# Towards the development of a non-bioptic diagnostic technique for eosinophilic esophagitis using Raman spectroscopy

Hemanth Noothalapati[1], Suguru Uemura[2], Naoki Ohshima[1, 3], Yoshikazu Kinoshita[1, 3], Masahiro Ando[4], Hiro-o Hamaguchi[4, 5], and Tatsuyuki Yamamoto[1, 2]*

[1]*Raman Center for Medical and Biological Applications, Shimane University, 1060 Nishikawatsu-cho, 690-8504, Matsue, Japan*

[2]*Faculty of Life and Environmental Science, Shimane University, 1060 Nishikawatsu-cho, 690-8504, Matsue, Japan*

[3]*Faculty of Medicine, Shimane University, 89-1 Enya-cho, 693-8501, Izumo, Japan*

[4]*Consolidated Research Institute for Advanced Science and Medical Care, Waseda University, 513 Tsurumaki-cho, Waseda, Shinjuku, 162-0041, Tokyo, Japan*

[5]*Institute of Molecular Science and Department of Applied Chemistry, National Chiao Tung University, 1001 University Road, 30010, Hsinchu, Taiwan*

Tel+Fax: +81-(852)-32-6551

E-mail: tyamamot@life.shimane-u.ac.jp

( Received )

keywords: Raman spectroscopy; eosinophilic esophagitis; non-bioptic diagnosis; eosinophil peroxidase

## Abstract

Eosinophilic esophagitis (EoE) is believed to be a rare pathological condition that is characterized by dense infiltration of eosinophils in esophageal epithelial layer. Occurrence of this disease worldwide has started increasing rapidly in the last decade. Routine endoscopic observations can help in diagnosis only if clear longitudinal furrows or multiple concentric rings are observed but does not give any definitive conclusion in the early stages. Hence esophageal tissue samples are collected from multiple sites by biopsy and the number of eosinophils is counted after staining. Such a procedure is time consuming and has an inherent risk of bleeding, eventually damaging esophagus. Hence we developed a resonance Raman spectroscopy based approach to detect eosinophils in esophagus using mouse models. Our results show the presence of eosinophils in mice esophageal tissues suffering from inflammation by administering interleukin-33 while there are none in control mice. We believe this method can be used in clinical application for diagnosing EoE in the near future.

## 1. Introduction

Eosinophils, one of the three types of granulocytes, are polymorphonuclear leukocytes that make up about 2-6% of white blood cells. They play key roles in regulating inflammation in addition to fighting parasites. Though inflammation is indispensible with a defensive role in isolating and controlling a disease site, unnecessary or over inflammation leads to a variety of inappropriate immunological responses resulting in allergic diseases eventually causing tissue damage. Recently, cases of eosinophil related inflammation of airway mucosa including sinuses, esophagus and lungs are reported to be rapidly increasing. Eosinophilic gastrointestinal disorders, characterized by dense infiltration of eosinophils in esophago-gastro-intestinal mucosa, can be categorized into eosinophilic esophagitis (EoE) and eosinophilic gastroenteritis. Ever since Landres et al. first reported EoE over 30 years ago, it was found to be prevalent only among pediatric population[1-3]. However, a growing trend of EoE in adults worldwide has been observed in the last decade, especially more so in western countries. Also, it develops more frequently in males than females (76 % of the patients are male in Europe or North America and 80% in Japan) with the average age of these patients being 51 years [4].

Predominant symptoms of patients with EoE are dysphagia, heartburn, chest discomfort, throat discomfort and food impaction which is primarily triggered by dense eosinophil infiltration into the upper mucosal layer of esophagus resulting in chronic inflammation[4-6]. This leads to increase in the permeability and growth of esophageal squamous layer while also promoting edema or fibrotic response in submucosal layer, which are difficult to observe in patients suffereing from EoE at the early stage. Effective remedy for this disease is already established with local action steroid or with anti-interleukin-5 or -13 antibody. However, the differentiating from reflux esophagitis, which shows similar symptoms to EoE is difficult by simple diagnosis using endoscope[7]. But definite diagnosis, which is done by counting number of eosinophils in mucosal epithelial tissue by staining, forces the patients to undergo biopsy of the mucosal tissue of esophagus. Since there is no guide for determining the appropriate position, multiple biopsies are considered superior to a single biopsy (sometimes more than five biopsies are done) in diagnosing EoE. This increases the risk of bleeding, especially when the biopsy sampling are done in multiple areas in esophagus[5, 8]. Such diagnosis gets even

more complicated in patients undergoing treatment with antithrombotic drugs. Adding to the complexity is important fact that many of these patients also simultaneously suffer from other difficulties such as allergies or asthma. Hence alternative technologies that do not depend on biopsy are need of the hour.

We propose to develop Raman spectroscopy based diagnostic method for EoE, for the advantages are manifold. First, Raman spectrum, which can be thought of as a chemical fingerprint, gives rich chemical information. Secondly, it does not require any external dye probes. Thirdly, it is a non-invasive technique that is suitable for *in vivo* applications[9-11]. It is well known that granulocytes are different in their chemical make up, especially in the heme peroxidases, and that they can be distinguished by Raman spectroscopy[12-14]. In fact, we have successfully automated distinguishing each white blood cell type using Raman spectroscopy and multivariate curve resolution analysis recently[15]. Particularly in the case of eosinophil peroxidase, there is an absorption band at 640 nm and the nearby ± 20 nm region can be used for resonance enhancements of this heme proteins[14, 16]. With this background, we set out to explore the possibility of applying such a method to detect eosinophil infiltration in esophageal mucous membrane in model mice using eosinophil peroxidase as a marker. We prepared model disease mouse with EoE by inducing inflammation in esophagus using interleukin-33 (IL-33), an IL-1 family of proteins that produce T helper-2 associated cytokines[17-19]. Pathologically, in the case of EoE, eosinophils are observed only in the esophageal epithelial layer but not in gastric or intestinal mucosa. Our results on successful detection of eosinophils during such inflammation in esophageal tissues of IL-33 administered model mouse by resonance Raman spectroscopy is reported in this article.

## 2. Materials and Methods

### *2.1 Preparation of esophageal tissue*

Six week BALB/c female mice (Charlesriver) were used to prepare two kinds of model mice, control and diseased (IL-33 administered). One control mouse (wildtype) was prepared by administering 25 μl of PBS while three EoE model mice (diseased) was prepared by injecting IL-33 (2 μg in 150 μl PBS) intraperitonealy for seven consecutive days from day 7 to 13. On day 14, mice were euthanized and esophagus dissected in order to carry out Raman measurements. The mice were anatomized after euthanasia according to the guide for

the treatment of laboratory animals of Shimane University. The dissected esophageal tissues were first thoroughly rinsed using PBS. The cylindrical shaped esophageal tissue was then cut lengthwise so as to expose the inner membrane. The cut esophageal tissues of control or IL-33 injected mice were kept in a bath of cooled PBS until further measurements. The desired tissue was then carefully transferred onto a plate with little PBS to avoid sample drying to perform Raman spectroscopic measurements. To check infiltration of eosinophils after Raman experiments, hematoxylin and eosin staining (HE) was done on both control and IL-33 treated mice.

### *2.2 Preparation of granulocytes obtained from human blood*

Granulocytes of human blood were extracted from one of the authors to compare and discuss the Raman spectra for esophageal tissue of mice. Polymorphprep was purchased from COSMO BIO for the purpose of blood separation. Red Blood Cell Lysis Solution was purchased from Miltenyi Biotec K.K. for hemolyzing red blood cells. 5 ml of human blood was collected and treated with heparin to prevent aggregation. It was then slowly added to equal amount of blood separating solution kept prior in a centrifuge tube, to give two layers. The tube was centrifuged at 500x g for 30 minutes at room temperature to get six separated layers. Granulocytes separated in the fourth layer from top. 200 μl from this fourth layer was collected into another centrifuge tube and 5 ml of PBS was added followed by centrifugation at 400x g for 10 minutes at room temperature to give white pellet at the bottom. The upper layer was removed and rinsed with PBS. Then 1 ml of PBS and 9 ml of diluted Red blood Cell Lysis Solution was added to the centrifuge tube and left to stand still for 10 minutes after ultrasonic agitation. It was again centrifuged at 400x g for 10 minutes at room temperature to give pure white pellet. The aqueous layer was removed and 1 ml of PBS was added to get a solution of granulocytes. The final concentration of granulocytes used for Raman measurements was $1 \times 10^6$ cells/ml.

### *2.3 Raman spectroscopic measurements*

Raman spectra of esophageal tissues and isolated eosinophils were measured with a home made confocal Raman microspectrometer. In brief, the 632.8 nm output of a He-Ne laser (used as the Raman excitation light) was coupled to an Olympus inverted microscope IX71. An objective lens with magnification

ratio of 20X was used to focus light onto the sample. The back-scattered light collected by the same objective passes through a long pass filter to reject Rayleigh scattering and a 50 μm pinhole for confocal detection. The light is then dispersed using a polychromator (Chromex) and detected with a Princeton Instruments SPEC-10 CCD camera. The system was controlled with LabVIEW software (National Instruments) and data were analyzed using IGOR Pro software (HULINKS). The average power used for measurements was 2.0 mW at the sample point. In the case of space resolved experiments, an exposure time of 10 seconds with spatial intervals of 100 μm were used.

### *2.4 HE staining of esophageal tissues*

Marker pins were tagged to point out the positions of Raman measurements after which they were subjected to HE staining using standard staining protocols[20]. The infiltration of eosinophils was independently confirmed by HE staining.

## 3. Results and Discussion

Figure 1 depicts major steps involved in our experiment. Control and diseased mice were euthanized and esophagus dissected. After rinsing, the cut esophageal tissue is taken for Raman measurements as it is.

### *3.1 Raman spectroscopic analysis of mouse esophagus*

About 30 space-resolved Raman spectra were measured from esophageal tissue of wild type and IL-33 administered mice and their representative spectra are shown in Figure 2. Spectra obtained from wild type esophagus (Figure 2 a-c) were dominated by Raman bands characteristic to proteins. Prominent protein bands include amide I vibration at 1657 $cm^{-1}$, C-H bending modes of aliphatic chain at 1450 $cm^{-1}$and 1342 $cm^{-1}$, amide III band at 1245 $cm^{-1}$, Phe ring breathing vibration at 1004 $cm^{-1}$, C-C skeletal mode at 936 $cm^{-1}$ and Tyr residue at 853 $cm^{-1}$. Band position of amide I indicate presence of α-helix structure particularly[21]. In IL-33 injected case, two groups of spectra were observed. The first (Figure 2 d & e), which represents ~ 95% of measured spectra, is similar to typical protein spectrum as in the wild type. However ~ 5% of the spectra i.e., 2 out of 30, were different with sharp bands at 1609 $cm^{-1}$, 1547 $cm^{-1}$, 1448 $cm^{-1}$ and 757 $cm^{-1}$ in addition to the 1004 $cm^{-1}$

band (Figure 2 f), suggesting the presence of heme structures.

### *3.2 HE staining of esophageal tissues*

IL-33 is a pro-inflammatory protein that induces type 2 cytokine response in wide range of cells including eosinophils. In this particular case, intraperitoneal injection of IL-33 into mice is expected to results in infiltration of eosiniphils into esophagus. To confirm this infiltration phenomenon, the same esophageal tissue regions, where Raman measurement was carried out, were pinned and HE stained as shown in Figure 3. Esophagus from the wild type mouse did not show the presence of eosinophils (Figure 3B) whereas dense infiltration was observed in the case of IL-33 administered mouse (Figure 3C).

### *3.3 Raman spectrum of single eosinophil granulocyte*

Next, to assign the heme spectra from the diseased mouse, Raman spectra of granulocytes extracted from human blood (belonging to one of the authors) were measured followed by staining to particularly distinguish eosinophil from other granulocytes. Figure 4 compares the heme Raman spectrum from IL-33 injected mouse (same as Figure 2f) and a single extracted eosinophil. Eosinophils contain eosinophil peroxidase, a heme peroxidase concentrated in secretory granules, which has a strong CT absorption band centered at 640nm. Excitation wavelength of 632.8 nm in this study was chosen specially for this reason, to selectively resonance enhance Raman scattering from eosinophil peroxidase. All Raman bands characteristic to this heme peroxidase coincide very well. In fact, these spectra also corroborates well with Raman spectra from eosinophil peroxidase reported in literature. Thus these prominent Raman bands, especially the doublet at 1609 $cm^{-1}$ and 1547 $cm^{-1}$ along with sharp features at 1004 $cm^{-1}$ and 757 $cm^{-1}$ can be used as markers of eosinophil peroxidase. Other granulocytes such as basophil and neutrophil have other signatures as reported by Salmaso and co-workers.

## 4. Conclusion

In this work, we made use of resonance Raman spectroscopy of eosinophil peroxidase and have successfully detected the presence of eosinophils in esophageal tissues of mice suffering from inflammation induced by

administering IL-33. Although there is a huge potential for our method, there are several challenges to overcome if we are to translate this technique to clinical setting for non-invasive diagnostics of EoE. By definition, ≥ 15 eosinophils/HPF (1 HPF is ~ 0.2 $mm^2$) indicates EoE[2]. Considering the size of eosinophils (12 - 17 μm in diameter) and the step size used (100 μm), the probability of finding eosinophils is very low as observed. Application of statistical analyses was difficult to the current set of data due to limited size. Hence increasing the sample size and accumulating sufficient data should let us take advantage of multivariate analyses in the near future. Also, efforts to combine this method with endoscopy using fiber optics for *in vivo* applications are underway in our laboratory.

## Acknowledgement

This work was financially supported in part by the grant-in-aid for scientific research from Japan society for the promotion of science for YK (25460928) and TY (24310022, 26650133, 15H04763) and also by the budget for project research from Shimane University.

## References

[1] R.T. Landres, G.G. Kuster, W.B. Strum, Eosinophilic esophagitis in a patient with vigorous achalasia, Gastroenterology, 74 (1978) 1298-1301.
[2] G.T. Furuta, C.A. Liacouras, M.H. Collins, S.K. Gupta, C. Justinich, P.E. Putnam, P. Bonis, E. Hassall, A. Straumann, M.E. Rothenberg, F.I.G.E.R.S.F. Subcommittees, Eosinophilic esophagitis in children and adults: a systematic review and consensus recommendations for diagnosis and treatment., in, 2007, pp. 1342-1363.
[3] C.A. Liacouras, G.T. Furuta, I. Hirano, D. Atkins, S.E. Attwood, P.A. Bonis, A.W. Burks, M. Chehade, M.H. Collins, E.S. Dellon, R. Dohil, G.W. Falk, N. Gonsalves, S.K. Gupta, D.A. Katzka, A.J. Lucendo, J.E. Markowitz, R.J. Noel, R.D. Odze, P.E. Putnam, J.E. Richter, Y. Romero, E. Ruchelli, H.A. Sampson, A. Schoepfer, N.J. Shaheen, S.H. Sicherer, S. Spechler, J.M. Spergel, A. Straumann, B.K. Wershil, M.E. Rothenberg, S.S. Aceves, Eosinophilic esophagitis: updated consensus recommendations for children and adults, J Allergy Clin Immunol, 128 (2011) 3-20 e26; quiz 21-22.
[4] Y. Kinoshita, K. Furuta, N. Ishimaura, S. Ishihara, S. Sato, R. Maruyama, S. Ohara, T. Matsumoto, C. Sakamoto, T. Matsui, S. Ishikawa, T. Chiba, Clinical characteristics of Japanese patients with eosinophilic esophagitis and eosinophilic gastroenteritis, J Gastroenterol, 48 (2013)

333-339.
[5] S.F. Pasha, J.K. DiBaise, H.J. Kim, G. De Petris, M.D. Crowell, D.E. Fleischer, V.K. Sharma, Patient characteristics, clinical, endoscopic, and histologic findings in adult eosinophilic esophagitis: a case series and systematic review of the medical literature, Dis Esophagus, 20 (2007) 311-319.
[6] N. Ishimura, S. Shimura, D. Jiao, H. Mikami, E. Okimoto, G. Uno, M. Aimi, N. Oshima, S. Ishihara, Y. Kinoshita, Clinical features of eosinophilic esophagitis: differences between Asian and Western populations, J Gastroenterol Hepatol, 30 Suppl 1 (2015) 71-77.
[7] S. Shimura, N. Ishimura, T. Tanimura, T. Yuki, T. Miyake, Y. Kushiyama, S. Sato, H. Fujishiro, S. Ishihara, T. Komatsu, E. Kaneto, A. Izumi, N. Ishikawa, R. Maruyama, Y. Kinoshita, Reliability of symptoms and endoscopic findings for diagnosis of esophageal eosinophilia in a Japanese population, Digestion, 90 (2014) 49-57.
[8] K. Furuta, K. Adachi, K. Kowari, Y. Mishima, H. Imaoka, C. Kadota, K. Koshino, T. Miyake, Y. Kadowaki, K. Furuta, H. Kazumori, S. Sato, S. Ishihara, Y. Amano, M. Honda, Y. Kinoshita, A Japanese case of eosinophilic esophagitis, J Gastroenterol, 41 (2006) 706-710.
[9] Y.S. Huang, T. Karashima, M. Yamamoto, H. Hamaguchi, Molecular-level investigation of the structure, transformation, and bioactivity of single living fission yeast cells by time- and space-resolved Raman spectroscopy, Biochemistry, 44 (2005) 10009-10019.
[10] M.S. Bergholt, W. Zheng, K. Lin, K.Y. Ho, M. Teh, K.G. Yeoh, J.B. So, Z. Huang, In vivo diagnosis of esophageal cancer using image-guided Raman endoscopy and biomolecular modeling, Technol Cancer Res Treat, 10 (2011) 103-112.
[11] J. Wang, K. Lin, W. Zheng, K.Y. Ho, M. Teh, K.G. Yeoh, Z. Huang, Simultaneous fingerprint and high-wavenumber fiber-optic Raman spectroscopy improves in vivo diagnosis of esophageal squamous cell carcinoma at endoscopy, Sci Rep, 5 (2015) 12957.
[12] S.S. Sibbett, S.J. Klebanoff, J.K. Hurst, Resonance Raman characterization of the heme prosthetic group in eosinophil peroxidase, FEBS Lett, 189 (1985) 271-275.
[13] G.J. Puppels, H.S. Garritsen, G.M. Segers-Nolten, F.F. de Mul, J. Greve, Raman microspectroscopic approach to the study of human granulocytes, Biophys J, 60 (1991) 1046-1056.
[14] B.L. Salmaso, G.J. Puppels, P.J. Caspers, R. Floris, R. Wever, J. Greve, Resonance Raman microspectroscopic characterization of eosinophil peroxidase in human eosinophilic granulocytes, Biophys J, 67 (1994) 436-446.
[15] M. Ando, H. Hamaguchi, Automatic and Objective Cell Discrimination by Raman Spectroscopy with Multivariate Curve Resolution Analysis, in: 11th Australian Conference on Vibrational Spectroscopy and the 5th Asian Spectroscopy Conference, University of Sydney, 2015.
[16] A. Jorg, J.M. Pasquier, S.J. Klebanoff, Purification of horse eosinophil peroxidase, Biochim Biophys Acta, 701 (1982) 185-191.
[17] M.A. Rank, T. Kobayashi, H. Kozaki, K.R. Bartemes, D.L. Squillace, H. Kita, IL-33-activated dendritic cells induce an atypical TH2-type response, J Allergy Clin Immunol, 123 (2009)

1047-1054.
[18] B. Stolarski, M. Kurowska-Stolarska, P. Kewin, D. Xu, F.Y. Liew, IL-33 exacerbates eosinophil-mediated airway inflammation, J Immunol, 185 (2010) 3472-3480.
[19] K.D. Dyer, C.M. Percopo, H.F. Rosenberg, IL-33 promotes eosinophilia in vivo and antagonizes IL-5-dependent eosinophil hematopoiesis ex vivo, Immunol Lett, 150 (2013) 41-47.
[20] A.H. Fischer, K.A. Jacobson, J. Rose, R. Zeller, Hematoxylin and eosin staining of tissue and cell sections, CSH Protoc, 2008 pdb prot4986.
[21] F.S. Parker, Polypeptides and Proteins, in: Applications of Infrared, Raman, and Resonance Raman Spectroscopy in Biochemistry, Plenum Press, New York, 1983, pp. 83-153.

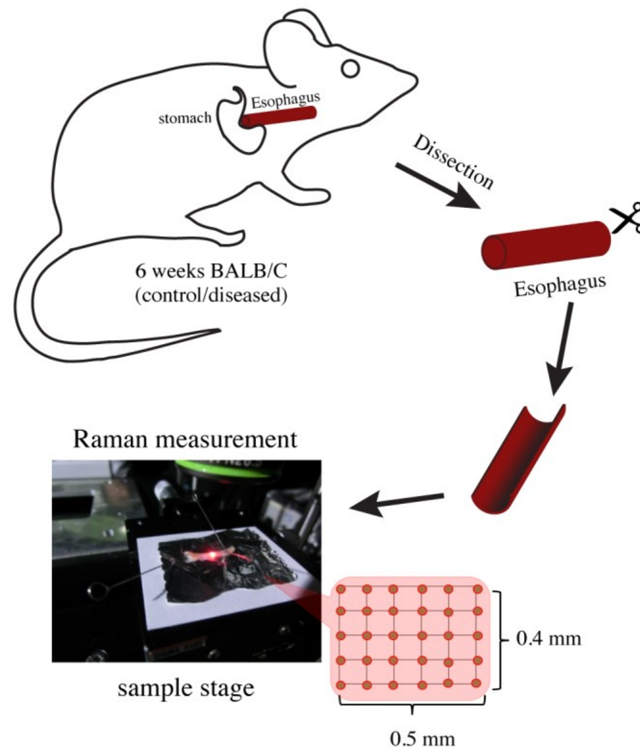


**Figure 1:** Steps involved in preparing esophageal tissue for Raman spectroscopic measurements.

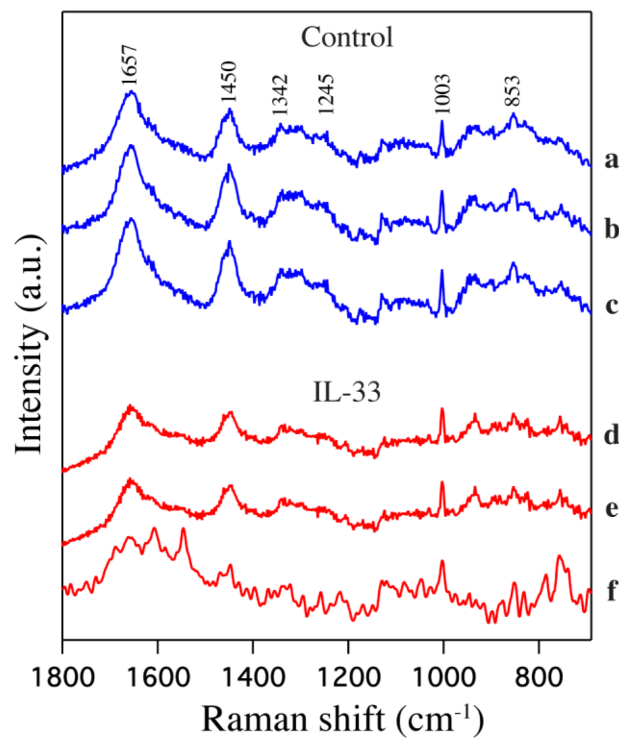


**Figure 2:** Space-resolved Raman spectra from esophageal tissue of mouse. Raman spectra obtained from; (a-c) a control mouse and (d-f) IL-33 administered mouse

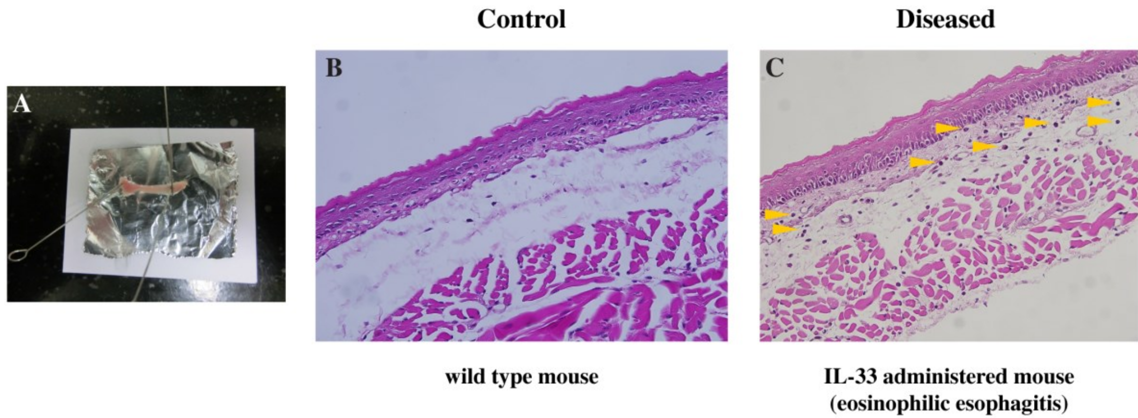


**Figure 3:** Photograph and photomicrograph of HE stained esophageal tissue. (A) photograph showing pinned tissue to identify same region as in Raman measurements for HE staining, (B-C) HE stained images from a control and IL-33 administered diseased mouse esophagus. Orange arrowheads in C indicate eosinophil infiltration.

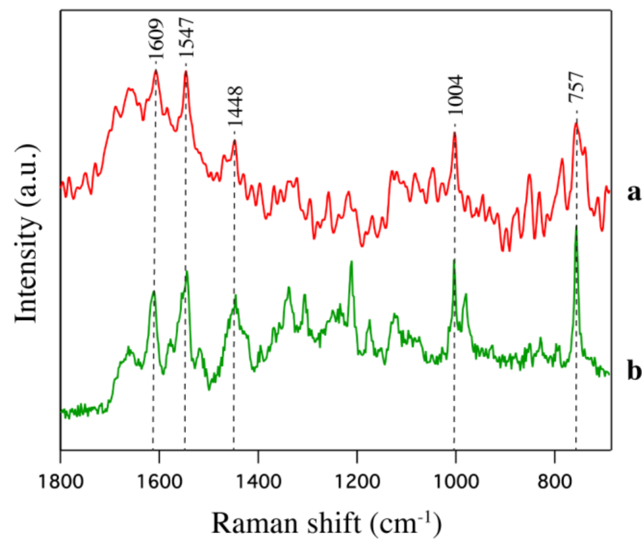


**Figure 4:** Comparison of Raman spectra. (a) IL-33 administered mouse esophagus [same as in figure 2-f] and (b) single eosinophil extracted from whole blood. Peak positions are as indicated. Excellent overlap of bands is observed indicating the molecular component in (a) is from eosinophil peroxidase.