen in vitro (Chintala et al 1996). In Bjerkvig et al (1989) showed that type IV collagen was strongly expressed in tumor spheroids from rat glioma cell line BT4C but was negative in monolayers, and fibronectin was strongly expressed in BT4C and BT4Cn cell lines, in an immunohistochemical study. In an immunofluorescence study, Bellon et al (1985) demonstrated that type IV collagen was localized to the subendothelial basement membrane of blood vessels in gliomas. Similar results were reported earlier by Rutka et al (1987).

These reported evidences may explain the observed results in the present study and it is possible to say that collagen is the dominating fluorophore in glioma tissues when compared to NADH and flavins. A comparison with the reported results on steady state fluorescence measurements indicates the need for detailed studies in this direction to confirm the observed results and to compare the absolute contribution of collagen in tumor tissues with respect to normal.

At 395-440 nm excitation, it is found that the autofluorescence in the green region around 520-540 nm possibly arising from flavins is more dominant in normal tissues than in tumor tissues. However, well defined emission peaks in the red region around 635 and 672 nm are more pronounced in the case of tumor tissues whereas in normal tissues they are significantly weaker. Also, the red emission peaks are found to be more intense in the tumor center than at the tumor peripheries. These characteristic red emission peaks may be indicative of the preferential accumulation of ALA-induced PpIX in tumor tissues. It is worth to mention here that this preferential accumulation of ALA-induced PpIX suggest the possible clinical applications of this technique for fluorescence guided tumor resection in surgery, as reported earlier (Stummer et al 1998 a and b).

The fluorescence images recorded at the normal and tumor sites on the cryosections of tissue biopsies from human glioma patients exhibit significant differences. At 365 nm excitation, the fluorescence images of tumor peripheries show bright blue fluorescence, which is more intense when compared to normal and tumor tissues. At 395-440 nm excitation, it should be noted that the green fluorescence is more intense in normal tissues when compared to
tumor periphery and center. Also, no red fluorescence is observed in the case of normal brain tissues. In the case of tumor periphery and tumor center, bright red fluorescence is observed indicating the preferential accumulation of ALA-induced PpIX. It is also seen that though the green fluorescence observed in the tumor center is not so intense in the case of normal tissues, it is more than that of tumor periphery. This is in agreement with the more intense fluorescence in the 520-540 nm region observed in the fluorescence spectra of tumor center when compared to tumor periphery.

The results of this pilot study clearly demonstrate the possible pitfalls of using only steady state fluorescence measurements in the characterization of normal and tumor tissues. Also, this study highlights wealth of information that may be provided by the time gated measurements in the fluorescence spectroscopic characterization of tissues. Hence, it may be suggested that time gated fluorescence spectroscopy and imaging offers supportive information to depict relative contributions of complex fluorophores contributing to tissue fluorescence, on the basis of their decay times. Also, the preferential accumulation of endogenous PpIX after oral administration of 5-ALA indicated by the bright red fluorescence in the fluorescence images and the fluorescence spectra at 395-440 nm excitation which are observed not only in the tumor center but also in the tumor periphery indicates potential clinical applications of this technique in identifying tumor margins during surgery of brain tumor.