**In vivo** detection of reduced scattering coefficient of C6 glioma in rat brain tissue by near-infrared spectroscopy

Lijuan Dai  
Zhiyu Qian  
Nanjing University of Aeronautics and Astronautics  
Department of Biomedical Engineering  
No. 29 Yuan Dao Street  
Nanjing, Jiangsu 210016, China

Kuanzhen L Li  
Tianming Yang  
Southeast University  
College of Medicine  
Nanjing, Jiangsu 210009, China

Hui Nan Wang  
Nanjing University of Aeronautics and Astronautics  
Department of Biomedical Engineering  
No. 29 Yuan Dao Street  
Nanjing, Jiangsu 210016, China

1 Introduction

Gliomas are a group of multiform, accumulative tumors that appear in the neuroectoderm. They generally induce perifocal edema and a massive increase of intracranial pressure for their compression, infiltration, and destruction on brain tissues. The C6 rat intracerebral glioma model is prevalent in glioma research for its good growth yields and low mortality rate. Perifocal inflamed symptoms and neonatal blood vessels occur at postoperative day (POD) 5. On POD 10, tumors increase highly in size with cellular exponential growth. During days 15–20, tumors reveal malignant gliomas with intratumoral hemorrhage and necrosis.1–2

Presently, near infrared spectroscopy is used widely in the differentiation tissue types.3–6 It is known that near-infrared (NIR) light in the 700–850 nm wavelength range is special to tissues as light scattering is more prominent than light absorption. There is a significant difference in light-scattering properties between different tissue types and between healthy and diseased tissues due to their differences in anatomical substructure; cellular and intracellular organelles of diseased tissues undergo changes in their density, morphological size, and shape.7–9 In this study, an effort was made to develop NIR technology as an alternate method for detection of certain characteristics of glioma.

The aim of this study is to determine the light-scattering patterns from rat cerebral glioma by using NIR reflectance spectroscopy. The C6 gliomas were induced in Sprague-Dawley rats (SD) by the stereotactic implantation technique.10 It has been reported that tumors generally have a lower scattering coefficient measured in vitro than normal tissue.7,9 Therefore, for this study, our hypothesis is that gliomas will lead to a decrease in light scattering of the brain and can be differentiated by the in vivo NIR measurement.

2 Materials and Methods

2.1 Cell Cultivation

The C6 glioma cells were grown in RPMI 1640 medium supplemented with 10% fetal calf serum (FCS), 2 mM L-glutamine, 50 µg/mL streptomycin, and 50 µg/mL penicillin. Cells were maintained in an incubator with 5% CO₂, 100% humidity at 37 °C. Before implantation to the rat brain, cells growing in an exponential rate were harvested by Trypsin for 5 min at 37 °C. Trypsin was inactivated by medium with 10% FCS, and the cells centrifuged at 600 g for 5 min. The pellets were then resuspended in RPMI 1640 medium without any supplement, at a concentration of 10⁶ cells/µL. Gentle manual agitation was applied to keep the cells in suspension before implantation.

2.2 Implantation Surgery

Thirty adult male Sprague-Dawley rats (230–280 g, Southeast University vivarium) were used in this study. The animals were kept in their habitual environment until the day of the experiment. All animals were anesthetized with 4% Nembutal (40 mg/kg, i.p.) and mounted into a stereotactic frame (incisor bar 2.4 mm below the interaural line) in a flat-skull posi...
After the periosteum was unmasked, two burr holes were performed with a 1 mm dental drill in the calvaria, respectively, on the left and right sides, 3 mm lateral from the midline, 1 mm posterior to the bregma. Suspension (10 μL) with $1 \times 10^6$ C6 glioma cells were stereotactically injected at a depth of 5 mm, by a 25 μL microamount syringe (the syringe was pricked 6 mm depth, and then was drawn 1 mm back) on the right side. The same volumes of sodium chloride were injected at the same depth on the left side. The holes were sealed with bone wax, and the operative field washed with saline solution and the skin sutured. The body temperature was maintained at $37 \pm 0.5 \, ^\circ$C using a homeothermic heating pad throughout the experiment. All the experimental procedures follow the institutional guidelines and were approved by the Southeast University, China.

2.3 NIRs and Experiments

NIR spectroscopy (NIRS) experiments were performed on POD 3, 10, and 17 with 10 rats each time. The homebuilt NIRS experimental system includes a light source (HL-2000, Ocean Optics, Inc., Dunedin, FL), a bifurcated needle probe (600 μm o.d. with two single mode fibers of 100 μm), a spectrometer (USB 2000, Ocean Optics, Inc.), step motor and its driver system, a laptop, as shown in Fig. 1. The bifurcated needle probe is composed of two branches. One branch is connected to the light source and the other to the spectrometer. The backscattered intensity of light depends strongly on the absorption and scattering properties of the tissue. The optic fiber probe was held perpendicularly above the exposed calvaria surface and mounted on a step motor, which was attached to a stereotaxic frame. This step motor drives the fiber probe deep into the brain at an interval of 0.2 mm.

For data acquisition, LabView (National Instruments, Austin, TX) was used to program the interface between the spectrometer and the computer. The integration time, which was taken by the detector to read out the intensity of backscattered light, was kept constant at 100 ms during the entire set of measurements. The measurements were taken on the left and right brain. On each side, 40 steps in each brain side were measured, starting from the cortex with a spatial interval of 0.2 mm. The data were recorded for a period of 10 s at 2 Hz at each step.

![Diagram of the NIRS system for measurements on brain tissue.](image)

**Fig. 1** Diagram of the NIRS system for measurements on brain tissue. 1—5 W halogen lamp as the light source. 2—Optic probe containing two silica fibers. 3—USB2000 spectrometer. 4—Computer and software. 5—MID-7604 driver. 6—PCI-7344 controller and step motor. a—the point of implant hole and the projection C6 glioma and b—symmetric point in the contralateral skull.

The reduced scattering coefficient ($\mu_s'$) serves as an index for the light-scattering property of the tissue. In principle, the intensity of backscattered light highly depends on light-scattering features of the tissue. Johns et al. had found the slopes of reflectance spectra curves between 700 and 850 nm could be used to differentiate cerebral gray matter and white matter. Qian et al. established a relationship among the reduced scattering coefficient, the profiles of the collected spectra from 700 to 850 nm, and the wavelength. In this study, an empirical formula between the reduced scattering coefficient and the slope between 700 and 850 nm was deduced by simulation experiment for the NIRS system before the rat experiments.

An intralipid solution of 8, 6, 4, 2, and 1% concentrations were chosen in the simulation experiment because these solutions yield light-scattering properties similar to those found in human tissues. The reduced scattering coefficients of these tissue simulation solutions (intralipid) were acquired by a standard Oximeter (model no. 96208, ISS, Inc., Illinois) working at 690 and 834 nm. At the same time, the reflectance spectra of these solutions were collected by the fiber-optic spectrometer of the NIRS system. Figure 2 shows the slopes and the relative reduced scattering coefficients.

The relationship between the slopes and the reduced scattering coefficients is obtained by the data fitting. Thus, an empirical formula for calculating the reduced scattering coefficient is derived as

\[
\mu_{s690}' = 4.5203 \exp(-0.3241 \times \text{slope})
\]

(1)

\[
\mu_{s834}' = 4.4645 \exp(-0.3103 \times \text{slope})
\]

(2)

The reduced scattering coefficients of intralipid solution measured by the Oximeter and calculated by Eqs. (1) and (2) and their differences are shown in Table 1, where the difference is equal to values calculated by the equations minus values measured by the Oximeter.
The accuracy of Eqs. (1) and (2) were further validated through the solid-tissue phantom experiment. Fifteen grams of Gelatin powder (Sigma, St Louis, MO, USA) made from porcine skin were added into 200 mL of boiling water and was dissolved completely by stirring. After the solution was cooled down, the intralipid solution with a certain concentration was added to the prepared gelatin solution. After that, the solution was frozen for the formation of the gelatin phantom. The percentage concentration of the intralipid used was varied, depending on the required $\mu_s'$. Five solutions with intralipid concentrations of 1–4% were used to create different $\mu_s$ values for gelatin phantoms, whose $\mu_s$ and $\mu_s'$ measured by the Oximeter and calculated by Eqs. (1) and (2) and their differences are shown in Table 2.

It shows in Tables 1 and 2 that the maximum difference is 3.1 cm$^{-1}$ and mean difference at 690 nm is bigger than at 834 nm. The maximum difference at 834 nm is 2.5 cm$^{-1}$. By combining the specific fiber-optic spectrometer with the empirical equations, an efficient method for real-time determination of the tissue reduced scattering coefficient is established.

<table>
<thead>
<tr>
<th>% Intralipid solution concentration</th>
<th>Slope</th>
<th>$\mu_s$ (cm$^{-1}$)</th>
<th>$\mu_s'$ (cm$^{-1}$)</th>
<th>Oximeter</th>
<th>Eq. (1)</th>
<th>Difference</th>
<th>Oximeter</th>
<th>Eq. (2)</th>
<th>Difference</th>
</tr>
</thead>
<tbody>
<tr>
<td>8</td>
<td>-8.54</td>
<td>73.2</td>
<td>70.0</td>
<td>63.2</td>
<td>63.2</td>
<td>0.0</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>-7.63</td>
<td>54.6</td>
<td>53.6</td>
<td>49.4</td>
<td>47.6</td>
<td>-1.8</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>-6.6</td>
<td>35.3</td>
<td>38.4</td>
<td>32.1</td>
<td>34.6</td>
<td>2.5</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>-3.68</td>
<td>16.5</td>
<td>14.9</td>
<td>15.3</td>
<td>14.0</td>
<td>-1.3</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>-2.08</td>
<td>8.4</td>
<td>8.9</td>
<td>8.1</td>
<td>8.5</td>
<td>0.4</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

2.5 Magnetic Resonance Imaging (MRI) and Histology Studies

MRI exams were performed at POD 10 and 17 on a 1.5-T MRI scanner (Philips Eclipse) using a human wrist coil after NIRs measurement. Gd-DTPA was applied intravenously at concentrations of 0.2 mmol/kg, 5 min before the scan. During the MRI scan, the rats were positioned in a plastic holder and anesthetized by 4% nembutal (40 mg/kg, i.p.). Standard multislice sagittal and cross images were obtained by a TSE sequence with TR=4113 ms, TE=96 ms, NSA=2, FOV=13 cm, slice thickness=3.0 mm, slice gap=0.5 mm, resolution=256X384, and seven slices.

After MRI, the rats were sacrificed with an anesthetic overdose and their brains were removed after the experiments. The brains were fixed in 10% formalin for more than 24 h and embedded in paraffin; consecutive 5 μm coronal sections were cut and stained with hematoxylin and eosin. A semiquantitative assessment of pathology was carried out by the neuropathologist for each individual specimen.
2.6 Statistical Analysis

The data reported here consist of only the reduced scattering coefficient at 690 and 834 nm. Spectral information between 700 and 850 nm for each sampling at each location in the brain was acquired. Data were collected 20 times at each measured point in a 10-s acquisition period and then averaged. The $\mu'_s$ data finally used in this study were the data at each point. For each side of a rat brain, there were 40 values because the probe went 8 mm deep into the brain at an interval of 0.2 mm. The $\mu'_s$ values were also averaged on 10 rats at each point of each side for POD 3, 10, and 17. Furthermore, all the 30 C6 glioma models were confirmed by the MRI and histology.

3 Results

3.1 Spectra and Light Scattering

Representative traces for the right brain (glioma) measured by the NIRS are shown in Fig. 3. They represent the different spectral value at different depths. The reduced scattering coefficients ($\mu'_s$) at 690 and 834 nm were calculated by Eqs. (2.1) and (2.2) using these spectral slopes.

Analysis of $\mu'_s$ showed that there were significant differences between left (control) and right (glioma) sides of the brain tissue (Fig. 4) on POD 10 and 17. Data in Fig. 4 are the average data at each point ($n=20$). $\mu'_s$ versus depth have the same trend as $\mu'_s$ versus depth. It shows that $\mu'_s$ of the right side within some range is lower than that of the left side at its corresponding position, and $\mu'_s$ of POD 17 is lower than of POD 10 in the right side. On POD 10, there was significant difference between the left and right from a depth of 3.2 to 6.8 mm. On POD 17, the difference between the left and right is from a depth 1.2 to 7.2 mm. The $\mu'_s$ values measured for the left (control) and right (glioma) brain side on POD 10 and 17 at depths of 1–8 mm are listed in Table 3 as mean ± SEM, respectively.

3.2 MRI Imaging

In images of T2-weighted spin-echo and gradient-echo with Gd-DTPA enhancement, there were ellipse high signals in the right hemisphere, and these high signals were inhomogeneous in some rats (Fig. 5).

3.3 Histology

The histopathological alterations of C6 gliomas were shown in Fig. 5. On a semiquantitative grading scale, all specimens had been classed into II/III grade by a neuropathologist. Prominent mitotic activity was observed under microscope on POD 10 [Fig. 6(a) and 6(b)], by which gliomas are classed into grade II. An amount of tumor cells necrosis and hemorrhage in the central region of tumors occurred on POD 17 [Fig. 6(c) and 6(d)], by which gliomas are classed into grade III.

4 Discussion

The in vivo measurements yielded $\mu'_s$ to be within the range 11–26 and 10–24 cm$^{-1}$, respectively, for 690 and 834 nm light in rat normal brain tissue, which is in good agreement with other in vivo measurements of $\mu'_s$ in mammalian brains. The upper and lower limits of the ranges can be taken as $\mu'_s$ values of white matter and gray matter, respectively, at 690 and 834 nm, according to their depth distributions in rat brain. For glioma on POD 10, the mean $\mu'_s$ and $\mu'_s$ are 19.3 cm$^{-1}$ (standard error 1.8 cm$^{-1}$) and 17.9 cm$^{-1}$ (standard error 1.6 cm$^{-1}$), respectively. For glioma on POD 17, the mean $\mu'_s$ and $\mu'_s$ are 16.0 cm$^{-1}$ (standard error
2.6 cm\(^{-1}\)) and 15.0 cm\(^{-1}\) (standard error 2.3 cm\(^{-1}\)), respectively. To our knowledge, in vivo measurements of \(\mu'_s\) of glioma at these two wavelengths have not been reported.

Gliomas are neuroectodermal tumors. It induces perifocal edema and a massive increase of intracranial pressure for their compression, infiltration, and destruction of brain tissues. It was reported that glioma cells had abnormal chromosome and genetic expression, lower cytochrome-oxydase, creatine phosphate, adenosine triphosphate, and higher deoxyribonucleic acid than normal tissues\(^{14-16}\). In the SD C6 glioma model, significant loss of both the mitochondrial enzyme activity and the mitochondrial protein concentration were reported\(^7\).

In principle, scattering properties of tissues vary with the light-scattering media in tissues such as the cell membrane and organelle membrane\(^8\), the nuclei\(^{17,18}\) and other intracellular organelles, including the mitochondria\(^,7,19-23\). Thus, light scattering can be a possible marker for tissue identification\(^{24-26}\) and cancer diagnosis\(^6\). A lot of studies have documented that the scattering properties of gray and white matter in the brain differ significantly in the near-infrared range\(^5,12,27-30\). Recently, scattering properties are used to monitor and understand neuronal functions and physiological changes in vitro and in vivo\(^{19,20,31,32}\). It becomes a more recognized and frequently utilized research approach in the area of neuroscience\(^{30-34}\).

### Table 3

<table>
<thead>
<tr>
<th>(\mu'_s) (cm(^{-1})) versus depth</th>
<th>Left (690 nm)</th>
<th>Right (690 nm)</th>
<th>Left (834 nm)</th>
<th>Right (834 nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>POD 10</td>
<td>12.1 ± 2.9</td>
<td>12.0 ± 2.0</td>
<td>11.5 ± 2.6</td>
<td>11.4 ± 1.8</td>
</tr>
<tr>
<td>POD 17</td>
<td>14.6 ± 3.3</td>
<td>14.7 ± 3.8</td>
<td>13.7 ± 3.0</td>
<td>13.8 ± 3.4</td>
</tr>
<tr>
<td></td>
<td>19.9 ± 4.8</td>
<td>17.4 ± 2.5</td>
<td>18.5 ± 4.3</td>
<td>16.2 ± 2.2</td>
</tr>
<tr>
<td></td>
<td>25.5 ± 5.2</td>
<td>16.5 ± 3.9</td>
<td>23.4 ± 4.6</td>
<td>15.4 ± 3.5</td>
</tr>
<tr>
<td></td>
<td>25.2 ± 5.7</td>
<td>19.4 ± 5.5</td>
<td>23.2 ± 5.0</td>
<td>18.0 ± 4.9</td>
</tr>
<tr>
<td></td>
<td>23.8 ± 5.4</td>
<td>21.9 ± 7.0</td>
<td>21.9 ± 4.7</td>
<td>20.2 ± 6.1</td>
</tr>
<tr>
<td></td>
<td>19.9 ± 5.2</td>
<td>18.3 ± 4.7</td>
<td>18.4 ± 4.6</td>
<td>17.0 ± 4.1</td>
</tr>
<tr>
<td></td>
<td>16.5 ± 4.6</td>
<td>16.5 ± 2.4</td>
<td>15.5 ± 2.1</td>
<td>15.5 ± 2.1</td>
</tr>
</tbody>
</table>

POD 10 = 10th postoperative day; POD 17 = 17th postoperative day.

### Fig. 5

(a) Emerge low signals in the center of high signals, which represent necrosis or hemorrhage. (b) shows inhomogeneous high signal in the position where implant C6 glioma cells on POD 17.

### Fig. 6

Microphotographs of C6 gliomas. A, C, D \(\times 200\) E-H; B \(\times 400\) E-H. (a) Shows grade I glioma (POD 10); (b) a mitotic phenomenon in the center of the photograph (arrows); (c) grade III, shows necrosis at low right; (d) grade III, hemorrhage in right side (POD 17).
Because cellular and intracellular organelles of tumors undergo changes in their density, morphological size, and shape, it is expected that light-scattering properties of tumors will differ from that of normal tissues. According to the existing reports,12,13,20,35–40 the reduced scattering coefficients of tumors were found to be greater or less than normal tissues, dependent on tissue type and detecting wavelength. In this study, we cultured C6 cells to induce cerebral glioma in SD rats. The NIR spectra reflected from glioma were measured by NIRS to determine the reduced scattering coefficient. The results show the reduced scattering coefficient of glioma decreased compared to normal tissues at the same position, which is in good agreement with the 632 nm results reported by Angell-Petersen et al.13

The differences in reduced scattering coefficient observed in our in vivo data could be the mixed results of the following aspects:

1. Mitochondrial protein concentration: $\mu'_s$ is found proportional to the mitochondrial protein concentration and decreases as the concentration falls.19 Loss of mitochondrial protein concentration is found in glioma cells.7

2. Cell nuclei size: Mie theory predicts a general decrease in scattering, increase in anisotropy, and a subsequently large decrease in reduced scattering as the radius of tissue scatterer (cell nuclei, etc.) increases. Tumor cell nuclei are often larger than normal,41 effectively decreasing the scattering from cell nuclei.

3. Cell nuclei density: The increased numbers of mitoses and multiple nuclei in a cell in tumors41 act as additional scattering centers, which can partially offset the decrease in scattering.

4. Absorption: The signal obtained by our NIRS is somewhat influenced by total hemoglobin concentration, which usually increases three to five times as a tumor develops.18 A simple experiment by adding two different concentrations of blood into 100 mL 4% intralipid solution was conducted. $\mu'_s$ measured by our NIRS decreased 1.3 and 1.1 cm$^{-1}$, respectively, when total hemoglobin concentration increased from 13.4 to 59.7 μmol/L. In the rat experiments, $\mu'_s$ measured by our NIRS decreased 5.8–8.5 and 5.2–7.6 cm$^{-1}$ in the center of the tumor (depth=5 mm), which shows absorption is not a main reason for $\mu'_s$ changes observed in the rat experiments.

Although it is expected that the tissue undergoes disorganization within hours after C6 glioma implantation, we were unable to show such a change by the NIRS technique on postoperative day 3. However, significant changes were found in our study on postoperative days 10 and 17, which correlate well with the expected pathological changes following glioma cells implantation. The following matters indicated the possibility by using in vivo NIRS techniques to identify glioma. First, abnormal changes and their boundaries can be deduced by the $\mu'_s$ curves. On the 10th day after C6 glioma cells were implanted at a depth of 5 mm in rat brain, there is significant difference between the left and right sides from depth 3.2 to 6.8 mm, which means the glioma enlarged, and depth 3.2 mm is its upper boundary and depth 6.8 mm is its lower boundary. On POD 17, the difference range between the left and right is from depth 1.2 to 7.2 mm, which means the volume of glioma is bigger. The upper boundary read from the $\mu'_s$ curve is approximately consistent with the data read from MRI images. Second, the $\mu'_s$ curves show the potential for differentiating a grading scale of gliomas. The histopathological alterations of C6 gliomas classified gliomas on POD 10 into grade II and gliomas on POD 17 into grade III. On the other hand, the $\mu'_s$ of glioma side on POD 17 is lower than that on POD 10; that is, $\mu'_s$ of gliomas of grade III is lower than grade II. However, more studies are needed to work out the differentiating number.

We did not observe the significant differences on POD 3. One possible explanation for the failure to detect the differences is due to the small volume of the glioma in its early stage. In the Sprague-Dawley rat C6 gliomas models, gliomas just begin to form a tumorous entity ~72 h after the implantation. Qian et al. in previous research found that the probe used here has a look-ahead distance at ~1 mm, which means the reduced scattering coefficient measured by this system is depending on the contribution of tissues located 1 mm under the probe. The percentage of intact versus diseased tissues in the 1 mm range may affect the scattering readings. It would be beneficial to develop a more sensitive NIRS probe with a better spatial resolution. A second possibility may be the positioning of the NIRS probe. A slight change in positioning angle could affect the reading. This possibility seems unlikely to account for a group difference because there was no systematic difference in probe positioning. However, increased accuracy in probe positioning could be obtained by developing an optic digital scanner that could provide a measurement for the distance around the center of the aiming surface. Such information could feed back to a microprocessor, which would adjust the angle of the micromanipulator.

In conclusion, this study reports $\mu'_s$ of normal brain tissue and glioma at two wavelength obtained using an NIRS method and demonstrates that NIRS has the ability to detect cerebral gliomas microinvasively. The results have proven our initial hypothesis and may suggest that the NIRS techniques have a potential for intraoperative application to identify gliomas within normal brain tissue. We believe that such a minimally invasive technique with simple, low-cost, and portable aspects could be utilized for multiple applications in the brain and inner body-studies, such as to measure dynamic light scattering changes during external stimulations. The possibility of characterizing light scattering under tumors in vivo may allow scientists to gain insight into tumor physiology from another angle besides tissue hemoglobin oxygenation, which has been done intensively in recent years.

Acknowledgments

This work was performed in Biophotonics Laboratory of Nanjing University of Aeronautics and Astronautics and supported by the National Nature Science Foundation of China (Grant No. 30671997).

References


