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ABSTRACT

Based on Visible Resonance Raman (VRR) method, we have developed a novel label-free portable VRR LRR2000™ Raman analyzer with a portable fiber-optic probe and used it for the classification of human gliomas ex vivo and for the analysis of changes in tumor chemical compositions in molecular level. The purpose of this study was to examine the performance of the LRR2000 Raman analyzer as an optical biopsy tool for detecting human brain tumors compared to the commercial laboratory HR800 and WITec300 micro confocal Raman spectroscopy instruments. As of 2018, a total 1,938 VRR spectra were collected using LRR2000, HR800 and WITec300 Raman system, ex vivo. Identification of the four grades of glioma tumors and control tissues was performed based on the characteristic native molecular fingerprints. LRR2000 demonstrated consistent diagnostic results with HR800 and WITec300 Raman systems. LRR2000 showed the advantages of high speed, convenience and low cost compared to the two confocal micro Raman systems. Using artificial intelligence (AI)-based analysis of part of the data, the cross-validated accuracy for identifying glioma tumors is ~90% compared with gold standard histopathology examination.

Key words: visible resonance Raman (VRR), handheld LRR2000 analyzer, human brain tissues, glioma tumor, optical biopsy, Optical molecular Pathology, principle component analysis(PCA), support vector machine (SVM).

1. INTRODUCTION

There is a search to develop a new armamentarium to detect cancer and identify the margin in brain for neurosurgeons. Visible Resonance Raman (VRR) offers an opportunity.

Human brain glioma tumors account for about half of the central nervous system (CNS) tumors and are the most difficult type to cure. 80.7% of malignant tumors of the brain are glomas. The survival times of patients with glioblastoma grade III and grade IV are only about 2 years and 1 year, respectively [1-3].

Effective and complete resection will reduce the recurrence rate of the tumor. Therefore, accurate judgment of cells on the margin of the tumor is very important. So far, surgeons have been relying on experience and visual observation to locate the margin of tumors, which are based on images of magnetic resonance imaging (MRI) tests before surgery. Neurosurgeons suffer from locating margins of tumors and the problem with the current technology which is not able to accurately identify the boundaries between brain tumors and normal tissues quickly and timely during surgery. In addition, they are blinded to cancer local seed sites outside the major growth region. Surgeons need a complementary technique to
aid them to both screen for the type and grade of the tumor during surgery and detect the form and extent of tumor cell invasion and the exact boundaries between the tumor and normal tissue.

In this paper, we report a visible resonance Raman (VRR) spectroscopy method using 532nm laser wavelength excitation [4-7]. This is a new label free optical molecular histopathology spectroscopy method that can evaluate brain normal, control (the negative margin of gliomas) and glioma tissues and tumor grades in real-time. The integration of VRR spectroscopy technology into neurosurgical multi-modality systems can achieve real-time, label-free, in-situ, and accurate identification of tumors and greatly enhance the routine invasive HE examination during surgery, and the sensitivity and specificity will be far superior to existing imaging techniques. For example, the traditional medical diagnostic techniques such as ultrasound (US), magnetic resonance imaging (MRI), positron emission tomography (PET), or x-ray imaging have achievable minimum resolution about 0.1–10mm. In contrast, VRR imaging 2D scan resolution is about 1μm. VRR technique helps doctors differentiate cancerous tissue from healthy brain tissue in less than a second, while traditional pathology biopsy often takes hours to days [8-14]. VRR spectroscopy technology has a high potential to improve the accuracy of brain biopsy in neuropathology diagnosis and to guide brain surgery with accurate margin judgment. The novel optical spectroscopic technique of VRR is based on the native molecular vibrational characteristics in Raman spectra of human brain tissues that we have found from our experiments since 2011 [4, 5,7,14].

VRR has significant advantages over the existing conventional Raman technologies due to the magic light of visible 532nm laser as excitation light source that matches the native bio-molecular resonance absorption or near-resonance absorption bands in biological tissue and cells, which can enhance the Raman signals from biomolecules by 10 to 1000 times. VRR method can be used to discover and study changes in the composition of biomolecules locally and reveal their variations in the concentration less than 1.0nM and spatial distribution by observation of the spectral fingerprints of the biochemical vibrational bands, e.g. tryptophan, DNA, amides, lipids and proteins. These advantages of VRR have led to a rapid progress in its applications in diagnosis of brain and other human cancers such as breast, skin, heart, GI, gynecologic and the endocrine system lesions [4-6,15,16] that are difficult to achieve by conventional non-resonance Raman method [17-20]. This work is a continuation of Alfano's group's optical biopsy (OB) research, which has made significant progress since the pioneering reports in 1984 and 1987 [21, 22] using optical spectroscopy to detect cancer. VRR was first reported in tissues in 1987 [22].

In this article, we will present a new design for a portable visible resonance Raman analyzer (VRR analyzer model LRR2000) with a hand-held optical fiber probe which can be used for analysis of glioma tumors in different grades and margins identification. For comparison with the commercial laboratory HR800 and WITec300 micro confocal Raman spectroscopy instruments, a total of 40 pieces of samples from 29 patients were investigated using LRR2000 analyzer, HR800 and WITec300 Raman systems. The four grades of glioma tumors and control states were identified based on the characteristic native molecular fingerprints using the ratios of peak intensities and peak positions in the VRR spectra reported previously. For example, the intensity of VRR vibrational modes at 1524cm⁻¹ from the lipophilic carotenoid, 1444, 2854 and 2888cm⁻¹ from saturated fatty acids revealed a rapid decrease in the high-grade of glioma brain tissues compared with the control tissues.

This VRR analyzer with LRR2000 model was developed by the research team of TRMTC and the company of JRMI in China. There is a collaboration between laboratory in China and the U.S. to address the cancer problems of the breast, lung and brain [1, 2, 21-23].

2. METHODS AND MATERIALS

Samples and Spectra collection: A total of 1,938 VRR spectra were collected using LRR2000 analyzer, HR800 and WITec300 Raman systems. The human brain glioma tumor samples were simultaneously tested, in order to compare the three systems. Among the VRR spectra collected, 1,513 were measured by LRR2000, 425 by HR800 and 95 from 38 mapping images by WITec300 Raman systems. Specimen Preparation: The specimens were obtained from the Air Force Medical Center, Beijing, China. The malignancy stages followed the World Health Organization (WHO) standards. Experimental procedures were approved by the committee of the Air Force Medical Center in Beijing, China. The specimens were kept in a snap-frozen condition in uncut and irregular shapes. Specimens were not chemically treated prior to the spectroscopic studies. Every sample was placed on the similar quartz plate for VRR spectral measurement [4].
VRR spectra measurements ex vivo: The VRR spectra of specimen were recorded from multiple sites (3 or more, depending on sample size). For each site the laser power was kept at 4mW, with acquisition time at 1s or 5s for LRR2000 Raman analyzer system, at 1mW with acquisition time at 1s or 30s for HR800 and at 3.5mW with acquisition time at 1s or 30s for WITec300 plus Raman systems. Twenty-one (21) specimens took re-examination for the tested site by H&E histopathology before and after VRR spectral measurements to increase veracity for classification in disease diagnosis.

Raman instrumentation: [4,8,10] The VRR-LRR Raman analyzer: A label-free portable Raman analyzer (model LRR2000) with an optical-fiber probe using 532nm excitation wavelength was used in the experiments shown in Fig. 1 (left). The laser power on the sample was kept at 3.5mW. The final spectral resolution was 8cm⁻¹ in the range of interest (200 to 4000cm⁻¹). A fiber cable with a 200μm focal point was used for focusing the laser beam, and collecting the scattered signals from the sample surface. The HR800 confocal micro Raman system: HR800 Raman system with 532nm excitation wavelength (JY-HR800 France). The WITec alpha300R confocal micro Raman imaging system: Using the 532nm excitation laser beam with a system resolution down to the optical diffraction limit of 200 nm, with a Zeiss 50X 0.55 objective, the final spectral resolution was 2cm⁻¹ in the range of interest. VRR spectra were collected with 1s integration time and 30 accumulations for a single scan.

![Diagram of the Raman system](image)

Fig.1 Photographs of the portable VRR-LRR2000 analyzer with a label free optical-fiber probe (left panel). The analyzer is 32cmx33cmx38cm in size and weighs 7.5kg, with a standard cable of probe of 2 meters. The screen displays recorded signals and test results, along with flickering color and ringtones in real time (right side of screen). When the surgical resection is hesitant, it may be at the border of the tumor with the real time display, the VRR-LRR handheld probe provides the results and helps a surgeon decide whether or not to remove tissue. A schematic diagram of VRR-LRR2000 analyzer (right panel). Both back-scattering micrograph and Raman spectrum are synchronously recorded from a sample site using white light and 532nm laser as the light sources respectively, as shown on the emulated computer screen in the upper left and lower right corners.

3. RESULTS AND DISCUSSION

In order to verify the results of the VRR spectral data and the functions tested using LRR2000 Raman analyzer, three Raman systems were used simultaneously in the experimental measurements on the same sample pathologically certified before and after the experiments. Four different grades of human brain tissue samples were tested. Here we analyze and discuss the part of typical VRR spectral results of the control, glioma grade I, and grade III samples measured by LRR2000 and HR800 Raman systems (WITec300 data is not shown here).

The photograph and schematic diagram of LRR2000 Raman analyzer: A hand-held optic-fiber Raman neuronavigation prototype with model LRR2000 which was developed based on the VRR technique is shown in the left of Fig. 1. The right of Fig. 1 shows the working principle diagram of model LRR2000.

Revealing VRR spectral characteristics of normal/control brain tissue using LRR2000 and HR800 Raman systems (1): VRR spectra of brain control tissues are shown in the left and right of Fig. 2 measured using LRR2000 and HR800 Raman system, respectively. Eight VRR feature peaks were observed that suggest to be arising from RR intrinsic molecular fingerprints and mainly overtone peaks of carotenoids (shown in the left of Fig. 2) when using LRR2000 Raman system. For the same sample, five VRR characteristic peaks were observed, and these peaks are thought to be arising from RR modes and overtones of carotenoids (right of Fig.1) when using the HR800 Raman spectrometer [12]. We believe that the two stronger VRR characteristic peaks at 1154 and 1511cm⁻¹ can be used as biomarkers to identify brain normal/control tissue [7,14]. Moreover, both systems worked with the same acquisition time 1 second, and the two strongest characteristic
peaks of 1154 cm\(^{-1}\) and 1511 cm\(^{-1}\) showed similar intensities. We can assume that these two systems have basically the same sensitivity when testing trace amounts of carotenoids in human tissues. The Raman spectrum of trace carotenoids present in brain tissue cannot be observed using 785 nm laser excitation, because 785 nm light is far from the absorption band of carotenoids, that is, in a non-resonant Raman excitation state [16,19].

![Figure 2: Typical VRR spectra of the control tissue from human brain glioma tumors measured at same exposure time 1s on the same sample surface which pathologically certified with before and after. The excitation laser wavelength at 532 nm, and with full scan range from 200 – 4000 cm\(^{-1}\) for analyzing VRR spectra. The left spectrum is collected by LRR2000 Raman analyzer. The right spectrum is collected by HR800 confocal micro Raman system.](image)

**Distinguish normal/control brain tissue from glioma grade I and grade III tumors using LRR2000 and HR800 Raman systems (2):** A clear decrease in the intensities of Raman peaks at 1157 and 1521 cm\(^{-1}\) was observed with the increase of the glioma grades that is shown in Fig. 3 (glioma grade I) and Fig. 4 (glioma grade III). This indicates a progression of the mutation process in the glioma tumors. When comparing VRR spectra of the control (Fig. 2) and glioma GII (Fig.4), these two VRR peaks of 1157 and 1521 cm\(^{-1}\) reduced significantly in grades III [4,7]. A dramatic decrease of carotenoid signal in gliomas versus the control brain tissue is quite remarkable. VRR modes of 1157 and 1521 cm\(^{-1}\) could be a significant marker to distinguish normal/control brain tissue from glioma tumors and diagnose other CNS cancers [7, 24-26].

**VRR spectral Characteristic peaks of glioma grade I and grade III and the intensity ratios measured using LRR2000 and HR800 Raman systems (3):** VRR spectra of glioma grade I (GI) tissues measured using LRR2000 and HR800 Raman systems shown in the left and right of Fig. 3, respectively. The relative intensity changes on the VRR spectral molecular fingerprints of glioma tumors at 1156, 1521, 1440, 1588, 2888 and 2932 cm\(^{-1}\) are observed. Differentiating the grade of glioma is evaluated according to the change at the intensity ratio of protein to lipids of the characteristic peaks. According to the criteria of glioma grading by VRR spectroscopy technique which we reported previously [7,14], from the VRR spectra of Fig. 3, the ratios of intensities calculated of RI was around 7.26, the ratio of R2 was around 0.93 for GI tested by LRR2000. The calculated ratio of RI was 3.31, the ratio of R2 was 0.96 for GI tested by HR800 system. The results of intensity ratios from VRR spectra of GI glioma tissues indicated that the ratio values basically fall in the database criteria with error region near 10% except RI of site of GI tested by LRR2000 Raman analyzer [7,14]. For the VRR spectra for all sites (in total 6 sites tested) of GI by LRR2000 Raman analyzer tested, the ratios of RI tends to be higher, but the ratio R2 is very stable and falls within the standard criteria. There may be two reasons for the presence of this singularity (the ratios of R1 tends to be higher). One is saturated fatty acids mode of 1440 cm\(^{-1}\) vibration that is not resonance active, and the other may be resonance enhanced vibrational mode around 1585 cm\(^{-1}\) from mitochondria and cytochrome c that is colored proteins located between the inner and outer mitochondrial membranes.

Fig. 4 shows a similar result. VRR spectra of glioma grade III (GIII) tissues measured using LRR2000 and HR800 Raman systems are presented in the left and right of Fig. 4, respectively. The relative intensity changes of characteristic VRR peaks of glioma tumors at 1156, 1521, 1440, 1588, 2888 and 2932 cm\(^{-1}\) are observed. According to the criteria of glioma grading by VRR spectroscopy technique which we reported previously, the ratios of peak intensities calculated for RI was around 7.9, the ratio of R2 was around 1.47 for GIII tested by LRR2000. The calculated ratio of RI was 5.7, the ratio of R2 was 1.78 for GIII tested by HR800 system. The results of intensity ratios from VRR spectra of GIII glioma tissues indicated that the ratio values basically fall in the database criteria with near 10% error [7, 14].

**Glioma identification using principal component analysis and support vector machines (4):** The spectral data were further analyzed using the statistical method based on machine learning for glioma identification [14,16]. The scatterplots of PC3 vs. PC2 are shown in Figs. 5(a) and (c) along with the SVM classifiers for HR800 and LRR2000, respectively. Leave-one-out cross validation (LOOCV) was also used to evaluate SVM classification performance. The receiver
operating characteristic (ROC) curves are also plotted in Figs. 5(b) and (d) for LOOCV for HR800 and LRR2000, respectively. To measure the classification performance, grade I and grade III are considered negative and positive, respectively.

**Fig. 3.** Typical experimental VRR spectral data of glioma grade I plotted: left plot data was tested by LRR2000 Raman system, the right plot data was tested from HR800 confocal micro Raman system. The ratios of intensity of main biomarkers set as $R_1 = \frac{I_{1585}}{I_{1443}}$ and $R_2 = \frac{I_{2933}}{I_{2888}}$ within the resolution range of the systems.

**Figure 4:** Typical experimental VRR spectral data plots of glioma grade III: the left VRR spectral data was tested by LRR2000 Raman analyzer, the right VRR spectral data was tested from HR800 confocal micro Raman system. The ratios of intensity of main biomarkers set as $R_1 = \frac{I_{1585}}{I_{1443}}$ and $R_2 = \frac{I_{2933}}{I_{2888}}$ within the resolution range of the systems.

**Figure 5:** (a) and (c) are scatter plots of PC3 vs. PC2 along SVM classifiers for HR800 and LRR2000, respectively. (b) and (d) are ROC curves for SVM with LOOCV.
4. CONCLUSION

By analyzing the VRR spectra of Figs. 2 to 4, LRR2000 analyzer demonstrated consistent diagnostic results with basically the same resolution and similar accuracy, and more prominently, the advantages of stability, high speed and convenience in quasi-clinical detection compared to HR800 and WITec300. Second, LRR2000 analyzer demonstrated tumor margin assessment. When the control tissues (Fig. 2) and GI glioma tissues (Fig. 3) are identified by molecular fingerprints of the VRR spectra that are shown in Figs. 2 and 3, this means the tumor boundary has been found that can help the surgeon make judgment, to cut out the tumor to the maximum without harming normal tissues. Third, this indicates glioma grading in situ and real-time. In this report, identification of GI and GIII by the intensity ratios of the molecular fingerprints are presented. It is revealed that the portable LRR2000 analyzer has potential to be used as an early diagnosis, general screening tool and to identify the malignancy of gliomas. In this study, PCA combined with SVM (PCA-SVM) was performed for glioma tumors classification and grading. The accuracy of glioma grades identification is ~90% compared with gold standard histopathology analyses which included both pre- and post- sample measurements.

Currently, the LRR2000 Raman analyzer is being used to test tumor diseases in adults and children such as human brain, gastrointestinal tract, etc. under quasi-clinical conditions. VRR-LRR2000 analyzer has the potential to be an optical neuro-navigator used in the surgical room in the future.

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REFERENCES


