

Rapid identification of anterior and posterior root of *cauda equina* nerves by near-infrared diffuse reflectance spectroscopy

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1 Introduction

End-to-end nerve repair remains the treatment of choice for nerve grafting and regeneration in surgical nerve repair. However, complete functional recovery cannot be achieved in most cases. It is difficult to get accurate coaptation of the nerves with the same function. The nerve root can be divided into an anterior root that consists of motor fibers and a posterior root that consists of sensory fibers. If the anterior and posterior roots in a mixed *cauda equina* nerve cannot be matched and coapted accurately during surgical nerve repair, the regenerated nerve fibers will fail to grow into the proper terminals, and the sensory and motor functions will be lost.¹ Thus, correct intraoperative identification of anterior and posterior roots of *cauda equina* nerves is important. However, it remains a great challenge in mixed nerve repair. Currently,

Abstract. A new rapid chemometric method has been developed to identify the anterior and posterior roots of *cauda equina* nerves by near-infrared (NIR) diffuse reflectance spectroscopy. NIR spectra of nerves were measured using a Fourier transform NIR spectrometer equipped with a fiber-optic probe. The result revealed no observable difference in the spectra between the anterior and posterior root samples, but the two roots could be identified by cluster analysis based on the differences of their spectral features. The overall accuracy of the cluster analysis model was 87.5%, and the accuracy for the actual anterior root and actual posterior root were 95% and 80%, respectively. The result suggested that NIR spectroscopy in combination with the chemometrics method (cluster analysis) could be used to classify the anterior and posterior roots of *cauda equina* nerves. The proposed method required only a few minutes, while classical methods commonly required at least one hour. It was demonstrated that the new method could provide a rapid, correct, nondestructive and low-cost potential means to quickly differentiate anterior and posterior roots in mixed *cauda equina* nerves, which would be helpful for surgeons to align nerve stumps correctly. © 2009 Society of Photo-Optical Instrumentation Engineers. [DOI: 10.1117/1.3086611]

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several methods have been developed to differentiate the two types of roots: anatomic identification,^{2,3} electrophysiological stimulation,^{4,5} radioisotopic method,⁶ histochemical method,⁷⁻¹⁰ and immunohistochemical technology.¹ Unfortunately, all of these methods share the common disadvantage that they take at least one hour or even more to finish the identification. It is urgent to develop a rapid and accurate technique to identify anterior and posterior roots. The method of near-infrared (NIR) diffuse reflectance spectroscopy used in this paper provides the feasibility and practicality for rapid (in several minutes) identification of anterior and posterior roots of *cauda equina* nerves.

NIR spectroscopy is a nondestructive technique and is also a rapid and accurate method for a wide range of analytical applications.¹¹ It can be used to identify and quantify many organic molecules from vibration absorptions at specific frequencies. This method is particularly effective in materials containing -CH, -OH, and -NH (and -SH) chemical functional groups, because the NIR spectroscopy region contains

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absorption bands corresponding to overtones and combinations of vibrations of these chemical groups.^{12,13} NIR spectra contain a lot of information about chemical constituents and physicochemical properties of the samples.^{11–14} Today, the NIR spectroscopy technique together with multivariate analysis is widely used not only in chemical, pharmaceutical, and food industries,^{12–14} but also in the field of noninvasive clinical diagnostics including the analysis of blood, brain trauma, tumor, skin, etc.^{15–17}

Generally speaking, the tissue constituents of anterior and posterior roots of *cauda equina* nerves are almost the same, including proteins, neurotransmitters (i.e., substance P, acetylcholine, etc.), neuropeptide polysaccharides, phospholipids, peptidoglycans, and nucleic acids, but the quantity and distribution differ between the two root types. Although the differences are obscure and hard to understand, they are implicit in the NIR spectra.

The aim of this study was to develop an NIR spectroscopy method to rapidly identify anterior and posterior roots of *cauda equina* nerves and to determine its feasibility for identifying anterior and posterior roots by using cluster analysis to extract the differences in NIR spectra.

2 Experiments

Forty nerves from four Beagle dogs were harvested and scanned by an NIR spectrometer. The collected spectra were then classified by clustering analysis, and the Fuminori enzyme-staining technique was used for comparison. The detailed process was as follows.

2.1 Materials

Acetylthiocholine iodide and tetraisopropyl pyrophosphoramidate were purchased from Sigma Chemicals (St. Louis, Missouri). Potassium ferricyanide and sodium citrate were purchased from Xilong Chemicals (Shantou, China). Copper sulfate was purchased from Zhengxing Chemicals (Shanghai, China).

2.2 Sample Preparation

Four adult Beagle dogs were anesthetized generally by intraperitoneal ketamine injection and sacrificed by intravenous injection of air. *Canalis vertebralis* were opened, and both anterior and posterior roots of the *cauda equina* nerves were harvested. Twenty anterior roots and twenty posterior roots were collected and numbered H1 to H40. These specimens were immediately frozen with liquid nitrogen for preservation.

2.3 Near-Infrared Spectra Measurement

NIR spectra were measured using a multipurpose Fourier transform NIR (FT-NIR) spectrometer (MPA, Bruker Optics, Inc., Ettlingen, Germany) equipped with a PbS detector and a fiber optic probe. The fiber-optic probe consists of a core, a cladding with a lower refractive index, and a protective jacket. One hundred transmitting and one hundred receiving fibers with active areas of 0.1 mm in diameter were packed into the optical fiber core randomly. The diameter of the tip of the bundle is 3.5 mm. The spectra were collected with OPUS software (Bruker Optics, Inc.). Each anterior root and posterior root sample was cut to a length of 2 mm after being

thawed to room temperature (25 °C) and was put directly onto the top of the fiber-optic probe. The nerve sample covered only a portion of the probe tip. The fiber-optic probe was kept in the vertical up position to maintain the same contact pressure between the nerve sample and the fiber-optic probe in all measurements. The measurements were performed in air and room temperature (25 °C). Each spectrum represented the average of 64 scans at a resolution of 16 cm⁻¹, over the wave number of 12,500 to 3600 cm⁻¹. Each sample was scanned three times. The average spectrum of each sample was used for further analysis.

2.4 Data Analysis

Cluster analysis was used to distinguish the anterior and posterior roots of the *cauda equina* nerves based on the differences in their NIR spectral features. The calculation was performed by OPUS/IDENT (version 5.0) multivariate statistical qualitative analysis software (Bruker Optics, Inc.). The spectra of the anterior and posterior roots were scaled by vector normalization before clustering. The Euclidean distance algorithm was used for intersample distance, and Ward's algorithm was used for interclass distance.

2.5 Fuminori Enzyme Staining

2.5.1 Preparation of the staining solution

The staining solution was prepared by mixing the fresh stock solutions before staining. Final concentrations were acetylthiocholine iodide (3.0 mM), phosphate buffer (65.0 mM), sodium citrate (10 mM), copper sulfate (30 mM), potassium ferricyanide (5 mM), and tetraisopropyl pyrophosphoramidate (0.6 mM). The pH of phosphate buffer was adjusted to 6.5. The final solutions were stored at 4 °C for 12 h. Fuminori⁸ reported a rapid and easy preparation of solution using three bottles and tubes. Acetylthiocholine iodide and tetraisopropyl pyrophosphoramidate (bottles B and C) were stocked as powders at -20 °C, and solutions (bottle A and all tubes) were stocked at 4 °C for more than 1 year.

2.5.2 Preparation of specimen and staining

The specimens used in the Fuminori enzyme-staining were the same as the ones used in the measurement of the NIR spectra of the nerve. These specimens were immediately frozen by liquid nitrogen. From each specimen, 12 frozen sections with a thickness of 10 to 20 μm were cut with a cryostat. The specimens were placed in 10% formal calcium solution and fixed for 15 min. The specimens were then washed three times for 1 min in distilled water. The 12 sections of each specimen were divided into six groups ($n=2$ each) and stained by Fuminori enzyme-staining techniques. The sections were placed under a microscope and observed at times of 1, 2, 4, 6, 8, and 12 h after staining.

3 Results and Discussion

3.1 Spectral Data Pretreatment Method

NIR spectroscopy was used to differentiate the anterior/posterior roots of *cauda equina* nerves based on the structural and biochemical differences of peripheral nerves as reflected by the variations in their NIR spectral patterns. Representative NIR spectra of five anterior roots and five posterior roots are

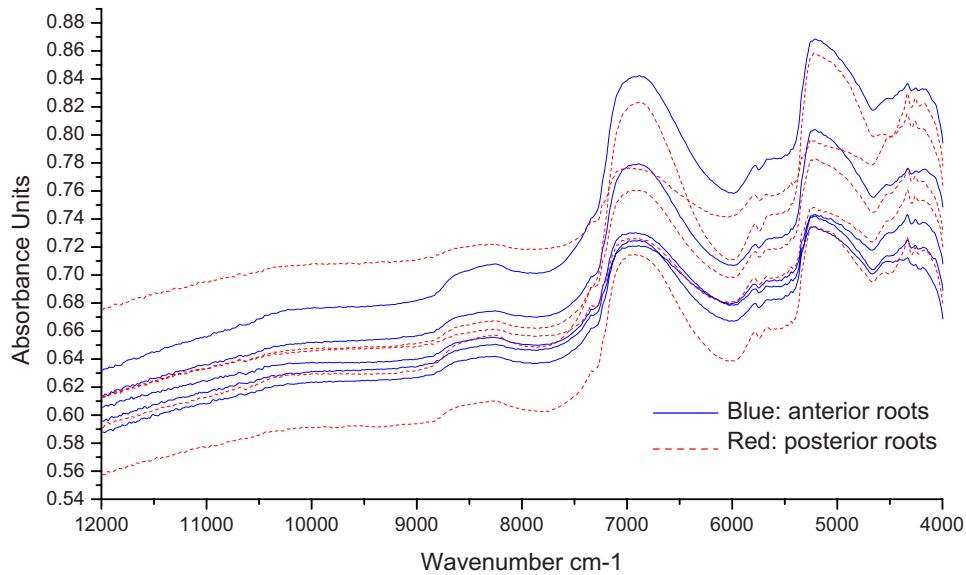


Fig. 1 Representative near-infrared spectra of anterior and posterior roots.

shown in Fig. 1. The spectra of the anterior and posterior roots were carefully examined, and it was found that there were not observable differences between the spectra of the anterior and posterior root samples. In this study, NIR spectroscopy was employed in combination with cluster analysis to classify the anterior and posterior roots.

Several spectral pretreatment methods were tested for cluster analysis, including no preprocessing, vector normalization, first derivative, first derivative combined with vector normalization, second derivative, and second derivative combined with vector normalization. The different wave number ranges, which also affected the clustering accuracy of nerve samples, were selected for spectral preprocessing and the clustering algorithm. Table 1 shows the clustering accuracy of the preprocessing method over the representative wave number ranges. The results suggested that the first derivative was better than the second derivative, and vector normalization over the range of 11,988 to 7112 cm^{-1} and 4953 to 4050 cm^{-1} was chosen as the optimal spectral data pretreatment method for this data set. The derivative technique is often used to process spectral data because it helps to separate overlapping absorption bands, remove baseline shifts, and increase apparent spectral resolution. But it adds noise to the spectra simultaneously and decreases the signal-to-noise ratio. The second derivative additionally removes baseline slope but further decreases the signal-to-noise ratio compared to the first derivative. Figures 2 and 3 show the difference between the two average spectra preprocessed by the first derivative and second derivative, respectively. First and second derivatives of the spectrum were calculated by using the Savitzky-Golay algorithm with nine smoothing points in OPUS. Vector normalization, a data enhancement algorithm, is always used for eliminating redundancy information and increasing the difference in samples to improve the predictive capability and robustness of the model. In order to include the strongest absorptions of the actual tissue but exclude water absorptions, the wave number bands at ~ 5200 and ~ 6800 cm^{-1} were not selected for vector normalization. The difference between the

two average spectra preprocessed by vector normalization over the range of 11,988 to 7112 cm^{-1} and 4953 to 4050 cm^{-1} was more significant (shown in Fig. 4). Therefore, the clustering accuracy of the spectra preprocessed with vector normalization over the ranges of 11,988 to 7112 cm^{-1} and 4953 to 4050 cm^{-1} was chosen as the optimum for this data set.

3.2 Cluster Analysis

Cluster analysis was employed to identify the anterior and posterior roots of *cauda equina* nerves based on the differences of their spectral features. Several clustering algorithms were tried to calculate interclass distance in cluster analysis, including single linkage, complete linkage, average linkage, weighted average linkage, median algorithm, centroid algorithm, and Ward's algorithm. The results showed that Ward's algorithm was the optimal clustering algorithm for this data set. The Fuminori enzyme-staining technique was used to validate the accuracy of cluster analysis. Most of the anterior roots of *cauda equina* nerves showed clear red-brown particles after staining for 1 h, and all sections of anterior roots were colored completely after staining for 2 h. All sections of the posterior roots were still not colored after 12 h. Figure 5 shows the cluster analysis results. The dendrogram suggested that 19 of 20 anterior roots and 16 of 20 posterior roots were correctly classified—that is, the model correctly classified 35 nerves of the 40 *cauda equina* nerves, and the total accuracy ratio was 87.5%. The accuracy for the actual anterior root and actual posterior root were 95% and 80%, respectively. The results demonstrated that NIR spectroscopy in combination with cluster analysis methods could be used to classify anterior and posterior roots of *cauda equina*. It was suggested that NIR spectroscopy may provide a quick method to differentiate anterior/posterior roots in mixed *cauda equina* nerves. However, the data set may be too small to be representative of the range of biochemical and morphological properties of

Table 1 Clustering accuracy of the preprocessing method over the representative wave number ranges.

Preprocessing method	Wave number range for derivative (cm ⁻¹)	Wave number range for normalization (cm ⁻¹)	Wave number range for clustering algorithm (cm ⁻¹)	Actual posterior/classified anterior	Accuracy of classified anterior (%)	Actual anterior/classified posterior	Accuracy of classified posterior (%)	Accuracy of method (%)
No processing	—	—	12000–4000	8/24	66.7	4/16	75.0	70.0
Vector normalization	—	12000–4000	12000–4000	4/21	81.0	3/19	84.2	82.5
First derivative	12000–4000	—	12000–4000	8/22	63.6	6/18	66.7	65.0
Second derivative	12000–4000	—	12000–4000	9/21	57.1	8/19	57.9	57.5
First derivative+ vector normalization	12000–4000	12000–4000	12000–4000	5/18	72.2	7/22	68.2	70.0
Second derivative+ vector normalization	12000–4000	12000–4000	12000–4000	7/19	63.2	8/21	61.9	62.5
No processing	—	—	11988–7112, 4953–4050	6/17	64.7	9/23	60.9	62.5
Vector normalization	—	11988–7112, 4953–4050	11988–7112, 4953–4050	4/23	82.6	1/17	94.1	87.5
First derivative	11988–7112, 4953–4050	—	11988–7112, 4953–4050	8/21	61.9	7/19	63.2	62.5
Second derivative	11988–7112, 4953–4050	—	11988–7112, 4953–4050	9/20	55.0	9/20	55.0	55.0
First derivative+ vector normalization	11988–7112, 4953–4050	11988–7112, 4953–4050	11988–7112, 4953–4050	4/13	69.2	11/27	59.3	62.5
Second derivative+ vector normalization	11988–7112, 4953–4050	11988–7112, 4953–4050	11988–7112, 4953–4050	10/22	54.5	8/18	55.6	55.0
First derivative+ vector normalization	12000–4000	5000–4000	12000–4000	11/27	59.3	4/13	69.2	62.5
Second derivative+ vector normalization	12000–4000	5000–4000	12000–4000	10/24	58.3	6/16	62.5	60.0
First derivative+ vector normalization	12000–4000	5000–4000	6000–4000	5/17	70.6	8/23	65.2	67.5
Second derivative+ vector normalization	12000–4000	5000–4000	6000–4000	10/23	56.5	7/17	58.8	57.5
First derivative+ vector normalization	12000–4000	5000–4000	5000–4000	5/16	68.8	9/24	62.5	65.0
Second derivative+ vector normalization	12000–4000	5000–4000	5000–4000	8/21	61.9	7/19	63.2	62.5
Second derivative	12000–4000	—	6000–4000	8/16	50.0	12/24	50.0	50.0
Vector normalization	—	5000–4000	12000–4000	8/22	63.6	6/18	66.7	65.0
Vector normalization	—	5000–4000	6000–4000	10/28	64.3	2/12	83.3	70.0
Vector normalization	—	5000–4000	5000–4000	16/35	54.3	1/5	80.0	57.5

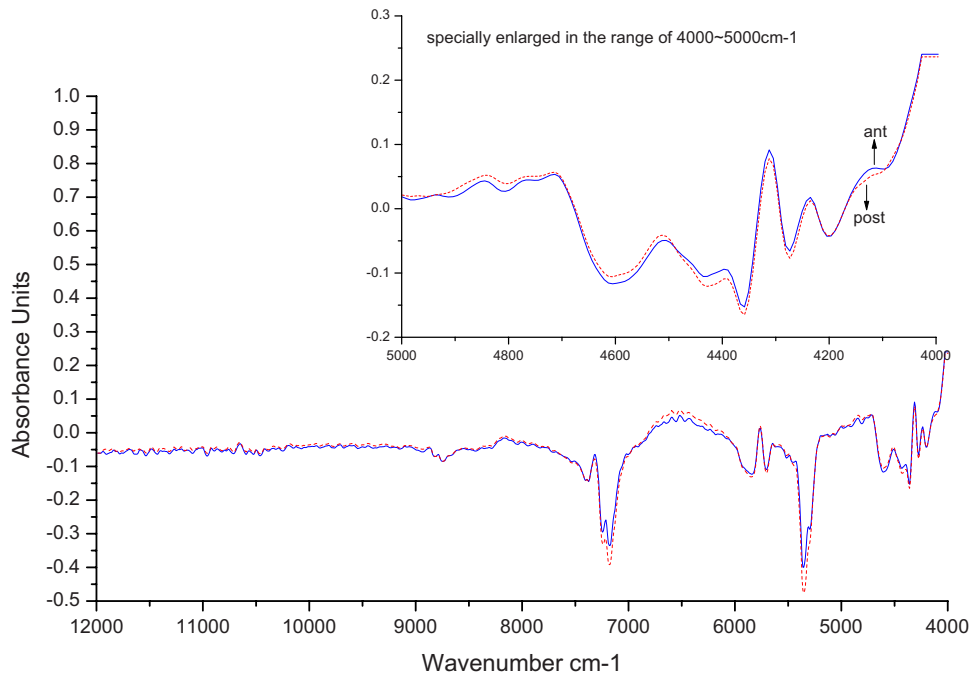


Fig. 2 Difference between two average spectra preprocessed by first derivative and vector normalization over the range of 4000 to 5000 cm^{-1} .

cauda equina nerves. A larger data set will be needed for the clustering model in further studies.

3.3 General Discussion

Rapid, simple, and low-cost identification of anterior/posterior roots in mixed *cauda equina* nerves is still very difficult. Generally, the anterior root is the motor nerve and the posterior root is the sensory nerve. Acetylcholinesterase (AChE) staining can successfully identify motor and sensory nerves, but it

requires at least 1 h for correct differentiation. The radioisotopic method requires 3 h or more to identify motor/sensory nerves, which may be more sensitive, while more expensive. Bright blue-labeled monoclonal (Blue-SAB) specifically reacts against the cell body and may be used to identify sensory fibers of nerve trunks by immunohistochemical technique. Unfortunately, these sophisticated methods are costly in both time and money, and they involve cumbersome preparation steps. Moreover, these identification methods are usually de-

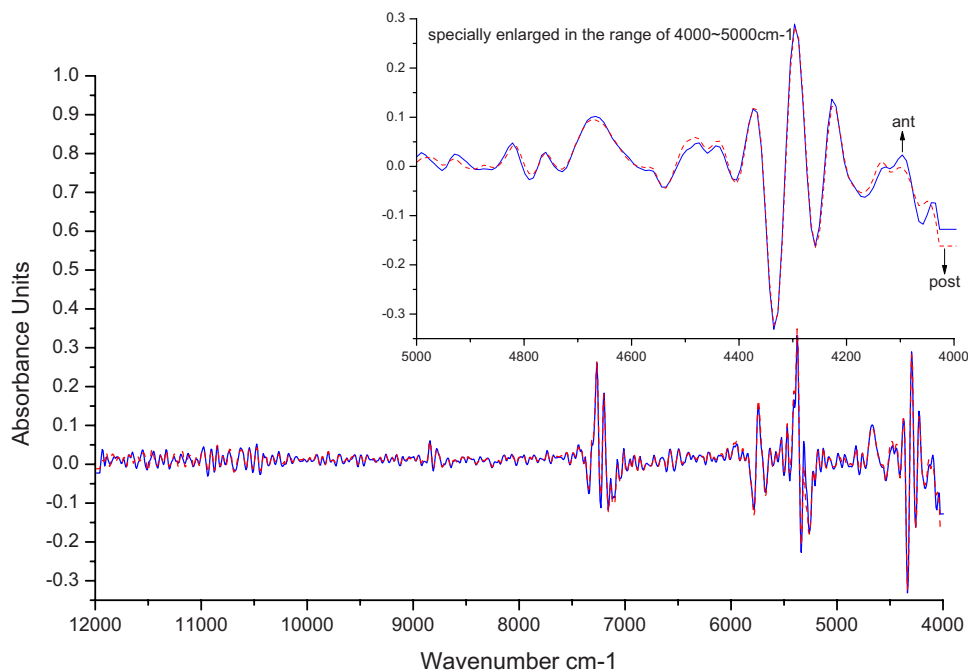


Fig. 3 Difference between two average spectra preprocessed by second derivative and vector normalization over the range of 4000 to 5000 cm^{-1} .

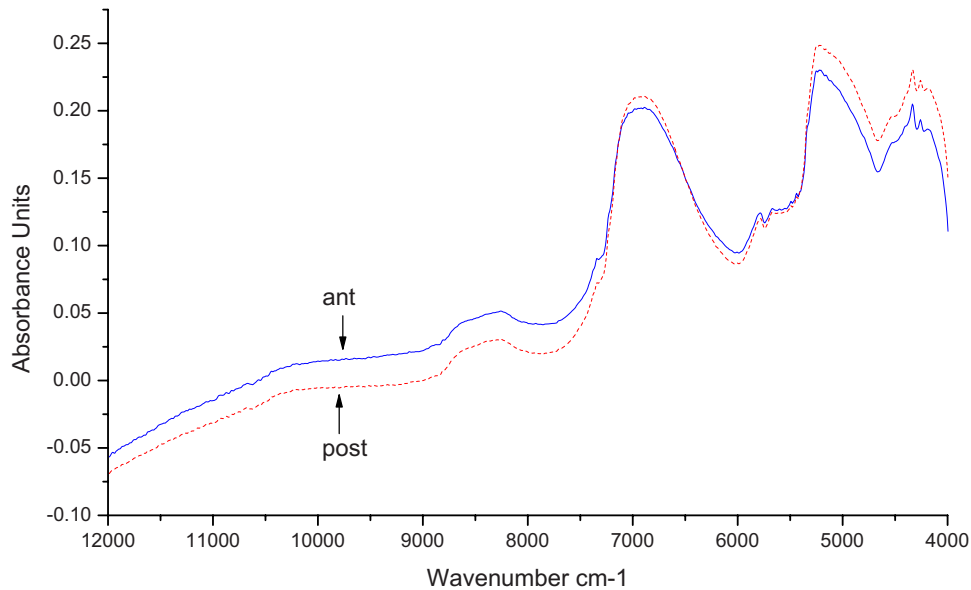


Fig. 4 Difference between two average spectra preprocessed by vector normalization over the range of 11,988 to 7112 and 4953 to 4050 cm^{-1} .

structive experiments to the samples. These disadvantages make them difficult to get an immediate correct matching of anterior/posterior roots in mixed *cauda equina* nerves during the surgery operation.

The most outstanding advantages of NIR spectroscopy over those conventional methods are little or no sample preparation and near real-time analysis. NIR spectroscopy is an extremely rapid method of measurement, with the capability

of performing an analysis in about one minute. Unlike most of the conventional analytical methods, NIR spectroscopy is nondestructive and involves no chemicals. It can simultaneously determine numerous constituents or parameters, and the instrument can be transported to nearly any environment—that is, true portability for fieldwork. Additionally, the NIR spectroscopy instrument is generally simple to operate. Despite the intuitive disadvantage of broad and

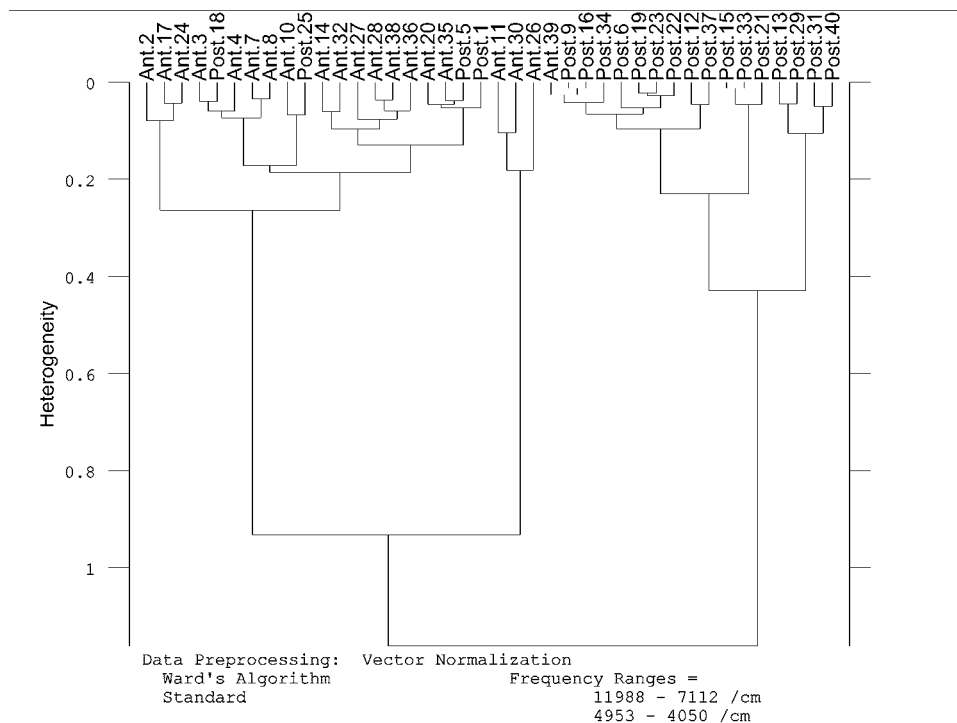


Fig. 5 Dendrogram showing clustering of anterior and posterior roots.

overlapping absorption bands, sophisticated chemometric techniques can extract meaningful information from the complex NIR spectra. And the reproducibility of NIR method is much better than the other methods.

The ability of NIR spectroscopy to differentiate samples involves detecting the differences in molecular vibrations of the examined species in the NIR region. A subtle difference in the constituents of a sample can lead to rapid characterization of the identity of that particular sample by giving a unique spectral fingerprint in the NIR region (12,500 to 3600 cm^{-1}). The main difference between the motor and sensory fibers is that the acetylcholine is the neurotransmitter of motor fibers, while substance P is the neurotransmitter of sensory fibers. That difference can be detected by NIR spectroscopy, which makes it possible to classify motor/sensory fascicles using appropriate multivariate analysis tools along with NIR spectroscopy. In this study, the model of NIR spectroscopy in combination with cluster analysis correctly classified 87.5% of the anterior and posterior roots. For the classification, the overall accuracy of NIR spectroscopy was lower than the classical methods, such as the AchE staining method, radioisotopic method, and immunohistochemical technique, but it may be more feasible in practical surgical nerve repair due to its fast analysis without sample preparation. The authors believe that the overall accuracy of the proposed method will be increased by enlarging the sample size of nerves in the future.

4 Conclusions

This study presented a rapid and effective method to identify anterior and posterior roots in mixed *cauda equina* nerves. The results showed unequivocally the potential of NIR spectroscopy for rapid, on-site and non destructive identification of the anterior and posterior roots. The accuracy of classification was 87.5% using the chemometric model (cluster analysis). Further studies based on a larger data set should be carried out to validate this method for the discrimination of anterior and posterior roots of *cauda equina* nerves.

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