Technological University Dublin
ARROW@TU Dublin

Conference Papers

School of Physics, Clinical and Optometric Science

2023

# In-Vitro Screening of Immune Response with FTIR Spectroscopy in a miRNA Murine Knock Out Model

Ryan Muddiman
*Technological University Dublin, Ireland*, ryan.muddiman@tudublin.ie

Abigail Keogan
*Technological University Dublin, Ireland*, abigail.keogan@tudublin.ie

Daniel Cullen
*Technological University Dublin, Ireland*, Daniel.Cullen@TUDublin.ie

*See next page for additional authors*

Follow this and additional works at: https://arrow.tudublin.ie/scschphycon

 Part of the Medicine and Health Sciences Commons

## Recommended Citation

Muddiman, Ryan; Keogan, Abigail; Cullen, Daniel; Afzal, Remsha; Nally, Frances; McCoy, Claire; and Meade, Aidan, "In-Vitro Screening of Immune Response with FTIR Spectroscopy in a miRNA Murine Knock Out Model" (2023). *Conference Papers*. 34.
https://arrow.tudublin.ie/scschphycon/34

This Conference Paper is brought to you for free and open access by the School of Physics, Clinical and Optometric Science at ARROW@TU Dublin. It has been accepted for inclusion in Conference Papers by an authorized administrator of ARROW@TU Dublin. For more information, please contact arrow.admin@tudublin.ie, aisling.coyne@tudublin.ie, vera.kilshaw@tudublin.ie.

This work is licensed under a Creative Commons Attribution-Share Alike 4.0 International License.
Funder: This work was financially supported in part by Science Foundation Ireland Grant Agreements #13/RC/2106-P2 and 19/FFP/6772 at the ADAPT SFI Research Centre at TU Dublin. The ADAPT SFI Centre for Digital Media Technology is funded by Science Foundation Ireland through the SFI Research Centres Programme and is co-funded under the European Regional Development Fund (ERDF).

## Authors

Ryan Muddiman, Abigail Keogan, Daniel Cullen, Remsha Afzal, Frances Nally, Claire McCoy, and Aidan Meade

This conference paper is available at ARROW@TU Dublin: https://arrow.tudublin.ie/scschphycon/34

# In-vitro screening of immune response with FTIR spectroscopy in a miRNA murine knock out model

Ryan Muddiman[1, 2], Abigail Keogan[1, 2], Daniel Cullen[1,2], Remsha Afzal[3], Frances Nally[3], Claire McCoy[3], Aidan D. Meade[1,2]

1. Centre for Radiation and Environmental Science, FOCAS Research Institute, Technological University Dublin, Ireland.
2. School of Physics, Clinical & Optometric Sciences, Central Quad, Technological University Dublin, City Campus, Grangegorman, Ireland
3. School of Pharmacy and Biomolecular Sciences, Royal College of Surgeons in Ireland, 123 St. Stephen's Green, Dublin 2, Ireland

Email: aidan.meade@tudublin.ie

**ABSTRACT**

MicroRNAs are small ~22 nucleotide RNA sequences that are guided to the 3' untranslated region (UTR) of protein-coding target mRNA sequences. One particular microRNA, miR-155, plays a remarkable role in the immune system, where it is essential for mounting appropriate immune responses. However, its dysregulation has been identified in multiple inflammatory disorders such as Multiple Sclerosis (MS), arthritis, psoriasis and colitis. More specifically, miR-155 has been found to be elevated in the serum and brain lesions of MS patients. Importantly, therapeutic inhibition of miR-155 can inhibit progression of the MS disease model. One of us has identified that macrophages are major contributor to miR-155 elevation in the MS disease model, whilst its inhibition specifically in macrophages can limit the disease.

Here macrophages were isolated from the femur and tibia of wild-type (WT) mice and mice with a knock-out (KO) of the gene regulating miR-155 production, and were cultured *in*-vitro and stimulated with lipopolysaccharide (LPS) to simulate an immune response. Cells were then prepared for spectral analysis by FTIR imaging with a Perkin-Elmer Spotlight 400 imaging microscope. After pre-processing the dimensionality of spectra were reduced using principal components analysis and classified using a support vector machine algorithm, delivering a classification performance approaching F1~0.89. Spectral features differentiating WT and KO classes were observed across the fingerprint region with no single spectral marker being the sole source of differentiation of the downstream molecular events. This study exemplifies the challenge in spectral discrimination of the complexity of molecular events in *ex-vivo* models of immune dysregulation.

**Keywords:** Multiple Sclerosis (MS), Fourier Transform Infrared (FTIR), micro-RNA (miRNA), principal components analysis (PCA), support vector machine (SVM).

## 1. INTRODUCTION

Biophotonic techniques are now widely used in biomedical research aiming to improve diagnosis, prognosis and surveillance of disease. Vibrational spectroscopy techniques like Fourier-transform infrared (FTIR) and Raman spectroscopy are used to observe the intramolecular vibrations and rotations of a sample when irradiated[1].

Despite their extensive use in cancer research[2], [3], FTIR and Raman spectroscopy have not been heavily utilized in the study of diseases which affect the central nervous system (CNS), such as multiple sclerosis (MS)[4][5].

MS is considered an autoimmune, neuro-inflammatory and degenerative condition, affecting the brain and spinal cord. Its exact etiology remains unclear, but genetic and environmental factors both contribute to an individual's susceptibility to develop MS[6]. The diagnosis of MS is usually based on the clinical presentation and the results of brain and spinal MRI, which reveals evidence of active and chronic lesions as well as focal and generalized atrophy[7].

This study was a pilot to determine the potential for the use of FTIR spectroscopy in the detection of an innate immune response to infection. A well-characterized murine model was utilized in which the gene encoding micro-RNA (miRNA) 155 was knocked out (miR155$^{-/-}$) [8], [9], and compared their macrophage responses to infection through *ex-vivo* stimulation with lipopolysaccharide (LPS), with reference to the response seen in wild-type macrophages (C57BL6/J). Our results suggest that spectroscopic profiling of macrophages with FTIR is insufficient alone as a diagnostic for MS in practice.

# 2. METHODS

## 2.1 Sample preparation

The overall experimental design is depicted in Figure 1. All animals were purchased from the Jackson Laboratories (Bar Harbor, Maine). Breeding of mice was approved by the Research Ethics Committee of the Royal College of Surgeons in lreland (REC-842) under licensing from the Health Products Regulatory Authority, Ireland (AE19127/001) and conforming to Directive 2010/63/EU of the European Parliament. All mice were bred and housed as described elsewhere[10].

For the isolation and differentiation of macrophages, bone marrow was isolated from 6-12 week old adult female littermates. Femurs and tibias were isolated in sterile conditions, and the bone marrow was flushed out using Dulbecco's Phosphate Buffered Saline (DPBS). Marrow was spun and incubated with red blood cell (RBC) lysis buffer (Sigma) to remove red blood cells. A single cell suspension was prepared by passing the cells through a 70 μm cell strainer (Corning).

Cells were then plated in 10 cm petri dishes in complete DMEM (Dulbecco's Modified Eagle Medium, Sigma-D5796) supplemented with 10% heat-inactivated Fetal Bovine Serum (FBS) (Sigma-F9665) and 1% Penicillin/Streptomycin (100 U/mL) (Sigma). A cell supernatant (20% L929) was also added to the culture to induce BMDM differentiation, after which cells were incubated for 6 days at 37 °C and 5% $CO_2$ levels. Cells were counted and tested for viability using Trypan Blue staining (Invitrogen) and a haemocytometer. Cells were then re-plated for experiments at $1x10^6$ cells/mL in complete DMEM supplemented with 10% L929 cell supernatant and the next day were either left untreated or stimulated with LPS (Sigma, E.*coli* O111:B4) at 100 ng/ml for 24 hours.

Cells were then rinsed with DPBS and scraped using a cell scraper, centrifuged at 1500 rpm for 5 min in DPBS. Any supernatant was removed and the cells were resuspended in 2% v/v paraformaldehyde (PFA) fixing solution (40 μl per sample). A total of 20 μL of each cell suspension sample was deposited onto a $CaF_2$ slide (Crystran, U.K.). The cells were fixed for 10 min at room temperature in a fume hood. Any excess fixing solution was removed using a pipette carefully. A drop of $dH_2O$ was added to each sample dose avoiding adding it directly onto the cells. After 5 minutes, the $dH_2O$ was removed and the step was repeated 2 more times. The samples were then allowed to air dry. The slides were stored in a desiccator at room temperature until needed.

## 2.2 Spectroscopic Acquisition

FTIR spectroscopy was performed using a Perkin Elmer Spotlight 400 imaging spectrometer (Perkin Elmer Inc.). Spectra were recorded over the wavenumber range from 900cm$^{-1}$ to 4000cm$^{-1}$ in imaging mode over a single 100μm × 100μm window using 8cm$^{-1}$ wavenumber resolution, 6.26 μm$^2$ spatial resolution and 16 scans per pixel. A single image was recorded for each replicate, mouse type and treatment, with spectra extracted from the imaging hypercube for pre-processing and analysis. In all 1600 spectra were acquired for each replicate, mouse and treatment, with a total of approximately 57600 FTIR spectra acquired for the study.

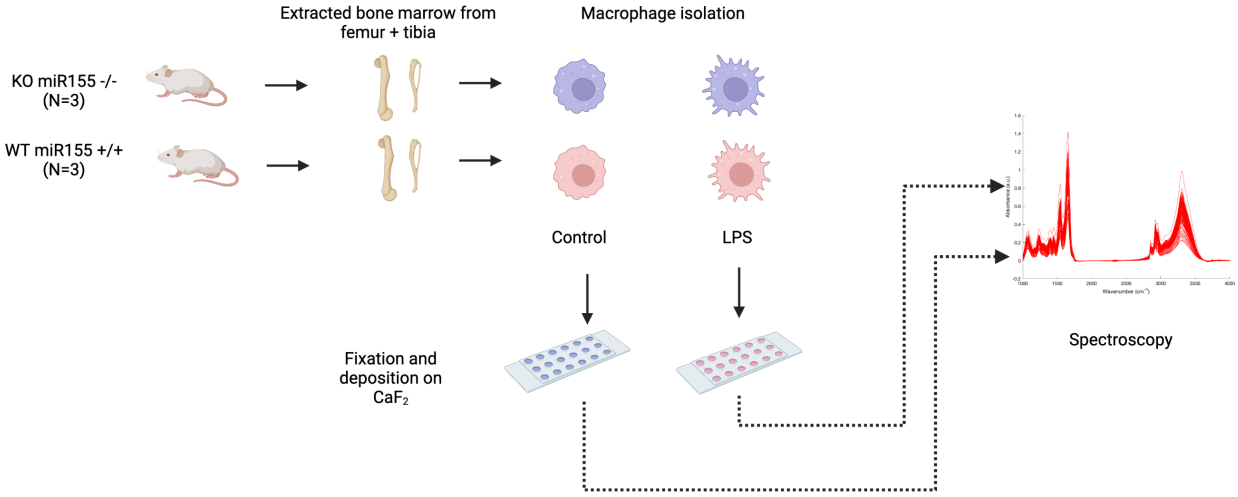


Figure 1. Overall study design. Created with BioRender.com

### 2.3 Spectral pre-processing and analysis

FTIR spectra were initially extracted from the manufacturers .fsm format using Matlab (R2022b, Mathworks, U.K. Ltd.) with all subsequent processing and analysis taking place in Python (v. 3.10.9) with scikit-learn (v.1.2.2). All subsequent spectral pre-processing steps (removal of atmospheric contributions, baseline, vector normalization) were conducted in Python using the OCTAVVS toolbox, as described previously [11].

For analysis all spectra were pooled, such that spectra were classified into 4 classes: wild-type control (WT CTRL), wild-type control with LPS (WT LPS), knock-out control (KO CTRL) and knock-out with LPS (KO LPS).

Classification of spectra utilised a dimensionality reduction followed by classification, with training of the algorithms using a leave-one-replicate-out cross-validation approach and classification performance being reported as the average of the performance over all CV folds. Dimensionality reduction was implemented using principal component analysis (PCA) and classification of spectra utilised a support vector machine (SVC) approach, as described previously. This process was implemented using the pipeline tool in scikit-learn with the SVC algorithm utilising a radial basis function kernel. Within this the optimal set of classification hyperparameters were determined using a grid search approach. The set of hyperparameters which were iterated over included the number of principal components (which varied from 1 to 30), the regularization parameter C (with values of 0.001, 0.01, 0.1 and 1) and the kernel coefficient, gamma (with settings of 'auto' (i.e. the inverse of the number of features, and 'scale' (i.e. the inverse of the product of the number of features and number of variables).

## 3. RESULTS AND DISCUSSION

Figure 2. depicts the mean spectra by class for each of the experimental classes. Visually there are few spectral locations where substantial spectral variation between classes is seen.

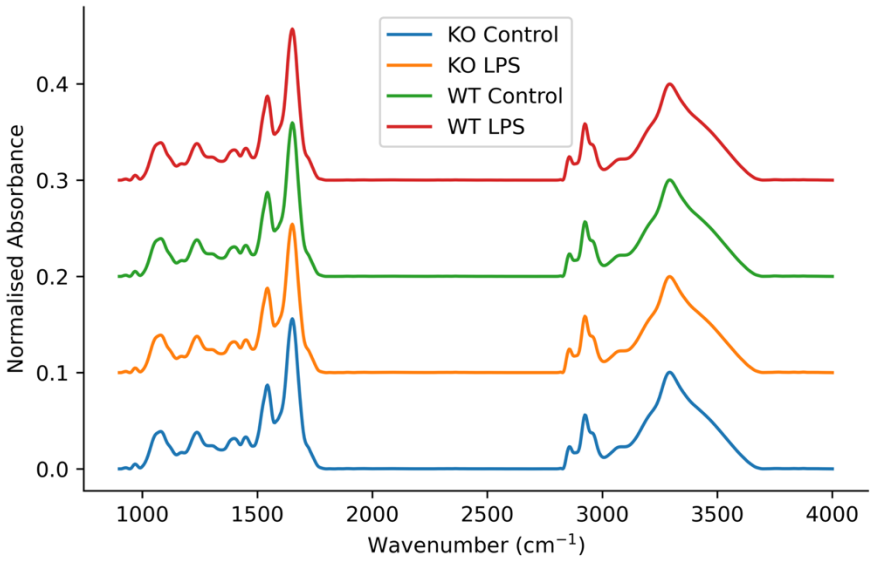


Figure 2. Mean spectra by cell class. Spectra are offset arbitrarily along the y-axis for visual clarity.

In Figure 3 the annotated PC loadings are presented together with a plot of the cumulative variance described by each PC. It is clear that there is substantial spectral variance within the dataset, such that the PC model requires upwards of 11 PCs to describe >95% of the variance within the dataset. However the PC loadings do allow a picture to emerge regarding the spectral origin of the variance within the dataset [12]. Firstly loadings at 1064 and 1060 are associated with the C-O stretching vibration and at 1232 cm$^{-1}$ are associated with the asymmetric stretching vibration of -$PO_2^-$ of nucleic acids (DNA/RNA). A series of loadings associated with vibrations of protein are seen at 1524, 1532 and 1544 cm$^{-1}$ (Amide II), 1628 and 1644 cm$^{-1}$ (Amide I), 3288 and 3296 cm$^{-1}$ (Amide A), while another set at 1460, 2852, 2920, 2924 and 2860 are associated with the bending and both symmetrical and asymmetrical stretching vibrations of -$CH_2$ moieties in lipid and protein. Loading to vibration of lipid moieties are also seen at 1072 cm$^{-1}$ (asymmetric and symmetric stretching vibrations of -CO-O-C) and 1728 cm$^{-1}$ (symmetric stretching vibrations of C=O in esters). Further loadings at 1012cm$^{-1}$ are tentatively assigned to C=C bending vibrations in alkene moieties while those at 1496 and 1600 cm$^{-1}$ are assigned to N-O stretching vibrations. This in totality suggests a complex series of molecular events differentiating the spectral response in cells exposed to an immunological stimulus in both wild-type and miR-155 deficient model systems.

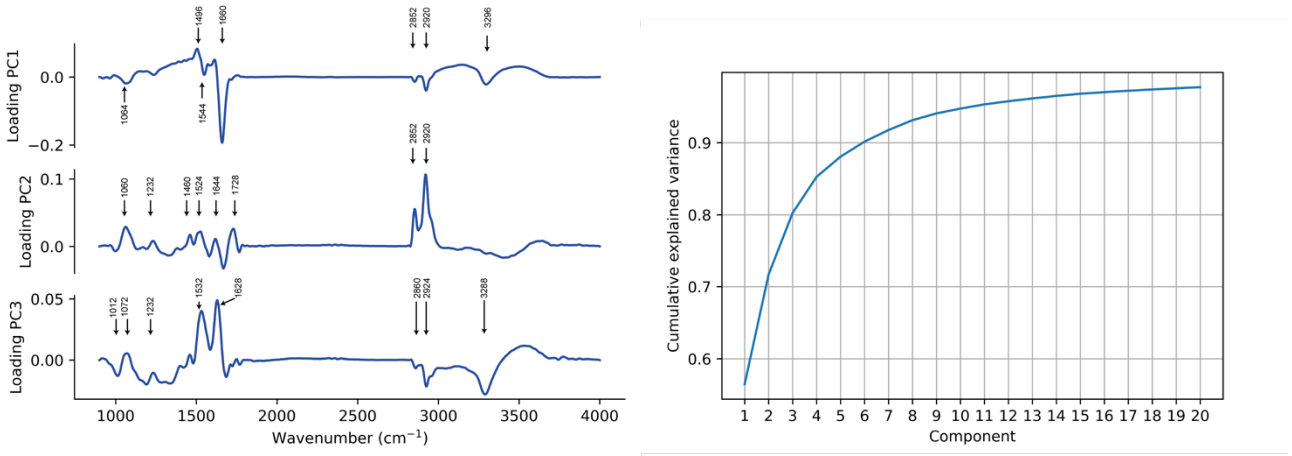


Figure 3. Left - Principal Component loadings on the first three PCs (describing, respectively 56%, 72% and 80% of the cumulative variance in the data). Right – scree plot of cumulative variance described in the first 20 PCs.

Further reinforcement of this picture emerges from a plot of scores of the first 3 PCs as shown in figure 4(a), where a significant degree of overlap is seen between the scores from each of the 3 PCs. In figure 4(b) the performance PCA-SVM models with variation in model complexity and hyperparameter setting is shown. These models achieved a maximum classification performance of an F1-score of ~0.89, although requiring upwards of 25 PCs to achieve this level of performance, exemplifying the complexity of the molecular response in this model system, from a spectroscopic perspective.

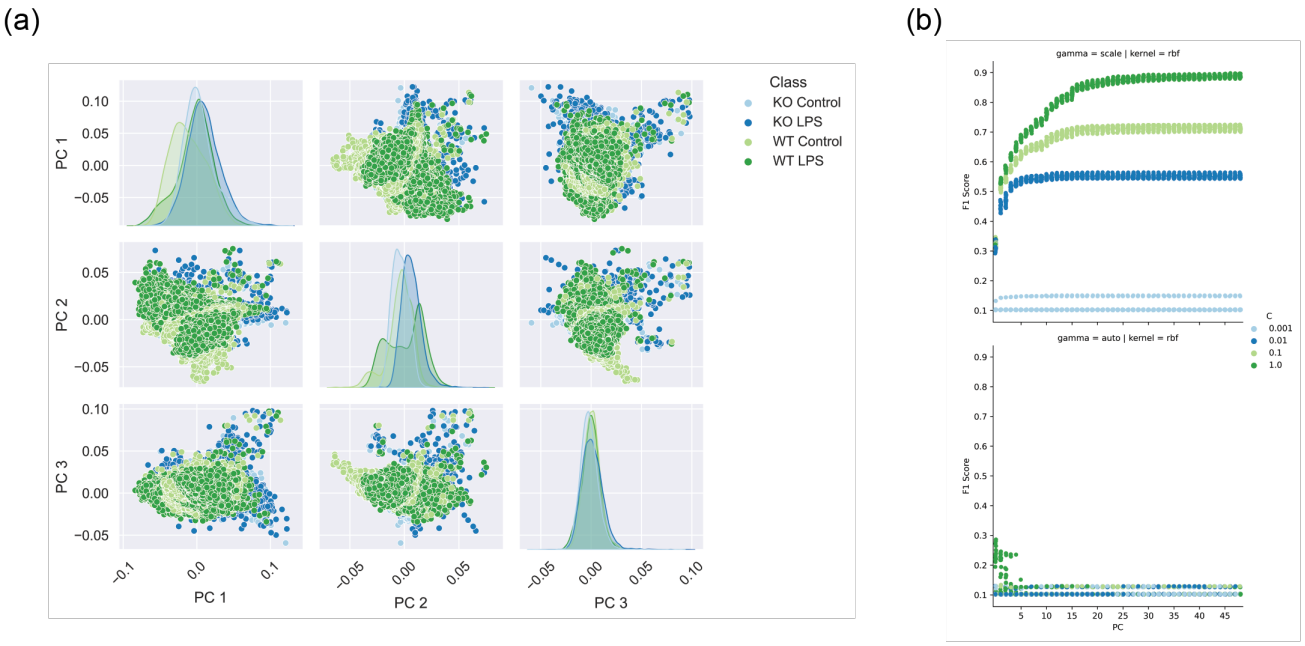


Figure 4 (a). Scores plots for the first 3 components in a PCA model of the FTIR spectral data in this study; 4 (b). Variation in performance of a PCA-SVM classification model with SVC hyperparameter and model complexity.

## 4. CONCLUSIONS

This study sought to determine whether spectral analysis with FTIR could differentiate the immune response in a mouse model of multiple sclerosis. While application of PCA-based spectral decomposition coupled to a support vector machine could deliver classification performances in the region of F1~0.89, very high model complexities were required achieve this level of performance. Therefore, we conclude that IR spectral data alone, even when coupled to powerful linear and decomposition methods and non-linear classification approaches, is not capable of disentangling the complexity of this molecular process without guidance from parallel biological measurements.

## 5. ACKNOWLEDGEMENTS

This work was financially supported in part by Science Foundation Ireland Grant Agreements #13/RC/2106-P2 and 19/FFP/6772 at the ADAPT SFI Research Centre at TU Dublin. The ADAPT SFI Centre for Digital Media Technology is funded by Science Foundation Ireland through the SFI Research Centres Programme and is co-funded under the European Regional Development Fund (ERDF).

## REFERENCES


[1] P. Lasch and J. Kneipp, *Biomedical Vibrational Spectroscopy*. John Wiley & Sons, 2008.

[2] A. L. Mitchell, K. B. Gajjar, G. Theophilou, F. L. Martin, and P. L. Martin-Hirsch, "Vibrational spectroscopy of biofluids for disease screening or diagnosis: translation from the laboratory to a clinical setting," *J. Biophotonics*, vol. 7, no. 3–4, pp. 153–165, 2014, doi: 10.1002/JBIO.201400018.

[3] M. Diem *et al.*, "Molecular pathology via IR and Raman spectral imaging," *Journal of Biophotonics*, vol. 6, no. 11–12. pp. 855–886, Dec. 2013, doi: 10.1002/jbio.201300131.

[4] S. Caine, P. Heraud, M. J. Tobin, D. McNaughton, and C. C. A. Bernard, "The application of Fourier transform infrared microspectroscopy for the study of diseased central nervous system tissue," *Neuroimage*, vol. 59, no. 4, pp. 3624–3640, Feb. 2012, doi: 10.1016/J.NEUROIMAGE.2011.11.033.

[5] I. R. Ramos, F. M. Lyng, I. U. Rehman, B. Sharrack, and M. N. Woodroofe, "The use of vibrational spectroscopy to study the pathogenesis multiple sclerosis and other neurological conditions," *Appl. Spectrosc. Rev.*, vol. 52, no. 10, pp. 868–882, 2017, doi: 10.1080/05704928.2017.1336450.

[6] S. Sawcer *et al.*, "Genetic risk and a primary role for cell-mediated immune mechanisms in multiple sclerosis," *Nature*, vol. 476, no. 7359, pp. 214–219, Aug. 2011, doi: 10.1038/NATURE10251.

[7] M. P. Wattjes *et al.*, "Evidence-based guidelines: MAGNIMS consensus guidelines on the use of MRI in multiple sclerosis--establishing disease prognosis and monitoring patients," *Nat. Rev. Neurol.*, vol. 11, no. 10, pp. 597–606, Oct. 2015, doi: 10.1038/NRNEUROL.2015.157.

[8] C. E. McCoy, "miR-155 Dysregulation and Therapeutic Intervention in Multiple Sclerosis," *Adv. Exp. Med. Biol.*, vol. 1024, pp. 111–131, 2017, doi: 10.1007/978-981-10-5987-2_5.

[9] C. E. McCoy *et al.*, "IL-10 inhibits miR-155 induction by toll-like receptors," *J. Biol. Chem.*, vol. 285, no. 27, pp. 20492–20498, Jul. 2010, doi: 10.1074/JBC.M110.102111.

[10] J. K. Dowling *et al.*, "Mitochondrial arginase-2 is essential for IL-10 metabolic reprogramming of inflammatory macrophages," *Nat. Commun. 2021 121*, vol. 12, no. 1, pp. 1–14, Mar. 2021, doi: 10.1038/s41467-021-21617-2.

[11] C. Slattery *et al.*, "Application of Advanced Non-Linear Spectral Decomposition and Regression Methods for Spectroscopic Analysis of Targeted and Non-Targeted Irradiation Effects in an In-Vitro Model," *Int. J. Mol. Sci.*, vol. 23, no. 21, Nov. 2022, doi: 10.3390/IJMS232112986.

[12] A. D. Meade, F. M. Lyng, P. Knief, and H. J. Byrne, "Growth substrate induced functional changes elucidated by FTIR and Raman spectroscopy in in-vitro cultured human keratinocytes," *Anal. Bioanal. Chem.*, vol. 387, no. 5, 2007, doi: 10.1007/s00216-006-0876-5.