INTRODUCTION:
Raman spectroscopy can probe the biochemical properties of bone tissue at a resolution of <5 microns, thereby providing compositional characterization of microscopic features and insight into bone quality. Traditionally, the ratio of the $\nu_1$ phosphate peak ($\nu_1$Phos) to the Amide I peak has been used to quantify the degree of mineralization. However, recent studies have found that the intensity of the Amide I band is affected by the orientation of the collagen fibrils relative to the incident Raman laser beam [1,2]. Since collagen orientation is heterogeneous in bone tissue [3], the peak ratio of $\nu_1$Phos/Amide I may not be sensitive enough to detect differences in tissue composition. To test this notion, we compared the ability of four Raman peaks related to collagen to differentiate non-fixed, fixed, and embedded bones because fixation in alcohol and plastic embedding has been found to affect peak ratios [5].

METHODS:
We collected a femur diaphysis (40 mm in axial length) from 7 donors (M94, M80, M48, F95, F86, F86, and F82) through the Vanderbilt Anatomy Lab. For each diaphysis, a diamond embedded, circular saw cut longitudinal strips from the medial quadrant (6mm x 6mm x 40mm). We then used the saw to transversely cut the medullary bone into 6 sections. Four sections per donor were fixed in 70% EtOH for 24 hours and then further dehydrated in 100% EtOH for 48 hours. Two sections per donor were then embedded in methacrylate (PMMA). Using a series of silicon carbide papers (800, 1200, 4000) on an Exact wheel, we ground the transverse side of one section and the longitudinal side of the other section. Thus, there were transverse cuts and longitudinal cuts (Fig. 1) for each donor and each processing technique (non-fixed, fixed, or embedded). Lastly, the ground sides were polished on cloth with 0.05 micron alumina slurry.

Raman spectra were acquired from the tissue in air using a confocal Raman microscope, with a 785nm laser diode source. A 50x objective focused the laser, and the inelastic light was collected with a Renishaw Raman microscope, with a 785nm laser diode source. A 50x objective.

RESULTS:
Tissue type, orientation of cut, and embedding affected $\nu_1$Phos/Amide I with no significant interactions. Tissue type and orientation of cut, NOT embedding, affected $\nu_1$Phos/Amide III and $\nu_1$Phos/CH$_2$ with no significant interactions; whereas, only tissue type affected $\nu_1$Phos/Proline with one significant interaction between type and orientation. Dividing the mineral peak ($\nu_1$Phos) by the Proline peak detected the greatest difference in composition between interstitial and osteonal tissue in the transverse cut (15%; Fig. 3). The traditional $\nu_1$Phos/Amide I detected the least difference between tissue types in this relevant orientation. On the other hand, this ratio was the most affected by orientation of the cut (Fig. 3B) with greater than 30% difference between transverse and longitudinal in both osteonal and interstitial tissue types.

DISCUSSION:
The Raman spectra of bone have a number of distinct peaks representing the vibration modes of various biochemical bonds comprising the mineral and collagen phases. The intensity of each peak depends on the number of bonds within a given volume, association with neighboring compounds (e.g., PMMA), and relative orientation of the bonds to the incident light. Presently, we found that $\nu_1$Phos/Amide I was affected by embedding media and the orientation of the cut, and therefore may not be suitable in quantifying compositional differences. Of the organic moieties, Proline was the most suitable peak for normalizing the $\nu_1$ phosphate peak in order to quantify differences in mineralization or specifically the mineral-to-collagen ratio. $\nu_1$Phosphate per Proline, however, was not affected by orientation of cut (p-value=0.487) nor by fixation in alcohol and embedding (p-value=0.235). It detected the greatest difference in mineralization between interstitial and osteonal tissue and was the most sensitive in detecting this difference within donors. It had the lowest coefficient of variance (0.0212-0.0343 compared to 0.0370-0.0424 for CH$_2$). Therefore, calculating the $\nu_1$Phos/Proline ratio is perhaps the best way to detect compositional differences by Raman spectroscopy, irrespective of whether the bone is embedded or not, and investigating determinants of bone quality.

REFERENCES: