Raman Spectroscopy Differentiates Each Tissue from the Skin to the Spinal Cord

A Novel Method for Epidural Needle Placement?

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ABSTRACT

Background: Neuraxial anesthesia and epidural steroid injection techniques require precise anatomical targeting to ensure successful and safe analgesia. Previous studies suggest that only some of the tissues encountered during these procedures can be identified by spectroscopic methods, and no previous study has investigated the use of Raman, diffuse reflectance, and fluorescence spectroscopies. The authors hypothesized that real-time needle-tip spectroscopy may aid epidural needle placement and tested the ability of spectroscopy to distinguish each of the tissues in the path of neuraxial needles.

Methods: For comparison of detection methods, the spectra of individual, dissected ex vivo paravertebral and neuraxial porcine tissues were collected using Raman spectroscopy (RS), diffuse reflectance spectroscopy, and fluorescence spectroscopy. Real-time spectral guidance was tested using a 2-mm inner-diameter fiber-optic probe-in-needle device. Raman spectra were collected during the needle's passage through intact paravertebral and neuraxial porcine tissue and analyzed afterward. The RS tissue signatures were verified as mapping to individual tissue layers using histochemical staining and widefield microscopy.

Results: RS revealed a unique spectrum for all ex vivo paravertebral and neuraxial tissue layers; diffuse reflectance spectroscopy and fluorescence spectroscopy were not distinct for all tissues. Moreover, when accounting for the expected order of tissues, real-time Raman spectra recorded during needle insertion also permitted identification of each paravertebral and neuraxial porcine tissue.

Conclusions: This study demonstrates that RS can distinguish the tissues encountered during epidural needle insertion. This technology may prove useful during needle placement by providing evidence of its anatomical localization. (Anesthesiology 2016; 125:793-804)
serious, complications during epidural corticosteroid injection, the U.S. Food and Drug Administration convened a panel of experts to determine practices to decrease their incidence.5

As each tissue has different proportions of molecules with unique spectral properties, optical spectroscopy provides biochemical tissue information and a method to distinguish different tissues from one another. Optical spectroscopy involves delivering light onto tissue and collecting the back scattered light. The technology is based on intrinsic tissue signal without exogenous injection or treatment, raising minimal safety concerns when translating this modality for clinical purposes. Multimodal spectroscopy, the combination of diffuse reflectance, fluorescence, and Raman spectroscopies, provides complementary tissue information.46 Diffuse reflectance spectroscopy (DRS) is based on white light absorption. Molecules with distinctive diffuse reflectance spectra include oxy-/deoxyhemoglobin, β-carotene, and melanin. Fluorescence spectroscopy (FS) is based on ultraviolet (UV)-induced fluorescence,47 and collagen, elastin, keratin, nicotinamide adenine dinucleotide, and flavin adenine dinucleotide generate strong UV-induced fluorescence signals. Raman spectroscopy (RS) is based on inelastic light scattering; tissues with greater amounts of proteins, lipids, and collagen have unique Raman spectra. To date, no study has utilized DRS, FS, and RS to characterize each tissue encountered during neuraxial anesthesia and epidural corticosteroid injection techniques.

We, therefore, conducted our current study to evaluate whether some combination of DRS, FS, and RS differentiates each tissue from the skin to the spinal cord along the trajectory of epidural needles. Our hypothesis is that these spectroscopic techniques can identify and differentiate each tissue during probe-in-needle insertion into ex vivo porcine neuraxial and paravertebral tissues.

Materials and Methods

Study Design

This study was approved by the Institutional Animal Care and Use Committee of Massachusetts General Hospital, Boston, Massachusetts (Protocol: 2014N000209). The first part of this study was performed in dissected, individual, ex vivo porcine neuraxial and paravertebral tissues. DRS, FS, and RS data were collected from epidermis/dermis, adipose, skeletal muscle, supraspinous/infragluteus ligament (SL), ligamentum flavum (LF), epidural adipose, dura mater, and spinal cord. The second part of the study was performed in ex vivo porcine neuraxial and paravertebral tissue corresponding to the lumbar and lower thoracic spinal column excised en bloc. Data were collected as a RS probe-in-needle was inserted in a stepwise fashion from the derrmis to the spinal cord. Tissues were subsequently identified by a neuropathologist.

Part 1: Characterization of Spectral Signature Using Dissected Tissues

Animal tissue was obtained from 40- to 50-kg female Yorkshire swine euthanized with 100 mg/kg of pentobarbital given intravenously. The neuraxial and paravertebral tissues, including the spinal column from the lumbar to lower thoracic vertebrae, were excised en bloc and refrigerated at 4°C for less than 24 h before use or frozen at −80°C within 3 h of excision and thawed at 4°C for 24 h before scanning. Tissue stored by both methods was dissected and scanned to determine if meaningful signal differences were detected. Key tissue planes were dissected and excised. After spectroscopy scans, each tissue sample was fixed using 4% formalin in phosphate-buffered saline and embedded in paraffin. Subsequently, 4-μm-thick sections were dewaxed and stained with hematoxylin and eosin before examination by a neuropathologist.

Diffuse reflectance and fluorescence spectra were acquired by custom-built tissue scanners.48 A broadband laser-driven light source (EQ-99-FC-S LDLS; Energetiq Technology, USA) was the excitation source for DRS. A 355-nm UV laser (SNV-40F-000; Teem Photonics, France) was the excitation source for FS. Both used custom-built optical fiber probes (one excitation and six collection fibers) to deliver the broadband light and collect the backscattered light. XY scanning was performed by a motorized stage (MS-6000-XY; Applied Scientific Instrumentation, USA). Backscattered reflectance light was delivered to a miniature spectrometer (HR-2000+; Ocean Optics, USA). LabView (National Instruments, USA) and MATLAB software (MathWorks, USA) controlled the systems. Dissected tissues were placed on top of a quartz plate. From each tissue, 20 diffuse reflectance spectra were acquired from a 5-mm² area. 15 mW of power was delivered to the sample with a 10-μs integration time. For fluorescence, 20 spectra were acquired from identical tissue locations as the DRS spectra to guarantee the same probe–sample distance. 3 mW of power was delivered to the sample with a 100-μs integration time. No correction was made for photobleaching, and no filters were used for fluorescence measurements as full spectrum information is recorded from the spectrophotometer.

Raman spectra were acquired by a custom-built near infrared Raman microscopy as described previously (Supplemental fig. 1, http://links.lww.com/ALN/B299).49 A 785-nm wavelength Ti:sapphire laser was used as an excitation source; XY tissue scanning was performed by galvanometer mirrors. A 1.2 numerical aperture water immersion objective lens with 60× magnification was used to focus the laser light onto the sample and collect the backscattered light. Backscattered Raman light from the sample passed through two dichroic mirrors, was collected by a multimode fiber, delivered to the spectrophograph, and detected by a thermoelectric-cooled, back-illuminated, deep depleted charge-coupled device. LabView software (National Instruments), a 16-bit data acquisition board (PCI-6251, National Instruments), and MATLAB software (MathWorks) were used to control the system and acquire the data. Dissected tissues were placed on top of quartz coverslips (043210-KJ; Alfa Aesar, USA). Twenty-five spectra were acquired from a 1.444-μm² area to account for tissue inhomogeneities. 60 mW of power was delivered to the sample with a 5-s integration time.
Part 2: Real-time Characterization of RS In Situ

Raman spectra were acquired by a multimodal spectroscopy system using a 830-nm diode laser to generate Raman signal from tissue. The laser light is filtered and coupled to an optical fiber Raman probe that delivers light to, and collects light from, a sample. The 2-mm outer diameter Raman probe consists of a single excitation fiber surrounded by 15 collection fibers; specialized filters and a ball lens are at the distal probe tip. Return light is passed through a spectrograph and dispersed onto a charge-coupled device detector. Tissue was acquired as described in Part 1: Characterization of Spectral Signature Using Dissected Tissues. A 2-mm inner diameter, custom-build, stainless steel needle with a short, cutting bevel (Supplemental fig. 2, http://links.lww.com/ALN/B300) and stylette were inserted into the tissue and advanced at 1- to 2-mm intervals. After each 1- to 2-mm movement, the stylette was removed, the RS probe was inserted, and 10 RS spectra were acquired with 60 mW of power and a 1-s integration time. The stylette was reinserted between steps. The needle was advanced along trajectories that correspond to the midline and paramedian approaches to epidural space localization. The needle depth was measured and recorded for each distinct spectrum. After insertion of the probe-in-needle to the posterior longitudinal ligament, the needle was left in place. Dissection then occurred along the length of the needle, and tissue was removed at the depth of each distinct spectrum.

Spectrum Analysis

Diffuse Reflectance Spectroscopy. In order to compensate for the spectrum from the broadband source, the raw reflectance spectrum from each tissue was divided by the reflectance spectrum acquired from a 20% white reflectance standard (Spectralon; Labsphere, USA). For each tissue type, a mean spectrum was calculated from 10 of the 20 tissue spectra (fig. 1). For each remaining test spectrum, eight Pearson correlation coefficients were calculated between the remaining spectrum and eight mean spectra using MATLAB. A Pearson correlation coefficient is defined as

$$\rho(A, B) = \frac{1}{N-1} \sum_{i=1}^{N} \left( \frac{A_i - \mu_A}{\sigma_A} \right) \left( \frac{B_i - \mu_B}{\sigma_B} \right),$$

where $N$ is the number of scalar observations for each variable, $\mu_A$ and $\sigma_A$ are the mean and SD of $A$, respectively, and $\mu_B$ and $\sigma_B$ are the mean and SD of $B$, respectively. The correlation coefficient for each test spectrum and mean spectrum pair was calculated using the test spectrum and mean spectrum as variables $A$ and $B$, respectively. The resultant correlation coefficients represent the degree of similarity between each test tissue spectrum and the mean spectrum of eight tissue types, with higher correlation coefficients signifying greater similarity. Each test spectrum was thus classified to the tissue type with the highest correlation coefficient.

Fluorescence Spectroscopy. The FS data were analyzed in the same manner as that of DRS (fig. 2).

![Fig. 1.](image-url) For each tissue type, a mean spectrum was calculated from 10 of the 20 tissue spectra acquired from a 5-mm$^2$ area. Some of the reflectance spectra from dissected tissues can clearly be differentiated from one another, while the spectra from others have subtle or no differences. SL = supraspinous/intraspinous ligament.
Raman Spectroscopy. For quantitative analysis, the acquired Raman spectra from the tissue specimens were decomposed as a summation of basis components, albumin, actin, collagen, triolein, and phosphatidylcholine. Each acquired spectrum was represented by five fitting coefficients, representing the relative amounts of basis components. Albumin and actin were selected to represent plasma and cytoskeleton proteins. Collagen was selected as it is the main component of connective tissues. Triolein and phosphatidylcholine were selected as nonmembrane and membrane lipids, respectively. The acquired spectra from these agents were normalized and used as the basis spectra for spectrum analysis (fig. 3) and represented as a vector. Wavenumbers (Raman shift) represent the energy shift between excitation photons and scattered photons from a sample. Typically, wavenumbers between 600 and 1,800 cm\(^{-1}\) are used due to good molecular specificity. For biochemical decomposition of the tissue, the ordinary least squares fitting method was used (fig. 4). To avoid data overfitting, the fitting process was carefully monitored by restricting the fitting coefficient to nonnegative values. Acquired Raman spectra were normalized and decomposed as a summation of basis components as previously described.\(^{51-53}\)

A decision algorithm was developed based on the five fitting coefficients. Ten Raman spectra from each of the eight tissues were used to develop the decision algorithm, and 15 spectra were used for testing the algorithm.

Machine Learning Classification Model. As variation was evident in fitting coefficients between dissected and nondissected tissue, a machine learning classification model was used to enable autonomous identification of real-time acquired RS spectra. The use of a machine learning classification model allows for robust identification of tissue type that can algorithmically compensate for the variation seen in fitting coefficients between dissected and nondissected tissue. Acquired spectra were separated into a training data set and a testing data set; 10 spectra from each dissected tissue type were used for training, while 80 spectra from each tissue acquired during probe-in-needle insertion were used to test the tissue identification accuracy of the algorithm. The five fitting coefficients, representing the relative amounts of albumin, actin, collagen, triolein, and phosphatidylcholine in each spectra, were used as a five-dimensional vector representation of each spectrum. The classification model was programmed in Python based on a Gaussian Mixture Model algorithm, a type of machine learning algorithm that clusters data points using maximum-likelihood estimation.\(^{54}\) The model was trained using the Raman spectra fitting coefficients to create data clusters for each tissue type in five-dimensional vector space and then classified test spectra by identifying the most probable cluster to the fitting coefficient vector of the test spectrum (Supplemental fig. 3, http://links.lww.com/ALN/B301).

Statistics. MATLAB software (MathWorks) was used to analyze the diffuse reflectance, fluorescence, and Raman spectra. For each tissue type, a mean spectrum was calculated from 10 of the 20 tissue spectra acquired from identical tissue locations as the diffuse reflectance spectroscopy to guarantee the same probe–sample distance. Some of the fluorescence spectra from dissected tissues can clearly be differentiated from one another, while the spectra from others have subtle or no differences. SL = supraspinous/intraspinous ligament.
spectra data from dissected tissues and to calculate correlation coefficients. The fitting coefficient of each basis component represents the “relative” amount of a basis component within each tissue since each has a different Raman cross section. For example, the epidermis/dermis has a fitting coefficient of 0.09 for actin and 0.84 for collagen. This does not indicate that the collagen content is more than nine times the actin content as collagen and actin have different Raman cross sections. However, the 0.22 (actin) and 0.64 (collagen) fitting coefficients within the LF indicate that the actin content within the LF is 144% greater than within epidermis/dermis, and the collagen content is 24% less.

A RS decision algorithm (Supplemental fig. 4, http://links.lww.com/ALN/B302) was developed based on the five fitting coefficients for classification of the dissected tissue. The RS decision algorithm was empirically designed and verified as described. Ten Raman spectra (40%) from each of the eight tissues were used to develop the decision algorithm, and 15 spectra (60%) were used for testing the algorithm. First, the tissue spectra were divided into two groups based on the collagen content. The high collagen tissues (more than 0.5) are further categorized based on the relative ratios of collagen and total protein (actin + albumin). Tissues are further subdivided based on actin content.

The low collagen tissues (less than 0.5) are further categorized based on the total protein content (more than 0.5 and less than 0.5). Low collagen, low protein content tissues are differentiated from one another using the relative ratio of triolein and phosphatidylcholine.

**Results**

Tissue refrigerated at 4°C for less than 24 h before use or frozen at −80°C within several hours of excision and thawed at 4°C for 24 h before scanning did not have meaningful differences in DRS, FS, and RS signal.

**Part 1: Characterization of Spectral Signature Using Dissected Tissues**

**Diffuse Reflectance Spectroscopy.** The normalized reflectance spectra from each tissue (fig. 1) show visible differences as previously reported. Tissue prediction results using DRS spectra are summarized in table 1. The overall prediction accuracy of DRS is 89%. The sensitivity for accurate tissue prediction using DRS is less than 90% for two tissues.
(LF, 60%; epidural adipose, 80%), while the specificity is greater than 90% for all tissues.

**Fluorescence Spectroscopy.** Strong fluorescence signals are generated from collagen in connective tissues. Therefore, tissues containing greater amounts of collagen (skin, SL, LF, and dura mater) have a higher signal (fig. 2). Adipose, muscle, and spinal cord do not generate strong fluorescence signals. FS cannot effectively quantitate signal strength due to photobleaching and is not sensitive enough to accurately differentiate low collagen content tissues. Generated fluorescence signals are distorted by the optical absorption and scattering properties of overlying tissue. Therefore, acquired spectral shapes are different from pure fluorescence spectra. Tissue prediction results using FS spectra are summarized in table 1. The overall prediction accuracy of FS is 81%. Poor distinction is made between the collagen-rich layers (skin, SL, LF). The sensitivity for accurate tissue prediction using FS is less than 90% for two tissues (SL, 40%; LF, 20%), while the specificity is greater than 90% for all tissues except epidermis/dermis (84%).

**Raman Spectroscopy.** Unlike the previous DRS and FS prediction methods, where tissue prediction is based on the similarity between acquired and averaged, representative spectra, the RS tissue prediction method is developed based on a combination of molecular fitting coefficients. Figure 4 demonstrates the fitting result for the Raman spectrum from the skeletal muscle. The fitting spectrum (red line) is similar to the raw data (blue dots), and the five fitting coefficients provide the chemical composition of the measured tissue.56 Normalized averaged Raman spectra from the eight dissected tissues are shown in figure 5. The red-highlighted Raman band at 939 cm⁻¹ indicates a collagen-specific Raman signature, while the yellow Raman band at 1,450 cm⁻¹ represents a lipid-specific Raman signal. Strong collagen signals are found in the spectra of

| Table 1. Tissue Prediction by Diffuse Reflectance Spectroscopy, Fluorescence Spectroscopy, and a Fitting Coefficients-based Decision Algorithm for Raman Spectroscopy |
|---------------------------------------------------------------------------------------------------------------|
| **How Often Each of the 10 (DRS and FS) or 15 (RS) Spectra from Histologically Confirmed Dissected Tissue**  |
| **Were Correctly Identified, and When Incorrectly Identified, What They Were Labeled As**                  |
| **Diffuse reflectance** (accuracy = 89%)                                                                  |
| Epidermis/dermis                                            | Adipose | Muscle | SL | LF | EA | Dura | Mater | SC | Sensitivity (%) (95% CI) | Specificity (%) (95% CI) |
| 10                                                       | 9       | 1     |    |    |    |      |       |    | 100 (66–100)           | 97 (89–99)                |
| Epidermis/dermis                                            | 9       | 1     |    |    |    |      |       |    | 90 (54–99)            | 97 (89–99)                |
| Adipose                                                    | 10      | 1     |    |    |    |      |       |    | 90 (54–99)           | 100 (93–100)             |
| Muscle                                                     | 9       | 1     |    |    |    |      |       |    | 90 (54–99)           | 95 (87–98)                |
| SL                                                         | 1       | 3     | 6   |    |    |      |       |    | 60 (27–86)           | 98 (91–99)                |
| Ligamentum flavum                                          | 2       | 8     |    |    |    |      |       |    | 80 (44–96)           | 98 (91–99)                |
| Epidural adipose                                           | 10      | 10    |    |    |    |      |       |    | 100 (65–100)          | 100 (93–100)             |
| Dura mater                                                 | 10      | 10    |    |    |    |      |       |    | 100 (65–100)          | 100 (93–100)             |
| Spinal cord                                                | 10      | 10    |    |    |    |      |       |    | 100 (65–100)          | 98 (91–99)                |
| **Fluorescence** (accuracy = 81%)                           |
| Epidermis/dermis                                            | 10      | 10    |    |    |    |      |       |    | 100 (65–100)          | 84 (73–91)                |
| Adipose                                                    | 10      | 10    |    |    |    |      |       |    | 100 (65–100)          | 100 (93–100)             |
| Muscle                                                     | 9       | 1     |    |    |    |      |       |    | 90 (54–99)           | 100 (93–100)             |
| SL                                                         | 6       | 4     |    |    |    |      |       |    | 40 (13–72)           | 97 (89–99)                |
| Ligamentum flavum                                          | 5       | 2     | 2   | 1 |    |      |       |    | 20 (3–55)            | 93 (100)                 |
| Epidural adipose                                           | 10      | 10    |    |    |    |      |       |    | 100 (65–100)          | 100 (93–100)             |
| Dura mater                                                 | 10      | 10    |    |    |    |      |       |    | 100 (65–100)          | 98 (91–99)                |
| Spinal cord                                                | 10      | 10    |    |    |    |      |       |    | 100 (65–100)          | 99 (91–99)                |
| **Raman (accuracy = 100%)**                                 |
| Epidermis/dermis                                            | 15      | 0     | 0   | 0 | 0 | 0 | 0 | 0 | 100 (74–100) | 100 (95–100) |
| Adipose                                                    | 0       | 15    | 0   | 0 | 0 | 0 | 0 | 0 | 100 (74–100) | 100 (95–100) |
| Muscle                                                     | 0       | 0     | 15  | 0 | 0 | 0 | 0 | 0 | 100 (74–100) | 100 (95–100) |
| SL                                                         | 0       | 0     | 0   | 15 | 0 | 0 | 0 | 0 | 100 (74–100) | 100 (95–100) |
| Ligamentum flavum                                          | 0       | 0     | 0   | 0 | 15 | 0 | 0 | 0 | 100 (74–100) | 100 (95–100) |
| Epidural adipose                                           | 0       | 0     | 0   | 0 | 0 | 15 | 0 | 0 | 100 (74–100) | 100 (95–100) |
| Dura mater                                                 | 0       | 0     | 0   | 0 | 0 | 0 | 15 | 0 | 100 (74–100) | 100 (95–100) |
| Spinal cord                                                | 0       | 0     | 0   | 0 | 0 | 0 | 0 | 15 | 100 (74–100) | 100 (95–100) |

In the leftmost column are the dissected tissues as identified by histopathology. The next eight columns hold the number of the 10 spectra (diffuse reflectance spectroscopy [DRS] and fluorescence spectroscopy [FS]) or 15 spectra (Raman spectroscopy [RS]) from each tissue assigned as each of the eight tissues based on the one with the highest correlation coefficient (DRS and FS) or decision algorithm (RS). This table reports how often each of the 10 to 15 spectra from histologically confirmed dissected tissue were correctly identified by the 3 spectroscopic methods, and when incorrectly identified, what they were labeled as.

EA = epidural adipose; LF = ligamentum flavum; SC = spinal cord; SL = supraspinous/intraspinous ligament.
epidermis/dermis, SL, LF, and dura mater. Strong lipid signals are found in adipose, epidural adipose, and spinal cord.

Quantitative analysis results are shown in figure 6 and table 2. Figure 6 demonstrates the differences in basis component composition between each of the eight tissues dissected and scanned by RS.

Results of the RS decision algorithm can be seen in Supplemental figure 4 (http://links.lww.com/ALN/B302). The high collagen group (more than 0.5) includes epidermis/dermis, SL, LF, and dura mater. These tissues have low lipid content and are further categorized based on the relative ratios of collagen and total protein (actin + albumin). SL has a ratio of more than 100. LF has a ratio of less than 3.6. Epidermis/dermis and dura mater have collagen/total protein ratios between 3.6 and 100 and can be differentiated based on their actin content. 3.6 and 100 were found to be the optimal collagen:total protein ratios for differentiating the high collagen tissues from one another.

The low collagen group (less than 0.5) includes skeletal muscle, adipose, epidural adipose, and spinal cord. Skeletal muscle can be differentiated due to its high actin and albumin content (total protein more than 0.5). The remaining three tissues (adipose, epidural adipose, and spinal cord; total protein less than 0.5) are differentiated from one another using the relative ratio of triolein and phosphatidylcholine. Adipose has a ratio of more than 20; epidural adipose has a ratio between 5 and 20; and spinal cord has a ratio of less than 5. 5 and 20 were found to be the optimal triolein/phosphatidylcholine ratios for differentiating the low collagen, low total protein tissues from one another. Table 1 shows the tissue prediction results. All eight tissue layers can be differentiated.

Table 1 shows the RS tissue prediction results. All eight tissue layers can be differentiated from one another. The overall prediction accuracy of RS is 100%. The sensitivity for accurate tissue prediction using RS is 100% for all tissues, while the specificity is 100% for all tissues.
The tissues identified during dissection, and subsequently scanned by DRS, FS, and RS, were confirmed by histology. All tissues underwent hematoxylin and eosin staining and were independently assessed by a neuropathologist who confirmed the tissue types (fig. 5).

**Part 2: Real-time Characterization of RS In Situ**

Normalized, averaged Raman spectra acquired from *in situ* probe insertion are shown in figure 5. Overall the spectra have a qualitatively smoother profile than those from dissected tissues due to different spectral resolutions (4 cm⁻¹ for dissected tissue measurements vs. 16 cm⁻¹ for probe-in-needle measurements) of the two instruments. Nonetheless, the RS spectrum of each tissue is similar to those acquired from the individually dissected tissues. The acquired Raman spectra were processed in the same fashion as spectra from dissected tissues (fig. 7). The fitting coefficients were similar to those from the dissected tissues except for those of SL and LF. Both tissues have higher lipid content than the dissected tissues. Adipose, removed during dissection studies, is actually interspersed with the SL and immediately superficial and adjacent to LF in swine. Thus, as the RS probe samples a volume of tissue within 1 mm of the probe tip, some adipose is sampled along with these thin ligaments.

With the training spectra presented to the machine learning classification model in random order, the algorithm has 85.5% accuracy across the entire test data set. Specifically, the model produced 100% accuracy of identification of 7 out of 8 issue types, including epidermis/dermis, adipose, skeletal muscle, LF, epidural adipose, dura mater, and spinal cord. Because of similarity of SL spectra to LF spectra, the current model cannot be used to differentiate SL and LF with spectra classified in random order; real-time classification, accounting for the expected order of transition between layers, enables differentiation between all eight tissue types.

**Discussion**

While obstetric epidural analgesia, perioperative epidural analgesia, and epidural injection of corticosteroids are useful techniques for acute and chronic pain, both minor and serious complications do occur. Thus, a technique to aid with epidural needle placement may be beneficial if it can identify each tissue in the path of the needle, potentially preventing wrong site needle insertion and drug injection.

**Table 2.** The Fitting Coefficients of the Basis Raman Spectra Identify a Unique Combination for Each Dissected Tissue Layer

| Tissue Type          | Albumin | Actin | Collagen | Triolein | PC     |
|----------------------|---------|-------|----------|----------|--------|
| Epidermis/dermis     | 0.09 ± 0.03 | 0.00 ± 0.00 | 0.84 ± 0.03 | 0.10 ± 0.01 | 0.00 ± 0.00 |
| Adipose              | 0.00 ± 0.00 | 0.00 ± 0.00 | 0.01 ± 0.02 | 0.92 ± 0.01 | 0.03 ± 0.01 |
| Muscle               | 0.10 ± 0.03 | 0.85 ± 0.04 | 0.14 ± 0.01 | 0.17 ± 0.02 | 0.00 ± 0.00 |
| SL                   | 0.00 ± 0.00 | 0.00 ± 0.00 | 0.98 ± 0.03 | 0.04 ± 0.02 | 0.00 ± 0.00 |
| Ligamentum flavum    | 0.22 ± 0.03 | 0.18 ± 0.06 | 0.64 ± 0.06 | 0.02 ± 0.02 | 0.00 ± 0.00 |
| Epidural adipose      | 0.00 ± 0.00 | 0.00 ± 0.00 | 0.00 ± 0.00 | 0.82 ± 0.03 | 0.11 ± 0.02 |
| Dura mater           | 0.10 ± 0.03 | 0.04 ± 0.02 | 0.83 ± 0.04 | 0.05 ± 0.02 | 0.00 ± 0.00 |
| Spinal cord           | 0.09 ± 0.03 | 0.05 ± 0.02 | 0.00 ± 0.00 | 0.43 ± 0.01 | 0.32 ± 0.01 |

Values are represented as mean fitting coefficient ± SD.
PC = phosphatidylcholine; SL = supraspinous/intraspinous ligament.
Our studies show that RS identifies each tissue in the path of an epidural needle in an ex vivo porcine model. Characterization of the DRS, FS, and RS spectral signatures showed that the Raman spectrum from each tissue is unique. Further investigation in which a RS probe was inserted in situ corroborated these results. RS may be useful to safely and effectively aid epidural needle placement during steroid injection and catheter placement procedures.

Other methods have been used to aid with epidural needle placement. However, these methods lack resolution and are unable to distinguish between each tissue encountered at the epidural needle tip during insertion. Ultrasound is increasingly used to aid neuraxial anesthetic placement. A recent meta-analysis concluded that neuraxial ultrasound more accurately identifies the lumbar intervertebral space over palpation, accurately predicts the distance from the skin to the epidural space, and improves efficacy. However, with some practitioners, the actual depth from the skin to the intended space may differ by as much as 15 mm from that estimated by ultrasound. Significant training and experience is necessary before one can properly utilize ultrasound for assistance with epidural needle and catheter placement. In addition, the acquisition and maintenance of the equipment is costly. As bone is not penetrated by ultrasound, it is not possible to acquire a comprehensive view of the epidural space, but the practitioner is limited to small windows. Real-time use of ultrasound during epidural needle advancement is typically not possible, and ultrasound is most often used to assess the location and depth of neuraxial structures before blind needle advancement.

Fluoroscopy can help guide the needle to, and confirm placement of the needle tip in, the epidural space. However, its use is labor and time intensive, requires additional equipment and often manpower, and exposes the patient and medical personnel to radiation.

Despite the relatively low specificity of this technique, a number of LOR assistance devices have also been developed and studied, including the Episure Autodetect syringe (Indigo Orb, Inc., USA), Epidrum (Exmoor Innovations Ltd, United Kingdom), and CompuFlo injection pump (Milestone Scientific, USA). A pilot study found fewer epidural catheter failures when the Episure Autodetect syringe was used for the placement compared to a glass syringe. Similarly, use of the Epidrum device led to a lower epidural failure rate and less time to identify epidural space compared to use of the traditional LOR syringe.

Previously, reflectance spectroscopy was shown to distinguish between the LF and dura mater as well as aid with epidural space localization. Another study using visible and near-infrared spectroscopy found that upon entry of the epidural needle into the epidural space, an increase in the lipid content of the tissue was detected. No data from either technique were published on the other tissues that are encountered during epidural needle insertion. As midline LF is absent up to 74% of the time in the cervical and high thoracic region, it is important to identify all tissue layers encountered to prevent injury and improve accuracy. Moreover, reflection or image-based methods will identify two areas of the same tissue as separate tissue types if the areas have different tissue densities. Additionally, DRS is sensitive to tissue inhomogeneities, probe–sample distance, and pressure variation. FS only differentiates tissues with high collagen content from those with low collagen content. Thus, neither method is ideal to accurately and precisely distinguish each tissue in the path of an epidural needle. Conversely, RS is specific to the chemical composition of the tissue. Areas of different density within the same tissue will change the Raman intensity, but the spectral shape will not be altered. Furthermore, the sampling space of RS extends 1 mm from the tip of the probe, allowing identification of tissue before the tip of the needle enters it.

In this study, RS was found to have greater prediction accuracy in identifying and differentiating tissues than DRS and FS. Furthermore, the Raman spectrum of each tissue was found to be unique and can be identified based on the relative amount of albumin, actin, collagen, triolein, and phosphatidylcholine. This identification held for the Raman spectra collected during in situ probe-in-needle insertion as well. Neither DRS nor FS was able to distinguish between all tissues of interest. Additionally, the machine learning classification model developed based on Raman spectra demonstrates the capability for autonomous tissue identification that can be used for real-time classification of tissues.

There are potential limitations to use of this technology during epidural needle placement. RS differentiated each dissected tissue but did not clearly distinguish between SL and LF during probe-in-needle insertion secondary to adipose interspersed within the undissected tissue. Although all tissues can be differentiated during real-time probe-in-needle insertion if the expected order of transition between layers is taken into account, future collection and use of a large training data set from in situ measurements are likely to allow differentiation of these two tissues with use of the machine learning classification model. Additionally, a smaller RS probe, necessary for clinical purposes and currently being built in our laboratory, will sample a smaller tissue volume at the tip of the needle and is less likely to include information from multiple adjacent tissues. While RS can identify tissue at the tip of the needle during neuraxial procedures, it cannot be used to guide the needle trajectory. It may be beneficial in the future to use RS for needle tip tissue identification along with ultrasound or fluoroscopy to aid with needle trajectory. Our current analysis model includes the five basis components as outlined in Spectrum Analysis under Raman Spectroscopy. If other compounds that strongly absorb Raman signal are present in these tissues, such as heme from minor bleeding during needle insertion or calcified ligaments in elderly patients, detection accuracy may be affected. The solution to this potential issue is to include the new component such as hemoglobin and/or calcium salts into the basis model. For example, 12 basis components are used for breast cancer diagnosis. Intravenous medications and electrolyte changes will not affect RS signal since their concentration is much lower.
than the major compounds responsible for Raman spectra within tissues. Raman signal depends on temperature, but this is extremely minor effect from the small temperature changes in biologic tissues. Patient movement may affect the tissue density and RS signal strength, but the spectral shape, and hence prediction accuracy, will not be affected.

It remains to be seen whether or not use of this technology along with the traditional LOR technique results in a change in procedure time, number of needle passes, procedure success, or complication rates. Future studies include miniaturization of the RS probe for use in an epidural needle and RS characterization of neuraxial and paravertebral tissues in a live animal model and in human cadaveric tissue in order to support the application of this technology for use in a human trial.

In conclusion, we have described a novel method, using RS, to identify each tissue at the tip of neuraxial needles. RS can potentially be used along with current techniques for epidural space localization. It remains to be seen if use of this technology decreases epidural needle complication rates.

Acknowledgments

The authors thank the staff of the Knight Surgical Research Lab at the Massachusetts General Hospital (Boston, Massachusetts), especially Dr. Michael Duggan, D.V.M., and John Beagle, B.S., for their time and expertise as well as the generous donation of swine tissue. Luis Galindo, B.S., a senior engineer within the Massachusetts Institute of Technology Laser Biomedical Research Center (Cambridge, Massachusetts), helped perform probe-in-needle experiments.

Research Support

Supported by the National Science Foundation 1549613 (Arlington, Virginia); funded research time from Jeanine Wiener-Kronish, M.D., Anesthetist-in-Chief, Massachusetts General Hospital Department of Anesthesia, Critical Care and Pain Medicine (Boston, Massachusetts); to Dr. Anderson; National Institutes of Health 9P41EB015871-28 (Bethesda, Maryland; to Drs. Kang, Dasari, and So); Massachusetts Institute of Technology (MIT) SkolTech initiative (Cambridge, Massachusetts; to Drs. Kang, Dasari, and So); Samsung Advanced Institute of Technology (Seoul, South Korea; to Drs. Kang, Dasari, and So); DP3-DK101024 01, 1-U01-NS090438-01, 1-R01-EY01756-06A1, 1R21NS091982-01, 1-R01-HL121386-01A1, and 1-U01CA202177-01 (to Dr. So); the Singapore-MIT Alliance 2 (Cambridge, Massachusetts; to Dr. So); the Biosym IRG of Singapore-MIT Alliance Research and Technology Center (Cambridge, Massachusetts; to Dr. So); MIT SkolTech initiative (to Dr. So); Hamamatsu Corporation (Hamamatsu City, Japan; to Dr. So); the Koch Institute for Integrative Cancer Research Bridge Initiative (Cambridge, Massachusetts; to Dr. So); and Connecticut Children’s Medical Center (Hartford, Connecticut; to Dr. So).

Competing Interests

Drs. Anderson, Kang, Dasari, and So are named as authors on patents related to several multimodal spectroscopy medical devices designed for needle-tip tissue identification. Drs. Anderson, Kang, Dasari, and So have equity interest in a for-profit company (Medisight Corporation, Cambridge, Massachusetts) designed to commercialize multimodal spectroscopy technology for needle tip tissue identification. The other authors declare no competing interests.

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Shakespearean scholar G. Q. Colton (1814 to 1898) revived Americans’ anesthetic use of laughing gas. Preferring to deaden dental pain rather than the dental patient, Colton apparently agreed with Shakespeare’s First Gaoler (or Jailer, from the Bard’s comedy Cymbeline) who opined that “he that sleeps feels not the tooth-ache….” Although administration of 100% nitrous oxide was reported as safe by Colton, dentists delivering hypoxic “Colton gas” could render patients “untimely smothered,” to quote Dowager Queen Margaret from Shakespeare’s historical play Richard III. She despised King Richard III, who was played onstage by actor David Garrick. In 1745, painter William Hogarth immortalized Garrick (above) playing Richard III. (Copyright © the American Society of Anesthesiologists’ Wood Library-Museum of Anesthesiology.)

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