LABEL-FREE HAEMOGRAM USING WAVELENGTH MODULATED RAMAN SPECTROSCOPY FOR IDENTIFYING IMMUNE-CELL SUBSET

Praveen C. Ashok^{1*}, Bavishna B. Praveen^{1*}, Elaine C. Campbell^{2*}, Kishan Dholakia¹ and Simon J. Powis².

¹ SUPA, School of Physics and Astronomy, University of St Andrews, Fife, UK.

² School of Medicine, University of St Andrews, Fife, UK

* Authors have equal contribution

ABSTRACT

Leucocytes in the blood of mammals form a powerful protective system against a wide range of dangerous pathogens. There are several types of immune cells that has specific role in the whole immune system. The number and type of immune cells alter in the disease state and identifying the type of immune cell provides information about a person's state of health. There are several immune cell subsets that are essentially morphologically identical and require external labeling to enable discrimination. Here we demonstrate the feasibility of using Wavelength Modulated Raman Spectroscopy (WMRS) with suitable machine learning algorithms as a label-free method to distinguish between different closely lying immune cell subset. Principal Component Analysis (PCA) was performed on WMRS data from single cells, obtained using confocal Raman microscopy for feature reduction, followed by Support Vector Machine (SVM) for binary discrimination of various cell subset, which yielded an accuracy >85%. The method was successful in discriminating between untouched and unfixed purified populations of CD4+CD3+ and CD8+CD3+ T lymphocyte subsets, and CD56+CD3- natural killer cells with a high degree of specificity. It was also proved sensitive enough to identify unique Raman signatures that allow clear discrimination between dendritic cell subsets, comprising CD303+CD45+ plasmacytoid and CD1c+CD141+ myeloid dendritic cells. The results of this study clearly show that WMRS is highly sensitive and can distinguish between cell types that are morphologically identical.

Keywords: Immunology, Raman spectroscopy, Fluorescence suppression, Flow-cytometry, Multivariate analysis, Lymphocytes, Dendritic cells

1. INTRODUCTION

Blood is an important body fluid which plays the key role of carrying nutrients and waste to and from different parts of the body. Blood cells can be broadly classified within three main categories, the red blood cells (erythrocytes), white blood cells (leukocytes) and the platelets (thrombocytes) as shown in Figure 1. Among these corpuscular elements, the leukocytes play the role of immune system of the body defending against infectious diseases and foreign materials. The leukocytes can be broadly sub classified into granulocytes (polymorphonuclear leukocytes) and agranulocytes (mononuclear leukocytes) based on the granule content of the immune cells. The granules in the granulocytes are predominantly lysosomes.

Based on the difference in the staining properties on the granules, the granulocytes are classified into neutrophils, basophils and eosinophils. Neutrophils defend against microbial infections, while the basophils deal with parasitic infections such as allergic reactions. Basophils play a major role in antigen response where they release histamine causing vasodilation. Agranulocytes contain lysosomes in their cytoplasm and all basically classified into lymphocytes, monocytes and macrophages. Lymphocytes are predominantly found in the lymphatic system, these cells are characterised by eccentric nucleus with small amount of cytoplasm. The lymphocytes are basically classified into three, the T cells, B cells and the natural killer (NK) cells. B cells belong to the humoral branch of the adaptive immune system. B cells have certain proteins on their outer surface known as B cell receptors (BCR), which allow these cells to attach to a specific antigen. The vital role of the B cells are to act as antigen presenting cells (APC), where they make antibodies against antigens and develop into memory B cells following activation by the antibody-antigen interaction.

Figure 1: Hierarchical depiction of the corpuscular elements in blood along with their major subpopulation

T cells mature in the thymus and have T cell receptors on their surface (TCR). T cells have several subsets which can be broadly classified into helper T cells, cytotoxic T cells, memory T cells, regulatory T cells and natural killer T cells. Each of these types of T cells has their own immune function which is deeply involved and is beyond the scope of this chapter. However, broadly the helper T cells, also called CD4⁺ T cells (due of the presence of CD4 glycoprotein on their surface), assist in the immunological processes of the white blood cells such as the maturation of B cells, activation of cytotoxic T cells and macrophages. Cytotoxic T cells also known as CD8⁺ T cells (due of the presence of CD8 glycoprotein on their surface) dose the role of destroying the virally infected cells and tumour cells. NK cells are critical cells of the innate immune system which have the unique ability to recognise stressed cells even in the absence of any antibodies and major histocompatibility complexes (MHC) thereby allowing faster immune response.

Monocytes, the next class of agraunolcytes, present pieces of pathogens for the T cells to recognise and kill the pathogens. Monocytes eventually leave the blood stream into the body tissues and differentiate into macrophages. Macrophages are part of both innate and adaptive immune system; they play an important role in phagocytosis of the cellular debris and pathogens along with stimulating immune cells to respond towards pathogens.

Another important class of immune cells are the Dendritic cells (DCs). DCs are antigen presenting cells that act as a messenger between the innate and adaptive immune system. The study of these immune cell subsets is an active area of research in the field of immunology since the exact origin; development and function of these cells are marginally understood at the moment. This is mainly because they are rare and hence difficult to isolate. However, broadly the DCs in the blood stream can be classified into myeloid DCs and plasmacytoid DCs.

The coordinated activities of these cells in the mammalian adaptive and innate immune system provide powerful protection against a wide range of potentially harmful pathogens. The numbers and type of immune cells alter in the diseased state, which makes it important to identify and distinguish different immune cell subsets present in blood. Currently this is realised using a variety of staining techniques for morphology, or staining with labelled antibodies for analysis by flow cytometry or fluorescence microscopy. These active methods often result in the destruction or potential alteration of cell behaviour. Hence the development of a reliable, label-free and passive method to identify immune cell subsets would be a highly useful tool in both the clinical and research environment.

In combination with appropriate statistical techniques, Raman spectroscopic tool has proved sensitive enough to distinguish between different types of cells and tissues [1]. Also, the fact that Raman spectroscopy allows label-free identification of the cells with minimal sample preparation helps maintaining the integrity and function of the cells intact. Hence Raman spectroscopy can be a sensitive tool for the identification and discrimination of immune cell subsets.

Raman spectroscopy has previously been used as a tool to study the dynamics of immunological cells [2, 3]. A recent study demonstrated that Raman spectroscopy is capable of discriminating between lymphocytes and neutrophils[4]. As can be seen from Figure 1, lymphocytes and neutrophils belong to completely different categories of immune cells based on the presence of granules in their cell body. As such these immune cell sub-categories of leukocytes differ significantly in their morphology and chemical composition.

The formidable challenge would be to identify the variety of subsets within lymphocytes that are known to exist. These immune cell subsets are morphologically similar. The conventional method of discriminating them involves fluorescence tagging followed by flow cytometry. This process is time consuming and is known to alter the cell behaviour. The external tagging can activate/deactivate the cell from its original state. Hence it is critical to develop a technique that can discriminate between these closely related cells without the need for any external tagging. This would require highly sensitive chemical fingerprinting to discriminate between such closely related cells. Here, Wavelength Modulated Raman Spectroscopy (WMRS) [5-8] is used to discriminate between different immunological cell types that are essentially indistinguishable morphologically. Further, the resultant differential Raman spectra were classified using a robust statistical classification algorithm based upon Principal Component Analysis (PCA) for feature selection and Support Vector Machine (SVM) for classification. WMRS was used to achieve highly sensitive discrimination between various sub-classes of lymphocytes including the two main classes of T lymphocytes (CD4+ versus CD8+), and the closely related CD56+ natural killer cell population. The technique was also used to demonstrate a much more challenging problem where WMRS is used to discriminate between two of the main categories of antigen-presenting and immune-regulating dendritic cells (DC) detected in the blood in the form of plasmacytoid (lymphoid) and myeloid DC [9, 10].

2. METHODOLOGY FOR THE LABEL-FREE FINGERPRINTING OF IMMUNE CELLS

2.1 Preparation of cells for performing Raman spectroscopy

Blood was taken after informed written consent, from a healthy male donor, with the approval of the University of St Andrews Teaching and Research Ethical Committee (UTREC). PBMC were isolated by centrifugation over Histopaque (Sigma, Poole, UK) and cells washed twice with 1 x PBS/0.1% BSA. Specific leukocyte subtypes were isolated using Dynabeads® Untouched™ Human CD4 T Cell Kit (depleting antibodies: anti-CD8, CD14, CD16a, CD16b, CD19, CD36, CD56, CD123 and CD235a) / Dynabeads® Untouched™ Human CD8 T cell kit (depleting antibodies: anti-CD4, CD14, CD16a, CD16b, CD19, CD36, CD56, CD123 and CD235a)/ Dynabeads® Untouched™ Human NK cell kit (depleting antibodies: anti- CD3, CD14, CD36, HLA Class II, CD123 and CD235a) (Life Technologies, Paisley, UK). Dendritic cells were isolated using MACS plasmacytoid dendritic cell isolation kit II and MACS myeloid dendritic cell isolation kit (Miltenyi Biotec, Bisley, UK).

The samples for the confocal Raman spectroscopic experiment were prepared using a pair of quartz slides (SPi Supplies, UK). A quartz slide whose dimensions are 25.4 mm x 25.4 mm, 1 mm thick, acted as the base on top of which a chamber well was prepared by pasting a vinyl spacer of 80 µm thickness. 20 µl of the cell sample in PBS was loaded into the well formed by the vinyl chamber. Another quartz slide of dimension 25.4 mm x 25.4 mm, 0.15 mm to 0.18 mm thick was used to seal this chamber. The sample was then allowed to sediment on the thicker slide for about 30 min. The slide was placed on the confocal microscope with the thinner slide at the bottom, closer to the objective.

2.2 Performing WMRS using a confocal Raman microscope

A home built confocal Raman system was used to chemically fingerprint the immune cells. The system was equipped with a 785 nm tunable diode laser for Raman excitation. For detection, the system was equipped with a monochromator with a 400 lines/mm grating, blazed at 850 nm and a deep depletion, back illuminated and thermoelectrically cooled CCD camera. The laser was focused through a 50x objective (Nikon, NA 0.9) onto the sample, with the power at the sample plane being 150 mW. By continuously acquiring Raman spectra with 5 s single acquisition time for 5 minutes, it was confirmed that the power we used does not cause any disruption to the cells.

A series of Raman spectra at different wavelengths were recorded to obtain WMRS spectra from a cell. This was achieved by recording five Raman spectra at equidistant excitation wavelengths amounting to a total modulation range of $\Delta\lambda=0.32$ nm. Each spectrum for this experiment was acquired with a single acquisition time of 5 s.

2.3 Processing the WMRS data

The data processing was performed using Matlab 2012b. Each set of acquired spectra from a single cell was normalised for area under the curve to compensate for the power fluctuation of the laser during wavelength modulation and PCA was performed on the dataset, considering each wavelength step as a parameter, resulting in the modulated Raman spectrum. The modulated Raman spectrum is devoid of the luminescence background and the zero crossing points of the differential Raman spectra give the peak position.

A training dataset was created from all the WMRS spectra recorded from immune cells. To achieve feature reduction on the obtained set of modulated Raman spectra, PCA was applied to the dataset, where wavenumbers were considered as parameters. The first 7 PCs were selected as it accounted for 98% variance in the dataset. The ability of this training dataset to distinguish between different cell subset was assessed using binary classification based on SVM. A 'linear' kernel was used for SVM based binary classification. The accuracy of the classifier was estimated using LOOCV.

2.4 Flow-cytometry characterization of immune cell subsets

In this study, the negative depletion of peripheral blood mononuclear cells (PBMC) with monoclonal antibodies was used to obtain the untouched populations of lymphocytes and dendritic cells. The reasoning behind this is that the labelling of the target populations of cells with antibodies may add artefacts in the Raman signature, or alternatively may lead to alterations, such as partial activation of the cells under study. The purified lymphocyte subsets were characterised by flow cytometry and also tested for activities corresponding with their phenotype [10].

Thus CD4+CD3+ T lymphocytes were obtained with 96 % purity (Figure 2a). CD8+CD3+ T lymphocytes were obtained at 77% purity (Figure 2b). CD56+CD3- natural killer (NK) cells (the majority of NK cells normally possess a CD56^{low} phenotype in healthy individuals) were obtained at 80% purity (Figure 2c. CD303+CD45+ plasmacytoid (also known as lymphoid) DCs were obtained at 93% purity and CD1c+CD141+ myeloid DC were obtained at 78% purity (Figure 2d and e) [10].

Figure 2: Characterisation of purified lymphocyte subsets and dendritic cells: [a] CD3/CD4 cell surface staining of CD4+CD3+ T cell isolates; [b] CD3/CD8 cell surface staining of CD8+CD3+ T cell isolates; [c] CD3/CD56 cell surface staining of NK cell isolates; [d] CD45/CD303 cell surface staining of plasmacytoid dendritic cell isolates; [e] CD141/CD1c cell surface staining of myeloid dendritic cell isolates. [10]

3. LABEL-FREE FINGERPRINTING OF LYMPHOCYTE CELL SUBSETS

As shown in Figure 1, the lymphocytes are mononuclear cells, which can be broadly classified into three subsets, T cells, B cells, and NK cells. As the natural process of T lymphocyte maturation occurs in the thymus precursor thymocytes begin to form two distinct populations. These precursor thymocyte populations are responsible for the expression of CD4 and CD8 antigens, which are formed in conjunction with the CD3 complex, and the formation of a T cell receptor.[11]. CD4+CD3+ T cells, also known as helper T cells (Th), recognise antigens presented on major histocompatibility complex (MHC) class II molecules, and usually function by the secretion of multiple types of cytokines[12]. CD8+CD3- T cells form the cytotoxic T cell population, which recognise antigens presented on MHC class I molecules and represent the main anti-viral cell population in the adaptive immune system[13]. CD56+CD3- NK cells are also lymphoid in origin, and are also key innate anti-viral cells, often responding to the frequent down modulation of MHC class I molecules on virally infected cells[14].

Figure 3: Raman spectra and analysis of purified lymphocyte cell subsets. [a,c,e] The vertical bands show the Raman band region that shows significant difference between each class. This was estimated using Student's t-test keeping the significance level as 10^{-7} . [b,d,f] The accuracy value of binary classification of each class was obtained through leave one out cross validation of the dataset where principal component analysis was used for feature reduction followed by binary classification using support vector machine. (a) CD4+CD3+ T cells versus CD56+CD3- NK cells, (b) cluster plots showing the first two principal components for CD4+CD3+ and CD56+CD3- NK cells, (c) CD8+CD3+ T cells versus CD56+CD3- NK cells, (d) cluster plots showing the first two principal components for CD8+CD3+ and CD56+CD3- NK cells, (e) CD4+CD3+ versus CD8+CD3+ T cells, (f) cluster plots showing the first two principal components for CD4+CD3+ and CD8+CD3- T cells. [10]

Discrimination of the cell subsets within the lymphocytes, which are morphologically similar, was demonstrated using three cell subsets such as CD4+CD3+ T cells, CD8+CD3+ T cells and CD56+CD3+/- NK cells. It should be noted here that CD4+CD3+ T cells and CD8+CD3+ T belong to the same subcategory of lymphocytes (T cells). The entire single cell Raman measurements were performed on a confocal Raman system based arranged with an inverted microscope to obtain Raman signatures from the immune cell subsets. WMRS was used to suppress the contribution of the fluorescence background in the Raman signature, resulting in differential Raman spectra being obtained[15]. The Raman signal was recorded from each of the isolated immune cell subsets with an acquisition time of 25 s for an individual cell. Raman spectra were collected for 40 to 60 cells per each cell subset. A comparison of the spectra collected from each of the lymphocyte cell subsets are shown in Figure 3. The spectra shown are the average spectra obtained for each cell subset. The differential Raman bands which are significantly different between the cell subsets are highlighted with vertical bars, which was estimated using student's T test with a significance level of $p < 10^{-7}$.

A training dataset was formed for the identification of different cell subsets from the obtained Raman spectra. PCA was applied to the dataset and the first seven PCs were selected to achieve feature reduction of the training dataset. Figure 3 shows the first two PCs of each cell subset. It can be seen that each cell subset forms its own cluster, proving that there are distinct Raman fingerprints, which can be used to identify each cell subset. The discrimination efficiency of the dataset using the first 7 PCs has been verified through leave one out cross validation where support vector machine (SVM) with a 'linear' kernel was used for classifying each data point. The accuracy of the classifier for discriminating between a pair of cell subsets is shown in Figure 3. The discrimination of NK cells from CD8+CD3+ and CD4+CD3+ cells yielded an accuracy of 90% and 91% respectively. Also discrimination between CD8+CD3+ and CD4+CD3+, which are within the T cell subcategory, was obtained with 86% accuracy [10].

4. LABEL-FREE DISCRIMINATION OF DENDRITIC CELLS

This section addresses a more challenging problem where the two main categories of the DCs – pDC and mDC were discriminated. DCs, which are rare in normal blood circulation, are commonly split into myeloid and lymphoid populations. Both of which develop from a common CD34+ haematopoietic progenitor lineage and express a range of different markers when resident in tissues, and are key antigen presenting and immunoregulatory cells that influence the outcomes of infections and autoimmune diseases[16].

The same method as described in previous section was followed for the discrimination of the DCs and it was found that these cells could be successfully classified with an accuracy of 90 % [10, 17-19].

Figure 4: Raman spectra and analysis of purified dendritic cells. The vertical bands show the Raman band region that shows significant difference between each class. This was estimated using Student's t-test keeping the significance level as 10^{-7} . The accuracy value of binary classification of DCs were obtained through leave one out cross validation of the dataset where principal component analysis was used for feature reduction followed by binary classification using support vector machine. (a) CD303+CD45+ plasmacytoid DC versus CD1c+CD141+ myeloid DC, (b) cluster plots showing the first two principal components for CD303+CD45+ plasmacytoid and CD1c+CD141+ myeloid DC [10]

5. CONCLUSION

This study demonstrates the potential application of WMRS to purified immune cells to accurately identify specific cell subsets. The purification method, specifically chosen for this study generated untouched and unfixed cell populations for analysis. This allowed defining non-activated homogeneous populations, since the significant alterations associated with cell activation have been noted. Future studies with WMRS, incorporating multiple activated and non-activated cell populations will permit live *in vivo* monitoring of cell cultures undergoing cell-cell interactions during on-going immune responses. Furthermore, by using positively selected cell populations based on antibodies conjugated to beads or fluorochromes, and directly comparing these to untouched cells, a full library of defined immune cells will be possible. This ability to positively select cells will also allow us to probe further subsets of cells within those presented here. For example within the CD4+CD3+ T lymphocyte population, the ability to distinguish Th1, Th2 and Th17 cells would be of great significance in both health and disease, and may be achieved by combining WMRS with Raman-tweezing and single cell RT-PCR cytokine profiling [20].

The cells analysed in this study are essentially similar in their morphology when in the bloodstream, and would normally require external labelling when using conventional detection methodologies. The results presented in this study clearly show that WMRS is highly sensitive and can distinguish between cell types that are morphologically and chemically closely related. Thus the further development of WMRS is likely to achieve completely label-free methods to identify complex immune cell subsets.

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REFERENCES

- [1] A. Mahadevan-Jansen, and R. R. Richards-Kortum, "Raman spectroscopy for the detection of cancers and precancers," *J Biomed Opt*, 1(1), 31-70 (1996).
- [2] A. B. Zoladek, R. K. Johal, S. Garcia-Nieto et al., "Label-free molecular imaging of immunological synapses between dendritic and T cells by Raman micro-spectroscopy," *Analyst*, 135(12), 3205-3212 (2010).
- [3] Z. J. Smith, J. C. E. Wang, S. A. Quataert et al., "Integrated Raman and angular scattering microscopy reveals chemical and morphological differences between activated and nonactivated CD8+T lymphocytes," *Journal of Biomedical Optics*, 15(3), (2010).
- [4] A. Ramoji, U. Neugebauer, T. Bocklitz et al., "Toward a Spectroscopic Hemogram: Raman Spectroscopic Differentiation of the Two Most Abundant Leukocytes from Peripheral Blood," *Analytical Chemistry*, 84(12), 5335-5342 (2012).
- [5] J. Funfschilling, and D. F. Williams, "CW Laser Wavelength Modulation in Raman and Site Selection Fluorescence Spectroscopy," *Appl. Spectrosc.*, 30(4), 443-446 (1976).
- [6] C. L. Tang, and K. Levin, "Wavelength-Modulation Raman-Spectroscopy," *Journal of the Optical Society of America*, 68(5), 646-647 (1978).
- [7] B. B. Praveen, P. C. Ashok, M. Mazilu et al., "Fluorescence suppression using wavelength modulated Raman spectroscopy in fiber-probe-based tissue analysis," *Journal of Biomedical Optics*, 17(7), 077006-6 (2012).
- [8] S. Dochow, N. Bergner, C. Krafft et al., "Classification of Raman spectra of single cells with autofluorescence suppression by wavelength modulated excitation," *Analytical Methods*, (2013).
- [9] R. I. Handin, S. Lux, and T. P. Stossel, [Blood: Principles and Practice of Hematology] Lippincott Williams & Wilkins, (2003).
- [10] P. C. Ashok, B. B. Praveen, E. C. Campbell et al., "Ultra-sensitive Label-free Identification of Major Immune Cell Subsets Using Wavelength Modulated Raman Spectroscopy," Under review.

- [11] W. Ellmeier, S. Sawada, and D. R. Littman, "The regulation of CD4 and CD8 coreceptor gene expression during T cell development," *Annu Rev Immunol*, 17, 523-54 (1999).
- [12] J. Zhu, H. Yamane, and W. E. Paul, "Differentiation of effector CD4 T cell populations (*)," *Annu Rev Immunol*, 28, 445-89 (2010).
- [13] P. Wong, and E. G. Pamer, "CD8 T cell responses to infectious pathogens," *Annu Rev Immunol*, 21, 29-70 (2003).
- [14] M. B. Lodoen, and L. L. Lanier, "Viral modulation of NK cell immunity," *Nat Rev Microbiol*, 3(1), 59-69 (2005).
- [15] M. Mazilu, A. C. De Luca, A. Riches et al., "Optimal algorithm for fluorescence suppression of modulated Raman spectroscopy," *Optics Express*, 18(11), 11382-11395 (2010).
- [16] G. J. Randolph, J. Ochando, and S. Partida-Sanchez, "Migration of dendritic cell subsets and their precursors," *Annu Rev Immunol*, 26, 293-316 (2008).
- [17] M. D. Mannie, T. J. McConnell, C. Xie et al., "Activation-dependent phases of T cells distinguished by use of optical tweezers and near infrared Raman spectroscopy," *Journal of Immunological Methods*, 297(1-2), 53-60 (2005).
- [18] J. W. Chan, D. K. Lieu, T. Huser et al., "Label-Free Separation of Human Embryonic Stem Cells and Their Cardiac Derivatives Using Raman Spectroscopy," *Analytical Chemistry*, 81(4), 1324-1331 (2009).
- [19] O. Inya-Agha, T. Davies, N. Elejalde et al., "Surface enhanced Raman spectroscopy of molecular motors," *Smart Structures and Materials 2005: Smart Electronics, Mems, BioMems, and Nanotechnology*, 5763, 117-125 (2005).
- [20] A. Kelso, P. Groves, L. Ramm et al., "Single-cell analysis by RT-PCR reveals differential expression of multiple type 1 and 2 cytokine genes among cells within polarized CD4(+) T cell populations," *International Immunology*, 11(4), 617-621 (1999).