

# A review of applications of Raman spectroscopy in immunology

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**Abstract.** Vibrational spectroscopic techniques have recently gained increasing clinical importance as non-invasive, rapid and inexpensive methods to obtain information on the content of biological samples. For some time Raman spectroscopy has been involved in preclinical applications, mainly in the cancer space, with evolving applications towards new horizons in the dermatology and companion diagnostics arena. It is attractive as an analytical technique due to its exquisite sensitivity, label-free operation and low water detectivity such that *in-vivo* applications are possible. In cytometry, Raman spectroscopy has been applied to the analysis of single cells providing a label-free alternative to cell classification approaches in the laboratory. In this review we collate *in-vitro*, *ex-vivo* and *in-vivo* examples of research using Raman spectroscopy for the detection, quantification and analysis of immune signaling at the cellular level. While cancer biology has recently focussed on the role of immunological signals in the development of the disease, it is timely to examine these applications as research in this space evolves.

Keywords: Raman spectroscopy, classification, innate and acquired immune responses, cytometry, auto-immune disease

## 1. The immune system

The immune system is the body's main defense against invading foreign pathogens and the diseases they cause. This protective role in vertebrates is achieved by two main immune strategies: one is the innate immune response which provides rapid and non-specific reaction to the presence of foreign pathogens but lacks memory and the other is the adaptive immune response which is characterized by a slow, challenge-specific response and creates an immunological memory after an initial encounter with a specific pathogen. It has long been accepted that there is significant bidirectional cooperativity between the innate and adaptive immune response, with many studies describing their crosstalk and the potential to exploit this to develop therapies for various diseases [16,24,27]. In a functional immune system, the biochemical events which transduce pro-inflammatory signals from infected tissue cause the recruitment of monocytes from the bone marrow. These monocytes differentiate into phagocytic macrophages and along with dendritic cells (DCs), enter the lymph nodes. The interaction between the antigen presenting cells (APCs) such as DCs and naïve T-cells, enhances the adaptive immune response against the pathogen [53].

The discovery of Toll-like receptors (TLRs) in 1997 demonstrated the specific recognition of foreign pathogens by the innate immune system [45]. TLRs are a class of the pattern recognition receptors

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which tailor innate and adaptive responses by rapidly detecting various pathogens possessing a structurally conserved pathogen-associated molecular pattern (PAMPs), such as viral and bacterial nucleic acids, lipopolysaccharide (LPS) and damage-associated molecular patterns (DAMPs; endogenous danger signals from dead and dying cells [29]). The TLR family can recognise a wide variety of PAMP expressing bacterial, viral and fungal components and are found on various innate and adaptive immune cells [29]. Extracellular pathogens are detected by the cell surface TLRs (TLR-1, -2, -4, -5 and -6) while intracellular pathogen-derived products are detected by intracellular TLRs (TLR-3, -7, -8 and -9) [2,29,31,33,62].

Each TLR is composed of extracellular ligand binding domains, leucine-rich repeat motifs which recognise PAMPs and DAMPs and a cytoplasmic toll/IL1 receptor (TIR) domain for intracellular signal transduction [34]. Upon recognition of PAMPs and DAMPs TLR signaling pathways are finely regulated by TIR domain-containing adaptors. Differential utilisation of these TIR domain-containing adaptors provides specificity of individual TLR-mediated signalling pathways [50]. The interaction between the adaptor protein and the TLR via TIR induces downstream signalling via the NF- $\kappa$ B and the interferon (IFN) pathways, which leads to the production of pro-inflammatory cytokines and type I IFNs that ultimately protect the host from foreign pathogens [34,50] (Fig. 1).

Many reports suggest that TLRs play an important role in both innate and acquired immune responses [20,28,48], however, inappropriate activation of TLR responses triggered by ‘self’-components can result in autoimmunity [19]. In autoimmune disorders such as Rheumatoid Arthritis (RA), Systemic Lupus Erythematosus (SLE), Multiple Sclerosis (MS) and Sjögren’s syndrome (SS), the presence of autoreactive immune cell subsets along with the loss of immunological tolerance results in aberrant activation

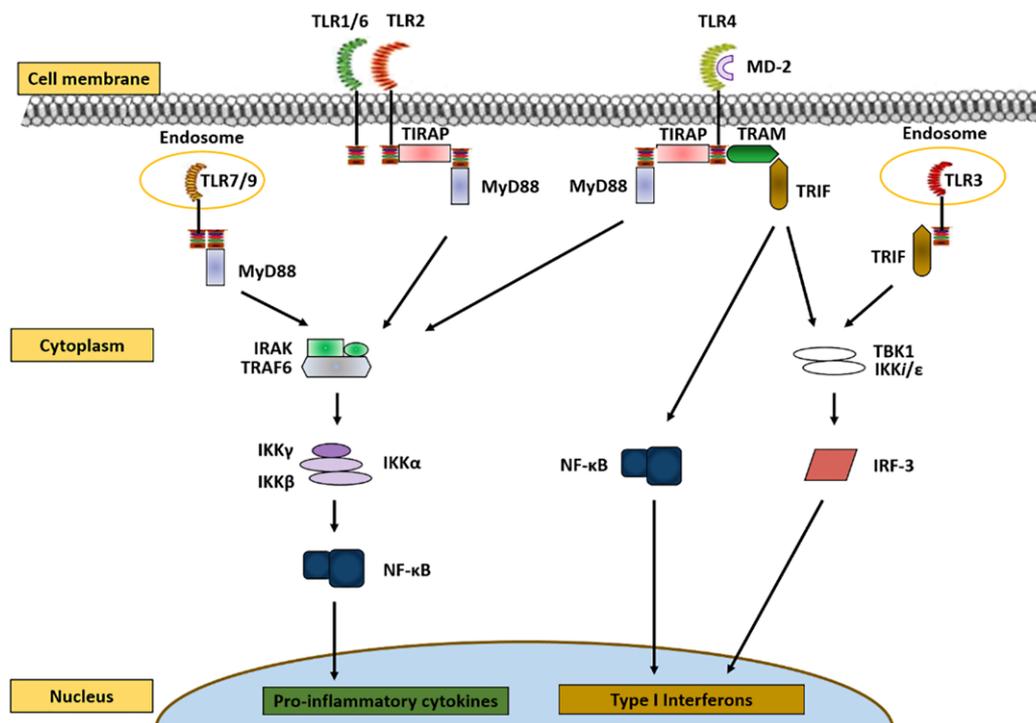


Fig. 1. Toll-like receptor signalling: there is crosstalk between different TIR domain-containing adaptors downstream of various Toll-like receptors resulting in pro-inflammatory cytokine or interferon production (adapted from [65]).

of a number of TLR pathways leading to the overproduction of proinflammatory cytokines and type I interferons which contributes to the pathology associated with these conditions [59].

### *1.1. Immune dysregulation*

The immune system's role is to defend the body against foreign pathogens through antibody and cell-mediated responses, and thus minimise the effect of foreign antigens on the immune response itself [53]. This dynamic communication has an inbuilt tendency to avoid attacking the body's own tissues and organs. At the beginning of the twentieth century, the concept of autoimmunity was first suggested by Noble laureate Paul Ehrlich who defined it as 'horror autotoxicus' [32]. Autoimmunity is the failure of the immune system to distinguish between the endogenous and exogenous antigens, and thereby its misdirection of the immune response against the body itself [21]. The breakdown mechanism responsible for self-tolerance or aberrant response of the body's immune system to endogenous-antigens leads to the pathogenesis of an autoimmune disorder [21]. Autoimmune disorders are the third-most prevalent category of disease in the industrialised world, following cardiovascular disease and cancer [15,60]. These disorders affect approximately 5–8% of the population in the United States and Europe. The dysregulation of immune response is highly prevalent in females [17,18] with environmental and genetic factors also playing a pivotal role in predisposition to the disease [17].

Each autoimmune disorder is unique in nature but the classic sign is systemic inflammation, which develops redness, swelling and pain [60]. These disorders are not restricted to one part of the body and may affect the joints, kidneys, brain, skin, muscles, connective tissues, blood vessels, and red blood cells [18,47]. Examples of inflammatory or autoimmune disorders with distinct manifestations include Common Variable Immune Deficiency (CVID), RA, MS, Fibromyalgia (FM), Antiphospholipid Syndrome, SS, SLE and Psoriasis [47]. The diagnosis of these chronic disorders is challenging due to the overlapping nature of the symptoms of each of the syndromes, with one disease often morphing into another [38,47]. Also, the mechanisms which lead to the dysregulation of immune responses are poorly understood, with diagnostic approaches often not having the capability to distinguish between autoimmune disorders [14].

## **2. Applications of Raman spectroscopy in immunology**

### *2.1. Application of Raman spectroscopy in identification and classification of immune cells*

Raman spectroscopy is a powerful analytical tool which delivers a biochemical fingerprint incorporating information regarding the molecular composition and morphology of a biological sample. As it relies on the intrinsic scattering characteristics of the molecular composition of the sample, it has the advantage that it does not require extrinsic labels such as dyes, stains or radioactive labels. Prior to its application in immunological research, Raman spectroscopy had been applied and proven in a range of disparate fields, including environmental and forensic sciences, archaeological studies, pharmaceutical detection and analysis, and semiconductor. Sophisticated applications of the technique for diagnosis of diseases including cancer [13,23,30,37,52] are now well established while novel applications to monitoring diabetes [39], detection of hyperbilirubinaemia [68], and observation of changes to the cervix during pregnancy [51] have emerged. In addition the sensitivity of Raman spectroscopy to biological effects occurring due to radiation exposure [43], radiotherapy [44] and chemotherapy [49] has also been demonstrated.

The first application of confocal Raman microspectroscopy (CRM) in the study of immune cells was the investigation of granulocytes by Puppels *et al.* [3]. They demonstrated that a CRM approach could spatially distinguish between the cytoplasm and nucleus of intact human eosinophilic granulocytes [55] and also between cell subtypes (neutrophils, eosinophils and basophils) [56]. Recent examples of the application of CRM in the differentiation of leukocytes have become more sophisticated. In one key example [11], CRM was employed with laser tweezers to distinguish normal human lymphocytes and transformed Jurkat T and Raji B lymphocyte cell lines. In this *in-vitro* and *ex-vivo* study, cultured cells of both lines were immobilised on a poly-L-lysine coated coverslip. The study discriminated transformed cells from normal cells on the basis of a significant increase in their protein and RNA features and a decrease in the cellular DNA. This work has been extended by Ramoji *et al.* [57] to develop a spectroscopic model classifying the two most abundant leukocytes (the neutrophilic granulocyte and the mononuclear lymphocyte), in healthy donors using principal components analysis (PCA) and hierarchical cluster analysis. Here a cross-validated PCA-linear discriminant analysis (LDA) model achieved an accuracy of 94% in discrimination of the two cell classes. Crucially, the model predicted the identity of unknown cells from a completely different donor with an accuracy of 81% when using single-cell Raman spectra. Similar work by Maguire *et al.* using *in-vitro* cell lines has confirmed the potential for spectroscopic differentiation of T-cells from myeloid cells with advanced machine learning approaches [40].

More detailed studies have been conducted [12] in which Raman spectra from CD4<sup>+</sup>, CD8<sup>+</sup> T-lymphocytes, CD56<sup>+</sup> natural-killer (NK) cells, myeloid dendritic cells (DCs) and plasmacytoid DCs isolated from healthy donors have been acquired. Similar analytical approaches to that employed by Ramoji *et al.* demonstrated high label free sensitivity and specificity in discriminating each cell type, and also confirmed no significant inter-donor variability between the Raman spectra of individual cell subsets [12]. Hobro *et al.* [26] have advanced this work further by successfully employing CRM for the discrimination of B- and T-lymphocytes. Automated systems have also been developed [42] which use CRM with Digital Holographic Microscopy (DHM) for distinguishing leukocyte subtypes (CD4<sup>+</sup> T cell, B cell and monocytes) isolated from the blood samples of a healthy cohort. These coupled approaches increase the sensitivity and specificity of the classification of each cell sub-type obtained by using CRM or DHM alone. Managò *et al.* [41] have extended this work via the development of CRM models for classification of leukocyte subpopulations (B, T and NK cells) with monocytes and granulocytes. Importantly they also demonstrated that CRM could also detect dose dependent effects (seen as a gradual decrease of nucleic acids and protein signal) in a MN60 B leukaemia cell line treated with methotrexate [41].

Smith *et al.* [64] have focused on analysing stimulus-dependent modifications in the Raman and elastic-scattering signals of activated CD8<sup>+</sup> T lymphocytes relative to their resting counterparts. In this work CD8<sup>+</sup> T cells were stimulated with either staphylococcal enterotoxin B (SEB) or phorbol myristate acetate (PMA) with rapid CRM spectral acquisition to avoid a decrease in cell viability. The Raman signatures showed a significant decrease in nucleic acid in SEB-mediated activation while lipid and protein features predominated in PMA-mediated activation. He *et al.* [25] have coupled CRM and imaging with atomic force microscopy (AFM) to demonstrate that T-cell receptor (TCR) mediated signal transduction is more effective than protein tyrosine kinase signal transduction to activate human T-lymphocytes. T-cells activated via the TCR signalling pathway exhibited increased variability in their diameter, while CRM signatures suggested the conformational modification of TCR secondary structure and a significant decrease in nucleic acid. Brown *et al.* have also examined T-cells stimulated with CD3/CD28 as a model of allogeneic activation to demonstrate the use of Raman spectroscopy in screening for renal

transplant rejection, demonstrating high specificity in this *ex-vivo* model [4,5]. A recent macrophage activation study [54] provided further insight into the LPS induced spectral changes in a macrophage-like cell line (RAW264), where morphological and molecular information through quantitative phase imaging and CRM were acquired. A time-course study [66] has also used CRM for the characterisation of LPS induced modification in a monocytic THP-1 cell line over a 16 hour timeframe, detecting significant changes in cellular DNA, mRNA, lipid and protein structure over this period.

## 2.2. Clinical applications of Raman spectroscopy in the diagnosis & discrimination of autoimmune disorders

CRM, and other optical and spectroscopic techniques, have demonstrated potential in the early diagnosis and classification of various autoimmune disorders [22,58]. In 2003, Canvin *et al.* [7] examined the synovium of the small finger joints in patients with both early stage and late stage RA using near-infrared spectroscopy. In this study, spectra were acquired from 28 early stage RA patients and 25 late stage RA patients via near infrared light directed through a fibre-optic probe to target joints on the hand, while the wavelengths of light absorbed by different structures of the joint were measured. A PCA-LDA model demonstrated that the absorption features of water, cytochrome and haemoglobin species, suggesting that a modification in oxidative status and water content within the joint are involved in the transition from early to late RA. Also, this LDA model could distinguish early from late RA with greater than 70% sensitivity.

Xue *et al.* [67] have demonstrated the potential of CRM to replace the lip biopsy in the diagnosis of SS. In that study minor salivary gland specimens from SS patients were differentiated from normal specimens. In a less invasive approach, Carvalho *et al.* [9] explored the use of CRM for diagnosing RA using the sera obtained from 24 RA patients and 16 healthy volunteers. The Raman spectra of RA showed significantly higher protein, amine, lipid and immunoglobulin features relative to the healthy individuals ( $p$ -value < 0.05). In this comparative study, the number of correctly identified patients was found to be superior to traditional clinical tests that monitoring C-reactive protein and rheumatoid factor. Further studies have confirmed that sera from healthy donors ( $n = 39$ ) and RA patients ( $n = 39$ ) contain spectral information that is useful for the diagnosis of RA [10].

Fourier Transform Infrared (FTIR) spectroscopy has also began to advance in this space. Carvalho *et al.* [8] further observed that RA patients ( $n = 47$ ) had elevated levels of IgM and albumin in serum in comparison to the healthy individuals ( $n = 47$ ) using FTIR spectroscopy. Another study by Lechowicz *et al.* [35] has confirmed that FTIR spectroscopy can discriminate serum from RA and healthy donors using a similar approach. Recently, Yonar *et al.* [69] have also used FTIR with cerebrospinal fluid for analysing the different progression patterns of MS. Microparticles (MPs) are membrane vesicles released from cells in response to activation and apoptotic processes. Altered levels of MPs are observed in RA, SLE and many other systemic inflammatory conditions [46,61,63].

FTIR spectroscopy has also been used to characterise MPs released upon immune activation, with one study validating its use as a quick method to investigate relative differences in biomolecular content of different MP populations that was complementary to traditional semi-quantitative omics approaches [36]. Using a LPS-induced monocyte model, Lee *et al.* used a FTIR approach to demonstrate differences in phosphatidylcholine and phosphatidylserine content of LPS stimulated MPs compared with MPs released from resting cells. LPS-stimulated monocytes had reduced concentrations of nucleic acids,  $\alpha$ -helical structured proteins, and phosphatidylcholine compared with resting monocytes but had an increase in total lipids. Autoimmune diseases affect about 20% of CVID patients and are often the first

manifestation of immune deficiency [1]. Diagnosis of CVID can take up to five years or more because of the lack of pathognomonic clinical or laboratory features. Recently FTIR has revealed spectral features capable of stratifying CVID patients from healthy controls with sensitivities and specificities of 97% and 93%, respectively for serum, and 94% and 95%, respectively for plasma [6]. In this study discriminating spectral biomarkers were observed in regions indicative of nucleic acids and a collagen-associated biomarker. To date, there are no reliable biomarkers for the diagnosis of FM, a chronic condition which is characterised by widespread musculoskeletal pain, mood alterations, memory loss and fatigue. A recent study demonstrated the potential of vibrational spectroscopy to differentiate patients with FM from those with RA, osteoarthritis (OA), or SLE [22]. Blood sample spectra were collected from patients with a diagnosis of FM ( $n = 50$ ), RA ( $n = 29$ ), OA ( $n = 19$ ), or SLE ( $n = 23$ ) using portable FTIR and FT-Raman microspectroscopy. Pattern recognition analysis allowed the clustering of all study participants into classes (FM, RA and SLE) with no misclassification. The spectra were also shown to correlate with FM pain severity. Taken together, these reports suggest that vibrational spectroscopy may provide a reliable diagnostic testing approach for differentiating inflammatory and autoimmune conditions with similar presentations and pathology.

### 3. Conclusion

This review has highlighted a range of developing applications of Raman spectroscopy in the immunology space where the evolution of the technique towards a potential spectroscopic approach to automated segregation and characterisation of immune cells, immune signalling and disorders of the immune system is gathering momentum. On the basis of the review, it is clear that this ultimate goal may be achieved with cytological and biofluid samples through the use of Raman spectroscopy with other microscopic and nanoscopic imaging techniques. In the long-term, the identification of the molecular species giving rise to the spectral demarcation between patient classes will be a key advance. In this context, the area is set to remain a fertile one for exploration into the future.

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