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Portable Microfluidic Raman System for Rapid, Label-free Early Disease Signature Detection

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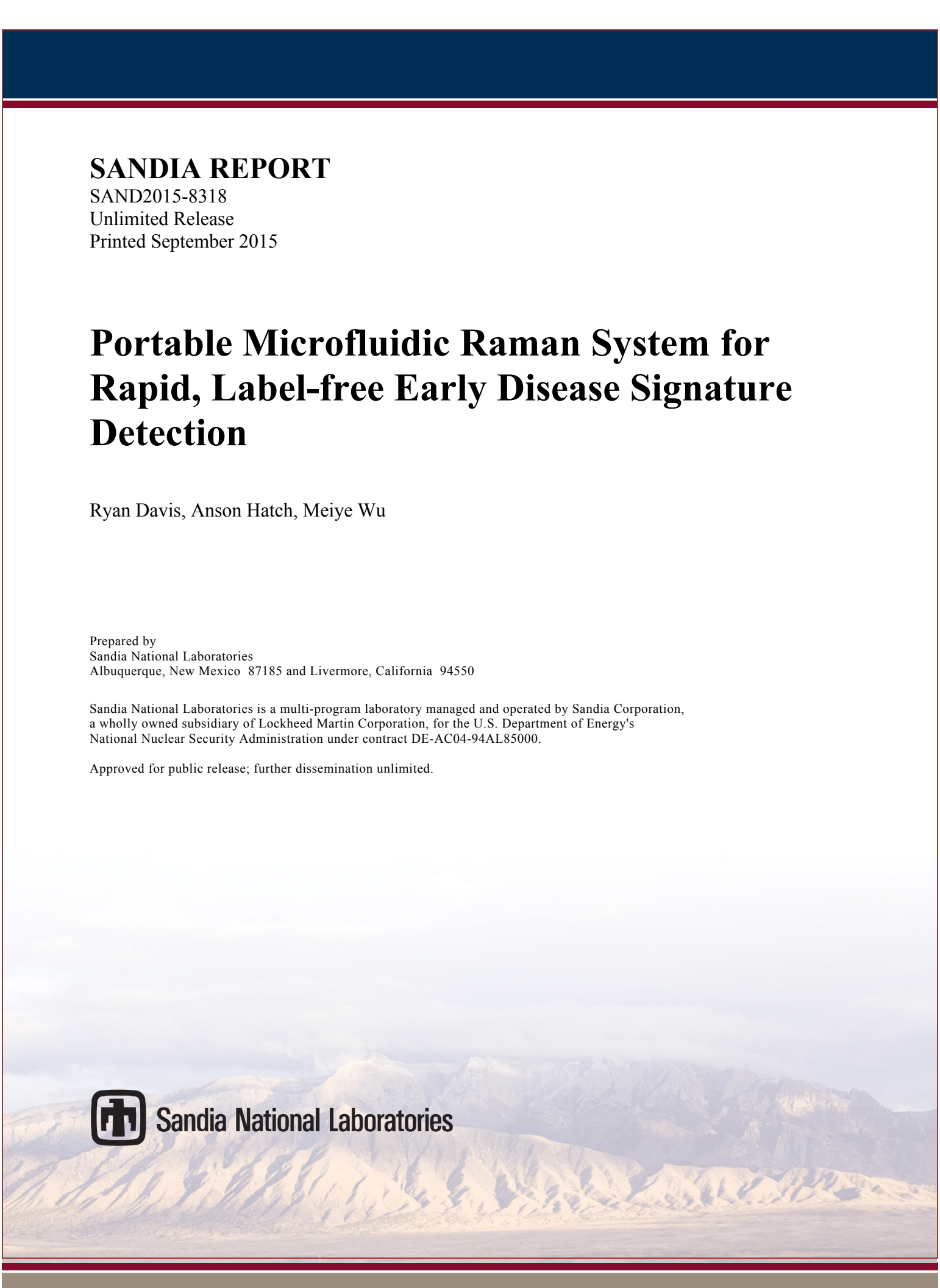

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Portable Microfluidic Raman System for Rapid, Label-free Early Disease Signature Detection

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Abstract

In the early stages of infection, patients develop non-specific or no symptoms at all. While waiting for identification of the infectious agent, precious window of opportunity for early intervention is lost. The standard diagnostics require affinity reagents and sufficient pathogen titers to reach the limit of detection. In the event of a disease outbreak, triaging the at-risk population rapidly and reliably for quarantine and countermeasure is more important than the identification of the pathogen by name. To expand Sandia's portfolio of Biological threat management capabilities, we will utilize Raman spectrometry to analyze immune subsets in whole blood to rapidly distinguish infected from non-infected, and bacterial from viral infection, for the purpose of triage during an emergency outbreak. The goal of this one year LDRD is to determine whether Raman spectroscopy can provide label-free detection of early disease signatures, and define a miniaturized Raman detection system meeting requirements for low-resource settings.

ACKNOWLEDGMENTS

The authors thank Dr. Paula Imbro for programmatic guidance and encouragement. We also thank Ariana Chin, an undergraduate summer intern for her data collection efforts.

CONTENTS

FIGURES

NOMENCLATURE

DOE	Department of Energy
DOD	Department of Defense
DHS	Department of Homeland Security
DTRA	Defense Threat Reduction Agency
SNL	Sandia National Laboratories
CDC	Center for Disease Control
NIAID	National Institute of Allergy and Infectious Diseases
NIH	National Institute of Healthy
DC	Dendritic cells
pDC	plasmacytoid dendritic cell
mDC	myeloid dendritic cell
TLR	Toll-like receptor
PBMC	peripheral blood mononuclear cells
PCR	polymerase chain reaction
SERS	surface enhanced Raman spectroscopy
FC	flow cytometry
IFNα	interferon alpha
TNFα	tumor necrosis factor alpha
Bfa	Brefeldin A
PFA	paraformaldehyde
PBS	phosphate buffered saline
DI	deionized

1. INTRODUCTION

1.1. Why label free diagnostics is needed

Early accurate diagnostics is essential for mounting rapid and effective response against bioterror attacks or emerging infectious disease outbreaks. For biodefense field applications, an ideal diagnostic device is a portable system that can be deployed in low resource settings, with minimal operator intervention. One possible candidate for the ideal portable infection detector is a portable Raman spectroscope system. Raman spectroscopy exploits the inelastic vibrational scattering of monochromatic light as it passes through a sample. It is a label-free, non-destructive detection method that can be used to monitor the chemical composition of liquid and solid biological samples. In the past two decades, Raman spectroscopy has seen wide usage in biomedicine, mostly as non-destructive quality assurance tests for pharmaceutical compounds and biological samples, with some initial studies using Raman spectroscopy to detect cellular changes in cancer and development***(3-5)***. The biggest advantage of a Raman based diagnostic device is that it will eliminate the need for affinity reagents, and provide access to an earlier diagnostic window currently unattainable using affinity based diagnostic tests (figure 1).

Upon initial exposure to pathogens, there is an incubation period where the body's innate immune system senses pathogen and responds by mounting a non-specific response, followed by initiation of adaptive immune responses several days later that ultimately leads to pathogen-specific antibody production. The current state-of-the-art diagnostic tests are affinity based tests (PCR or antibody) that detect pathogen sequences or proteins from clinical samples taken after onset of symptoms. While the affinity based tests perform superbly under ideal laboratory settings, they have two major shortcomings. First, PCR polymerase and antibodies requires cold storage and transport, the logistics of which has long been an issue for field PCR testing. Two, newly emergent pathogens mutate rapidly, often circumventing detecting by PCR or antibody due to the changes in the pathogen genome, producing inconclusive or false negative results, especially at titers near the limit of detection.

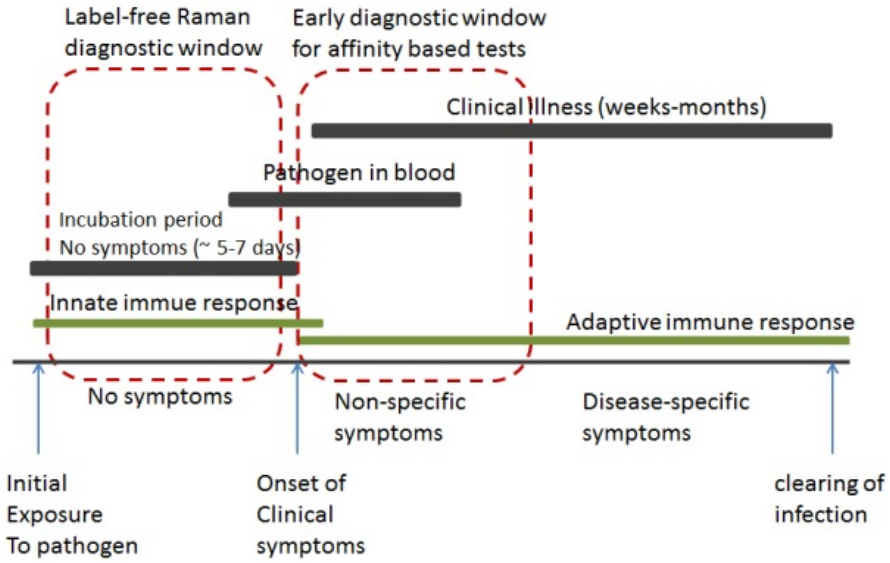

Figure 1. Timeline of infection by unknown pathogen. The proposed Raman based method focuses on label-free monitoring of the innate immune response to access the earliest possible diagnostic window.

1.2. Technical approach

In order to access the very earliest diagnostic window, immediately after initial exposure (figure 1), one will need to monitor the host innate immune responses prior to onset of symptoms. Of the innate immune sentry cells that respond to pathogens are dendritic cells (DCs). DCs represent an especially attractive target for early diagnostics because DCs act as sentry cells that detect and mount initial responses to pathogens, and link the innate to the adaptive immune system by activating T cells and presenting antigens. While the innate immune response shows no specificity towards any pathogen in particular, it does have distinct responses to different classes of pathogens. Peripheral blood DCs can be divided roughly into two categories- plasmacytoid dendritic cells (pDCs) and myeloid dendritic cells (mDCs). These two dendritic cell subpopulations sense pathogens through Toll-like receptors (TLRs) on the cell surface and endosomal compartments. The different TLRs recognize distinct physical motifs on pathogens, with TLR 2,4,5,6 recognizing bacterial components and TLR 3,7,9 recognizing viral components (figure 2, top). The pDC and mDC populations express complementary and non-redundant TLRs: pDCs express TLR7 and TLR9, mDCs express TLR2 and TLR5 (figure 2, bottom). In addition, pDCs will produce enormous amounts of type I interferons within 6 hours of exposure to virus, but will not respond to bacterial pathogens in the same time frame***(6)***, and mDCs lack the ability for rapid, early IFN production to viral pathogens. By performing label-free monitoring of the DC sub-populations in peripheral blood, one should be able to distinguish viral infection versus bacterial infection prior to pathogen titer build up that must precede affinity based diagnosis.

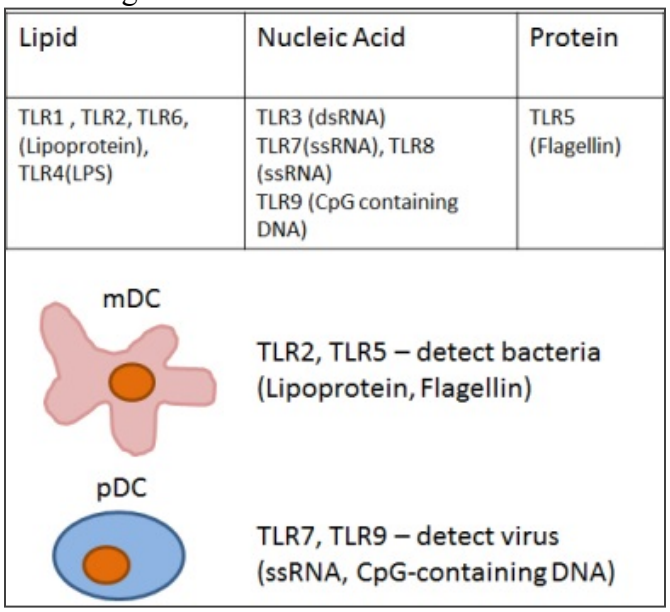

Figure 2. Pathogen specificity of TLR receptor family (top) ***(1)***. Bottom: mDC and pDC express non-overlapping sets of TLRs that sense different pathogens***(2)***.

The primary objective of this LDRD is to ascertain whether Raman spectroscopy monitoring of DCs can provide diagnostic information to differentiate between infected vs. non-infected, and bacterial vs. viral infection before any affinity based tests can be performed. Raman spectroscopy is very sensitive to changes in cellular nucleic acid, lipid, and carbohydrate contents, and the DC responses to pathogen involve upregulation of proinflammatory genes (nucleic acid changes), increase in production and exocytosis of cytokines (lipid changes), and global changes in dynamic glycosylation (carbohydrate changes) ***(7)***, therefore, Raman

spectroscopy should be a very suitable detection method for characterizing DC physiological states. To perform the proof-of-principle studies, primary human mDCs and pDCs isolated from human peripheral mononuclear cell fraction will be cultured, and first stimulated with chemical agonists to elicit characteristic responses to gauge Raman shifts as a result of those stimulations. The goal is to determine the timing and dosage of agonists to achieve maximal Raman shifts. Then, using the TLR agonist results as preliminary guide, primary DCs will be infected with pathogens known to be sensed by different TLR receptors: gram positive and gram negative bacteria (TLR2, TLR4, TLR5), single-stranded RNA virus (TLR7), and double-stranded DNA virus (TLR9) to determine whether unique DC Raman signatures that differentiate between these pathogens exist. For Raman spectroscopy, a laser line projection will be used to excite Raman emission from infected dendritic cells (100-1000) simultaneously (figure 3). The resulting Raman emission from the DCs will be analyzed to find characteristic shifts that can be correlated to the type of pathogen. Once the Raman bands associated with pathogen type have been identified, the detector format can be further simplified and miniaturized to focus on spectral bands of interest, greatly enhancing throughput. The unique Raman signatures correlating to bacterial and viral pathogens will be captured and cataloged as standards for further diagnostics development.

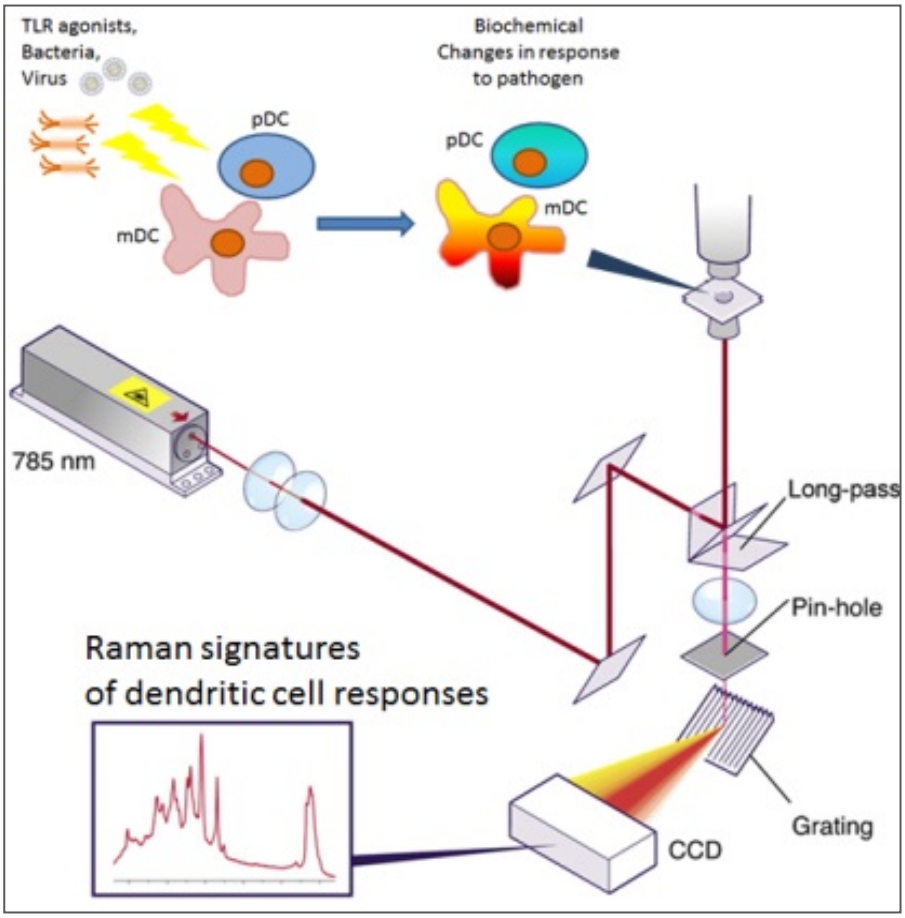

Figure 3. Experimental scheme. DC subsets will be isolated and stimulated with different pathogens to illicit innate immune responses. The infected DCs will be examined using Raman spectroscopy and the Raman signatures corresponding to each cell type and pathogen will be recorded and cataloged.

1.3. Relationship to prior and other on-going work

Sandia has significant track record in optical analysis of innate immune cell responses***(8-10)*** and Raman spectroscopy***(11, 12)***. The methods for cell culture, cell isolation, and sample preparation are well established. The Raman spectroscopy work proposed in this proposal will utilize hardware and software developed for bioprospecting for algae biofuels, with new adaptations for Raman spectroscopy of mammalian cells. Special focus will be paid to the lipid, carbohydrate, and nucleic acid specific Raman bands in the DC samples. In addition, Anson Hatch and Meiye Wu are currently working on a Bioscience IA LDRD exploring tunable electrokinetic step gradient (EKSG) based separation method for label-free cell separation from complex biological samples. The parameters for label-free separation of dendritic cell enrichment to prepare samples for Raman spectroscopy will be explored.

There are many commercially portable Raman detectors on the market. A brief survey of these systems determined that all hand-held Raman detectors are marketed for Pharmaceutical/chemical material analysis, and all larger portable Raman systems focus on chemical analysis as well, with only one system, the i-Raman from B&W Tek having been tested as a cervical cancer diagnostic for verification of cancer in biopsy samples. So far, Raman based medical diagnostic tools developed both in Academia and in industry are in the cancer diagnostics or cardiovascular imaging areas. No efforts in portable monitoring of immune responses or early infectious disease diagnostics exist. Since Sandia also boasts prototyping and systems integration capabilities, there is ample opportunity for Sandia to become the first to build a portable, perhaps even handheld Raman based early disease diagnostics system that can be used for biodefense as well as general biomedical diagnostic applications.

1.4. Tie to investment area and relevance to DOE and national security missions

The work proposed in this project will expand Sandia's portfolio of Biological threat management capabilities, and address the IHNS Biological Risk Management call to develop novel technologies and systems that provide warning and inform rapid and effective response should chemical or biological agents be used against US military forces or civilian populations. Label-free pathogen detection will access previously untapped windows of opportunity for passive monitoring of at-risk populations as well as provide triage in the event of a bioterror attack. This technology will greatly enhance ongoing DTRA, DOD, DHS efforts to develop technology for protecting American warfighters and their allies from threats posed by biological weapons. This work will also impact HHS (NIH/NIAID, CDC) and DOD (DTRA, USAMRIID) missions to protect civilians and military personnel from the threat of emerging infectious disease.

2. MATERIALS AND METHODS

2.2 Macrophage cell culture and stimulation

RAW 264.7 Macrophages were cultured in growth medium consisting of 450 mL of DMEM, 50 mL of FBS (Gemcell), 10 mL of HEPES, 5 mL of L-glutamine (200 mM), and1:100 penicillin/streptomycin (Gibco). For stimulation, RAW cells were seeded at 10^6/ml in growth media onto ultra low attachment dishes or plates (Corning) 1 hour prior to addition of stimulation agent or pathogens. The stimulated or infected cells were incubated for 5-6h in cell culture incubator and harvested by centrifugation.

2.2 Dendritic cell enumeration

1. Prepare 50mL buffer: Use Wash/Stain buffer from Biolegend
2. Make a foil tent, find racks
3. Dilute 5mL 10x Red Blood Cell Lysis Solution by adding 45 mL double distilled water.
4. Label 12 capped Falcon tubes, 1-6 for DC count, 7-12 for control.
5. Transfer 300 ul of whole anticoagulated blood into each tube, twice.
6. Add 20uL anti-BDCA Cocktail to tubes 1-6.
7. Add 20uL Control cocktail to tubes 7-12.
8. Mix gently and incubate at room temperature in horizontal position for 10min.
9. Add 4mL 1x RBC cell lysis solution to each sample
10. Mix gently and incubate at RT in the dark for 10 min, use foil tent.
11. Spin down cells at 300g for 5min. Aspirate the supernatant completely, and resuspend the cells in 4 mL of buffer.
12. Spin cells down at 300g for 5 min, aspirate supernatant and resuspend in 300uL of buffer.
13. Add 150uL Fix solution and 5uL discriminator stop reagent to each sample.
14. Transfer cells to eppendorf tubes and run by flow cytometry.

2.3 Whole blood stimulation

Stimulate fresh whole blood with HKLM 10^8/ml, LPS (100ng/ml), Imiquimod (10µg/ml), H_2O(endotoxin free) – all reagents purchased from Invivogen, and Sendai virus (Charles River, Maine) at 1:600 ~ 3.33µg/ml

1. Order blood from Allcells(Alameda, CA), get blood within 4 hours.
2. Morning before blood arrives:
3. Make 50 ml 1x BD lysis buffer (Cat no. 349202), store at 4C.
4. Make 100X stock solutions for stimulation: 1:10 dilution of LPS stock to make 10ug/ml 100x stock. Aliquot into 50ul tubes and store the rest in -20C.
5. Immediately prior to experiment, resuspend 1 tube of ssRNA40 in 500ul water, mix gently. Allow 15min to completely solubilize. Makes 50ug/ml stock, use at 1:100, or 0.5ug/ml.
6. dilute Imiquimod stock with water to make 10mg/ml 100x stock.
7. Aliquot 0.5 ml of fresh Whole blood to each of 12 wells in ultra-low attachment dishes (Corning), add 5µl of each agonist. Swirl to mix.
8. Incubate for 2 hours in incubator. If performing intracellular cytokine staining, proceed to steps 9-12, for Raman spectroscopy, go to step 13.

9. Add 0.5ul Bfa (BD Biosciences) to every well, swirl to mix.
10. Incubate for 3 more hours in incubator.
11. During incubation, aliquot 5ml of 1x BD lysis buffer in 15ml conical tubes for each sample.
12. At 3 hours, Pipet blood from plate into each of the 12 conical tubes containing 5ml BD lysis buffer. Vortex and incubate for 10min at RT. Store in -80°C for Flow Cytometry analysis next day.
13. For Raman spectroscopy, isolate PBMC using Lymphoprep (Stemcell) and SepMate (Stemcell) tubes.
14. Add 15ml lymphoprep to each SepMate tube by carefully pipetting it through the central hole of the SepMate insert.
15. Dilute 60ml of blood with 60ml of 2% FBS in PBS, mix gently
16. Keeping the SepMate tube vertical, add 30ml of diluted sample by pipetting it down the side of the tube. The sample will mix with the density medium above the insert.
17. Centrifuge at 1200g for 10min at RT with the Brake ON.
18. Pour the top layer into new 50ml conical tube. This supernatant contains the MNC, careful not to invert the SepMate tube for more than 2 sec.
19. Wash the enriched MNCs with 20ml PBS + 2% FBS , spin at 300g for 8 min at RT, brake ON.
20. Resuspend each pellet with 5ml PBS + 2% PBS, combine into 1 tube, spin down at 300g for 8min with the brake ON.
21. Count cells

2.4 Immunostaining and flow cytometry

Immunostaining for TNFα, IFNα, IL12 in pDC (CD123, CD303) and Monocyte/Macrophages(CD68) followed by Flow cytometric analysis:

1. Make 12 ml of 1X BD permeabilization solution in reagent grade water.
2. Make 1% PFA solution in PBS.
3. Turn on 37°C water bath
4. Make Ab and IC mix as follows:
5. CD303-PE, CD123-647, IFNα-Fluorescein (pDC/IFNα), staining buffer, use 50ul Ab cocktail/condition.
6. CD68-PE, IL-12/23-Alexa647, TNFa-Alexa488, Stain buffer
7. Isotype control-FITC, Isotype control – PE, isotype control – 647, stain buffer
8. Briefly thaw 1 set of stored WB in lysis buffer at 37°C.
9. Add 7 mL of wash buffer and centrifuge at 500g for 10 min at RT with brake on.
10. Decant the supernatant, and resuspend the pellet in 0.5ml of wash buffer.
11. Add 2.5ml of permeabilizing buffer to each tube. Vortex and incubate for 10min at RT.
12. resuspend pellet in 10 ml wash buffer, spin down at 500g for 10min at RT.
13. During incubation, label 12 x 1.7ml sterile microcentrifuge tubes with the stimulation conditions. 3 tubes for each agonist. (1A, 1B, 1C…etc.)

14. Decant supernatant, resuspend in 200µl wash buffer.
15. Aliquot 50µl cells to each microcentrifuge tube
16. Add 50ul of each Ab cocktail to each tube. Mix by pipetting up and down.
17. Incubate at RT in foil for 30min.
18. Add 1 ml of wash buffer to each tube
19. Spin tubes at 500g for 10min.
20. Resuspend in 200 ul 1% PFA and pipet up and down to mix. Incubate for 10min at RT.
21. Add 1ml wash buffer. Spin at 500g for 10min.
22. Aspirate supernatant and resuspend in 200ul of PBS and transfer to 96 well plate.
23. Run in cytometer, read 180ul.

2.5 Raman spectroscopy

All Raman spectroscopy of cell lines and primary blood cells were performed on the B&W Tek iRaman 1064nm system shown in figure 4. 3 readings were taken from each sample spot at different locations in the spot to represent 3 technical replicates.

Sample preparation protocol for Raman spectroscopy:

1. Fix cells with 1% paraformaldehyde (Electromicroscopy Sciences) for 10 min at room temperature.
2. Wash cells with 20 volumes of PBS twice, spin down at 300g for 10min.
3. Resuspend cells at $2x10^5$/µl PBS
4. Spot cell suspension onto glass slide, place inside sterile petri dish, with the lid on, but left ajar. Leave inside biosafety cabinet overnight to air dry.

Figure 4. The portable B&W Tek iRaman 1064nm.

iRaman 1064nm Protocol

1. Turn on laser (plug in power cord, turn the key on the back of the laser 90 degrees clockwise to the “on” position, plug in the safety interlock below the key, and flip the switch below the interlock to the “on” position).
2. Turn on computer and open the BWSpec4 software. If the software window does not read “Online” in the lower left corner, turn off the laser and restart it.
3. Select an integration time.
4. Open the laser’s manual shutter and take a dark scan by selecting the Dark Scan option (the black dot inside a blue circle).
5. Collect a spectrum. If overriding the last spectrum, select Acquire One Spectrum (the blue circle with one arrow). If keeping the last spectrum, select Acquire Overlay (the blue circle with an arrow and a horizontal line below the arrow). Turn the laser off when collection is finished by clicking the red circle with a vertical white line.
    a. If using the laser directly:
        i. Position the laser probe with the connected laser shaft directly over the sample at a distance of approximately 5.9 mm from the sample.
        ii. Focus the laser by viewing the sample through an Android phone camera. Raise and lower the laser to a position that will provide the smallest and most defined laser spot possible (a white/purple spot).
        iii. Set the laser power percentage then collect a spectrum.
    b. If using the laser with the microscope:
        i. Select the corresponding camera plug-in from the Plug-In Menu.
        ii. Connect the laser to the microscope without the shaft by using the adapter and tightening the port screws to view the laser spot (a white circle).
        iii. Turn the LED light on and position sample on the microscope stage.
        iv. Use the Camera View Screen on the computer from the camera plug-in to focus the image with the microscope knobs.
        v. Once focused, turn LED light off, set the laser power percentage, and collect the spectrum.
6. To perform a background correction, select Background Removal from the Plug-In menu. Then select the check box next to the collected spectrum in the Spectrum List Panel. From the Tools menu, select Background Removal then Do.
7. To perform a relative intensity correction, select Relative Intensity Correction from the Plug-In menu. From the Option menu, select Relative Intensity Correction and choose SRM 2244 (Raman) for Standard Material. Set Reference Data to Current Reference Data and select Create. From the Option menu, select Enable Relative Intensity Correction, locate the Ratio3_***.txt file, then select Set.

8. Save data in .txt and .csv format. Take a screenshot of the spectrum and save in .pdf or .jpg format for a reference visual.

2.6 Surface enhancement Raman spectroscopy (SERS) sample preparation

1) Seed 10 ml of RAW cells at $1x10^6$/ml in two10 cm diameter ultra low attachment dishes (Corning).
2) Stimulate first plate with 10ul endotoxin free water, the other with 10ul LPS (100ng/ml final)
3) Incubate for 5 hours.
4) Harvest and wash with 25ml PBS 2x.
5) Fix cells with 1% PFA for 10min.
6) Wash with 25ml PBS.
7) Make serial dilution of gold nanoshell: Nanocomposix, 980 nm Resonant Gold Nanoshells (NanoXact, 0.05 mg/mL, PEG, 25 mL) Lot # ECP1201
   a) Vigorously vortex gold nanoshell.
   b) Take 2 ml of 0.05mg/ml nanoshell and Centrifuge, aspirate supernatant
   c) Replace supernatant with water, resuspended at 110µl, comes out to be ~0.9mg/ml
   d) make 4 serial dilutions at 1:2
      i. from 0.9mg/ml, 50ul nanoshells + 400ul water = 0.5 mg/ml
      ii. 40ul 0.5mg/ml + 40ul water = 0.25mg/ml
      iii. 50ul water
8) resuspend the each cell pellet in 12.5ul water
9) label tubes with nanoparticle concentration and treatment, mix cells 1:1 (6ul cells, 6ul nanoparticle) with nanoparticles
10) vortex vigorously, pipet up and down before spotting onto slide (duplicates per condition).

2.7 Data analysis

The raw data generated from BWSpec4 software was saved as .txt files with the Raman frequency band and corresponding intensity readings in two columns. The .txt files corresponding to each experiment with multiple stimulation conditions were imported into Matlab(Mathworks) as a matrix and subjected to Principle Component analysis and regression analysis.

3. RESULTS

3.1 Feasibility results for Raman spectroscopy hardware configurations

The initial Raman feasibility testing was performed by B&W Tek, and 3 configurations of the iRaman spectrometer was tested to determine the optimal laser frequency, power, and integration time for collecting data from immune cells. RAW macrophage cells were stimulated with 100ng/ml LPS or vehicle for 6 hours, washed twice with PBS and resuspended at $5x10^5$ cells/µl, and spotted onto glass slides and air dried. The slides were shipped to B&W Tek and the feasibility test was performed on the iRaman 532nm, iRaman 785nm, and iRaman 1064nm systems. Raman data collected on the iRaman 532nm system was not informative at all (figure 5), and the fluorescence from the glass slide obscured specific cellular signal on the iRaman 785nm system (figure 6). The iRaman 1064nm system produced spectra indicative of cell signals (figure 7). The optimal Raman spectrometer configuration was determined to be 1064nm laser at 100% power with 2 min integration time.

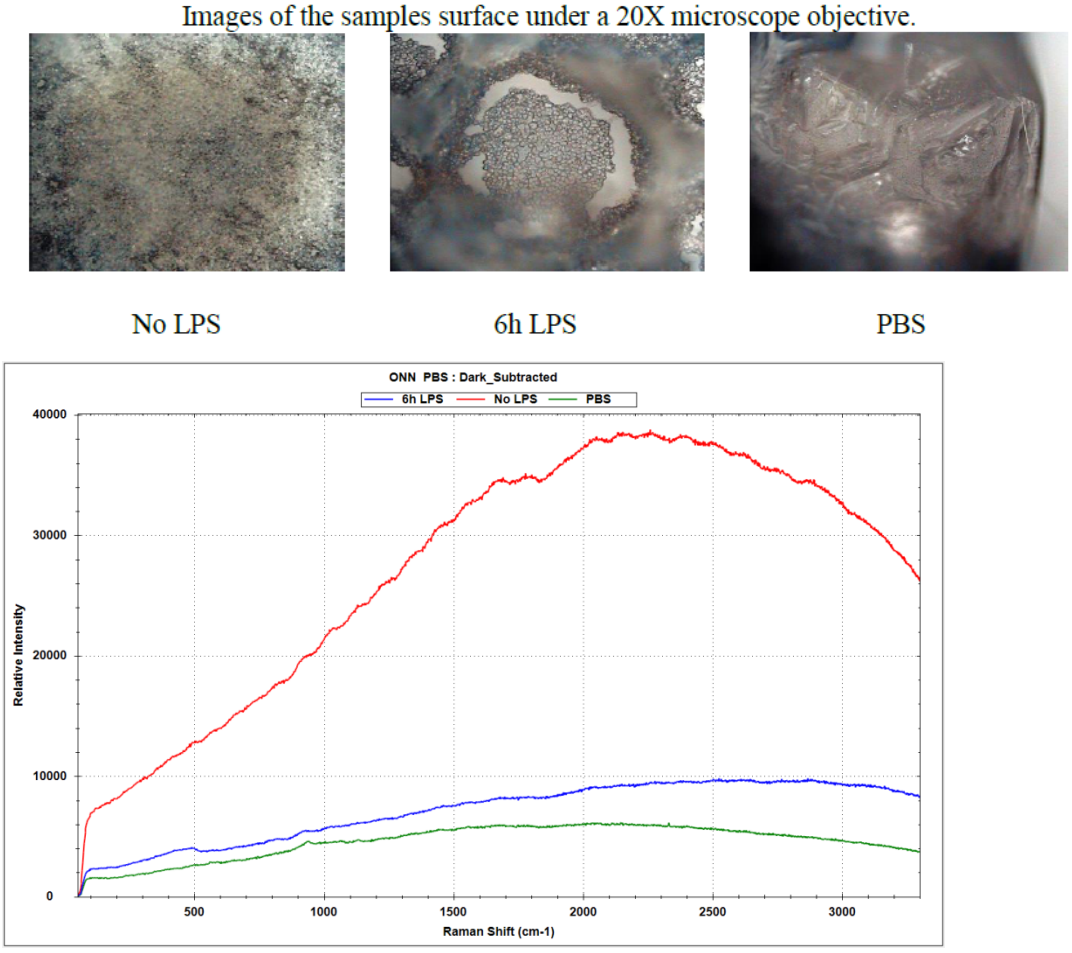

Figure 5 The 532nm system test parameters for No LPS and 6h LPS was 5 second integration time using 100% laser power (~50mW) averaged over 1 scan, and for PBS it was 30 second integration time using 100% laser power average over 1 scan.

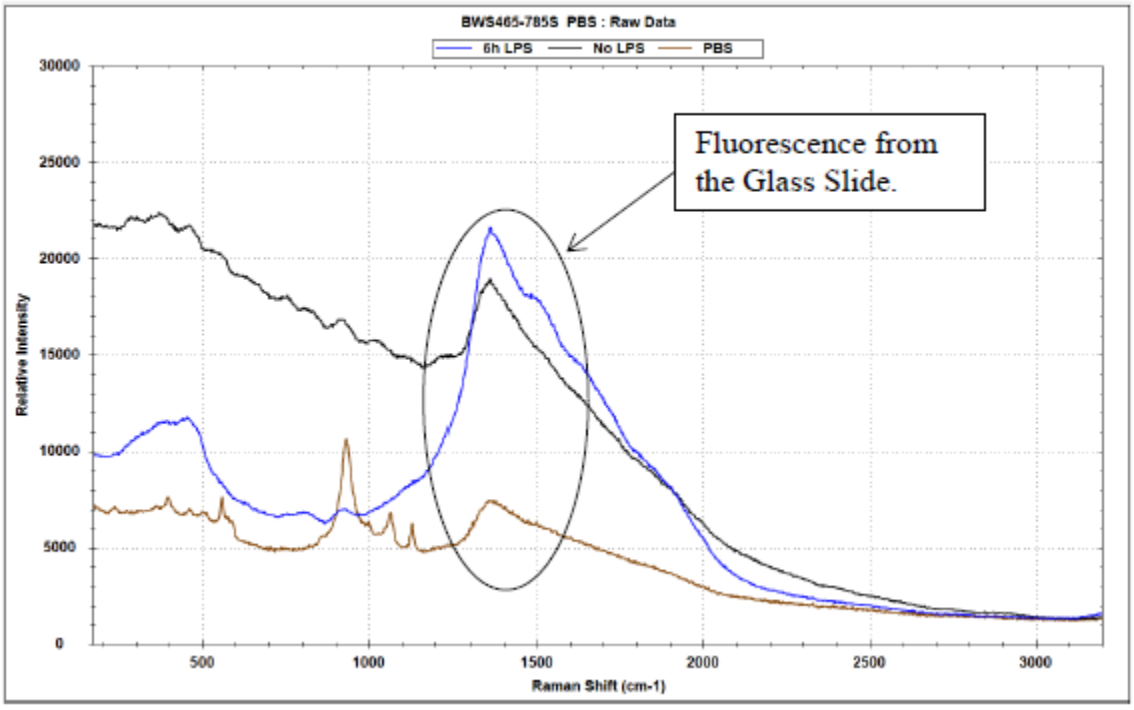

Figure 6 The 785nm system test parameters for No LPS and 6h LPS was 9 second integration time using 100% laser power (~300mW) averaged over 1 scan, and for PBS it was 30 second integration time using 100% laser power average over 1 scan.

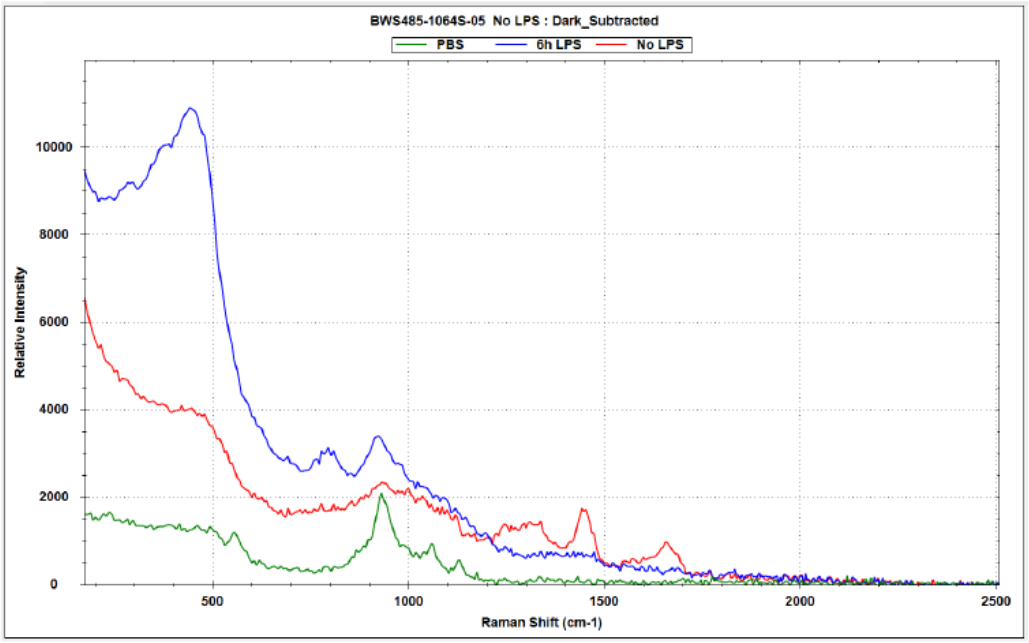

Figure 7. The 1064nm system test parameters for No LPS and 6h LPS was 120 second integration time using 100% laser power (~450mW) averaged over 1 scan, and for PBS it was 90 second integration time using 100% laser power average over 1 scan.

3.1. Sample preparation method optimization results from macrophage cell line

To optimize the sample preparation method, RAW 264.7 cells were seeded in triplicate at 10^6 cells/ml, and stimulated with 100ng/ml E. *coli* LPS or vehicle for 6 hours. Cells were pelleted and washed with PBS and resuspended at 3 different concentrations ($5x10^4$/µl, $2x10^5$/µl, and $1x10^6$/µl). The optimal cell density for label free Raman spectroscopy was $2x10^5$ cells/µl and air dried overnight (figure 8), where reproducible Raman spectra from RAW cells were collected and a clear difference between stimulated and unstimulated cells were readily detectable (figure 8). Further principle component analysis of the RAW data shown in figure 8 revealed that identified 15 Raman bands that distinguish the unstimulated from stimulated RAW cells and 99.9% of the variance can be explained by 2 principle components (figure 9). Attempts to collect Raman spectra from wet samples were unsuccessful due to instrument issues.

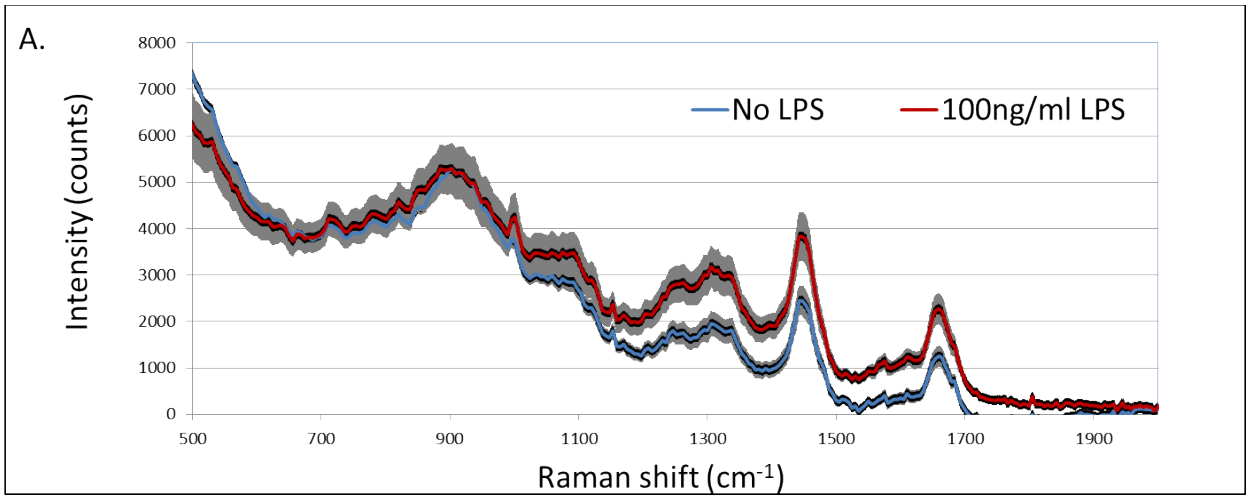

Figure 8. Raman spectra of RAW macrophage cells stimulated with water (No LPS) vs. 100ng/ml E. coli LPS.

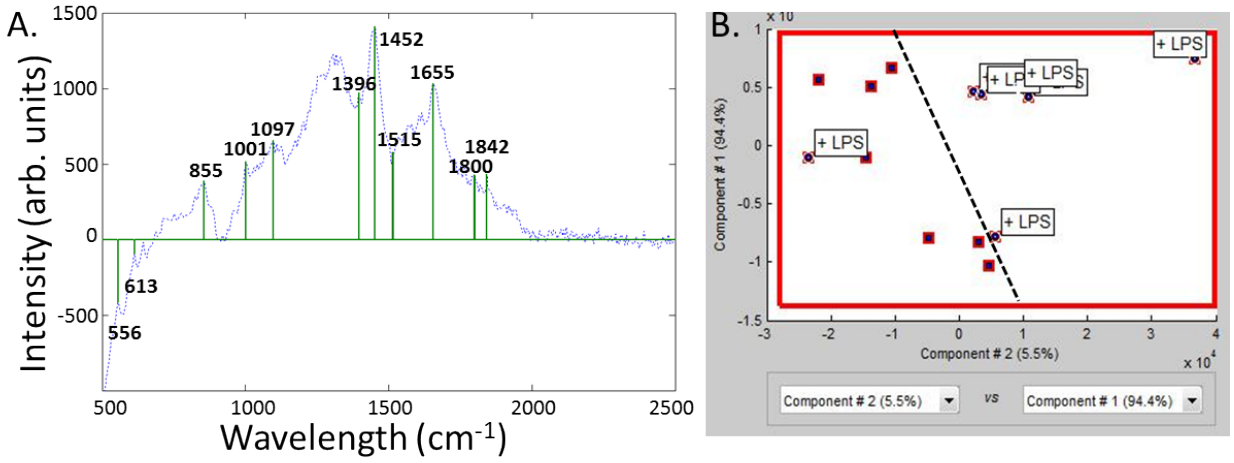

Figure 9. Immune cell activation state differentiation by bulk label-free Raman spectroscopy. (A).Raman fingerprint of macrophage activation. The Raman spectra from macrophages stimulated with 100ng/ml LPS was subtracted from the Raman spectra of unstimulated macrophages to generate the difference spectrum (blue dash). The highlighted Raman peaks have signal/noise ratio corresponding to 90% confidence interval. Data shown is the average of 3 biological replicates, each with 2-3 technical replicates (B). Principle Component Analysis on 15 total Raman spectra. 99.9% of the Raman spectral variance can be explained by 2 PCA components. LPS treated samples are labeled as "LPS", control samples are unlabeled squares.

3.3 RAW cell line infection results

To determine if label-free Raman spectroscopy can distinguish between different types of stimulation/infection, RAW cells were stimulated with E. coli LPS, Imiquimod, heat killed E. coli, and live Sendai virus, along with vehicle control. The stimulated/infected RAW cells were harvested by centrifugation, and washed with PBS, then fixed with 1% paraformaldehyde for 10 min to ensure compliance with laboratory safety regulations. The fixed cells were resuspended

at $2x10^5/\mu l$ and spotted onto glass slides. The data was analyzed by 5 principle component analysis, and the result is shown in figure 10. The Sendai infected cells were very clearly identified (figure 10, Red highlighted region) from all other conditions. In addition, the LPS, HKEC, and Imiquimod stimulated cells were also readily identified from vehicle and Sendai infected cells (figure 11). The classification and regression tree of the Raman spectra data from all stimulations identified 4 dominant bands for sorting causal agents (figure 12) with over 90% accuracy for each component, compared to the full regression tree with 16 identified Raman bands (figure 13).

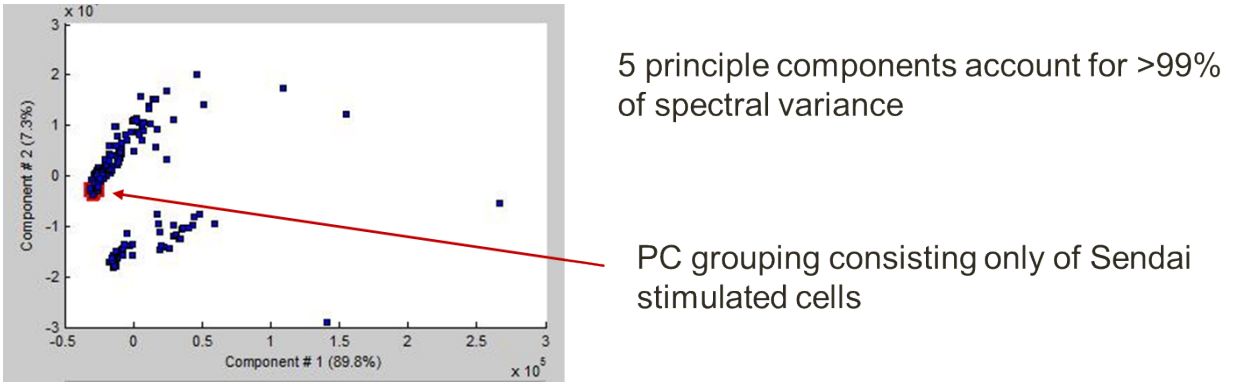

Figure 10. PCA analysis of RAW cells stimulated with water, LPS, Imiquimod, and Sendai virus.

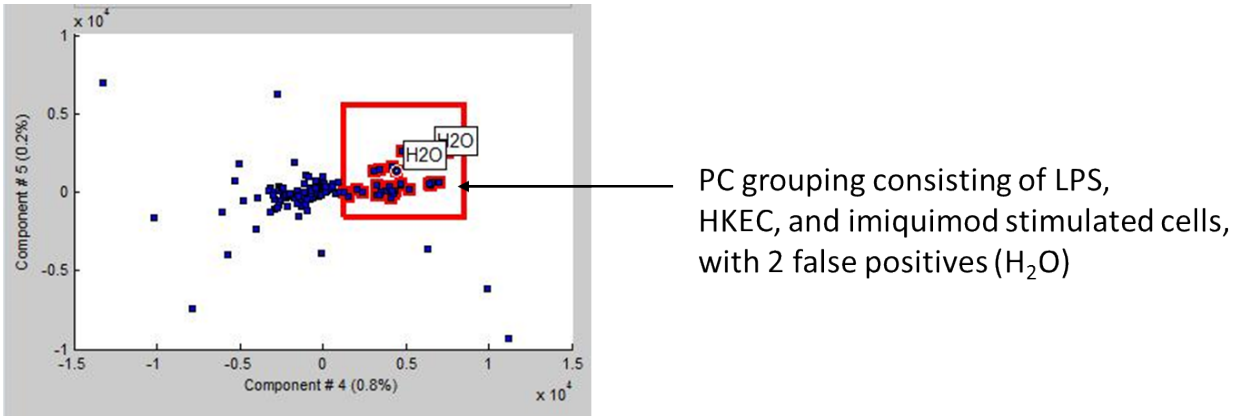

Figure 11. LPS, Heat killed E. coli, and Imiquimod stimulated cells were distinguished from vehicle and Sendai infected cells.

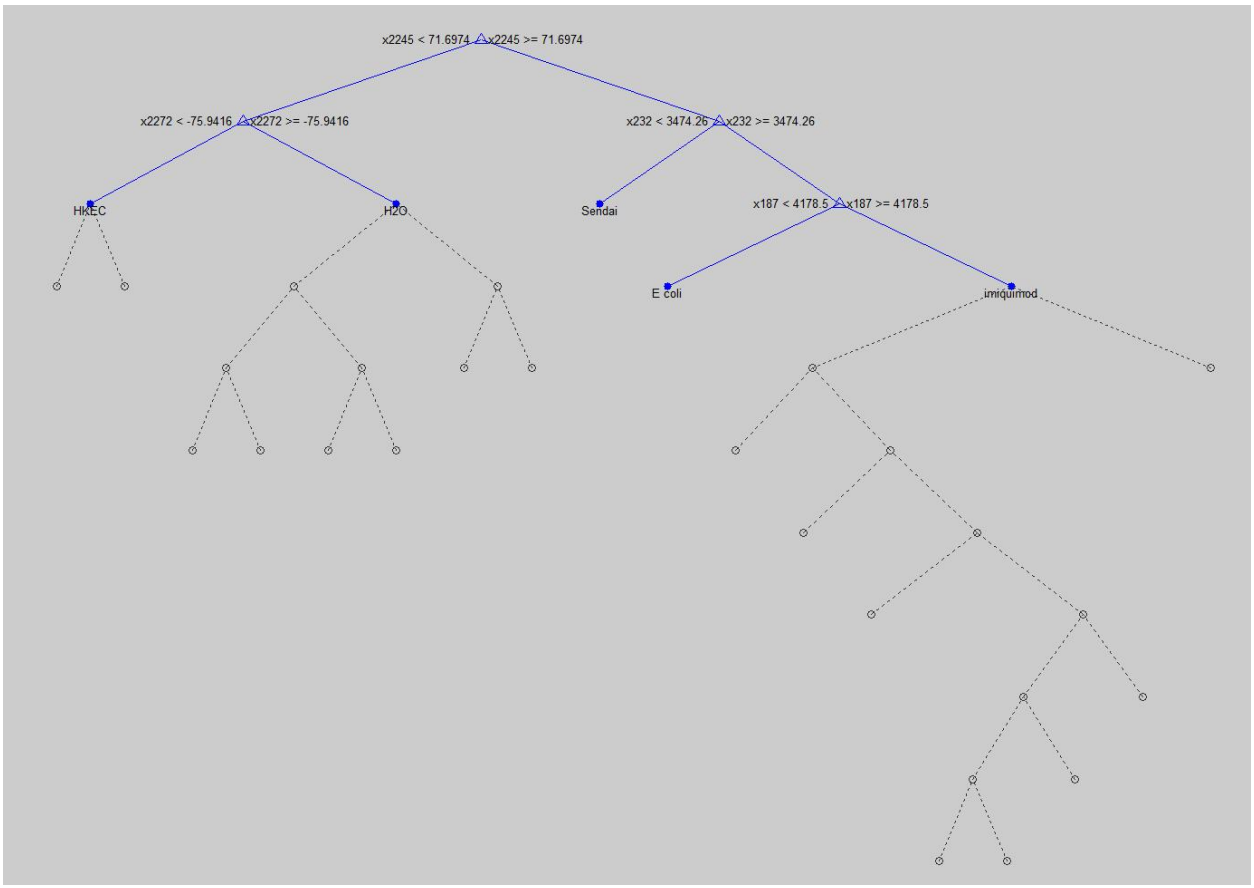

Figure 12 Minimum Classification & Regression Tree for Stimulation Agents

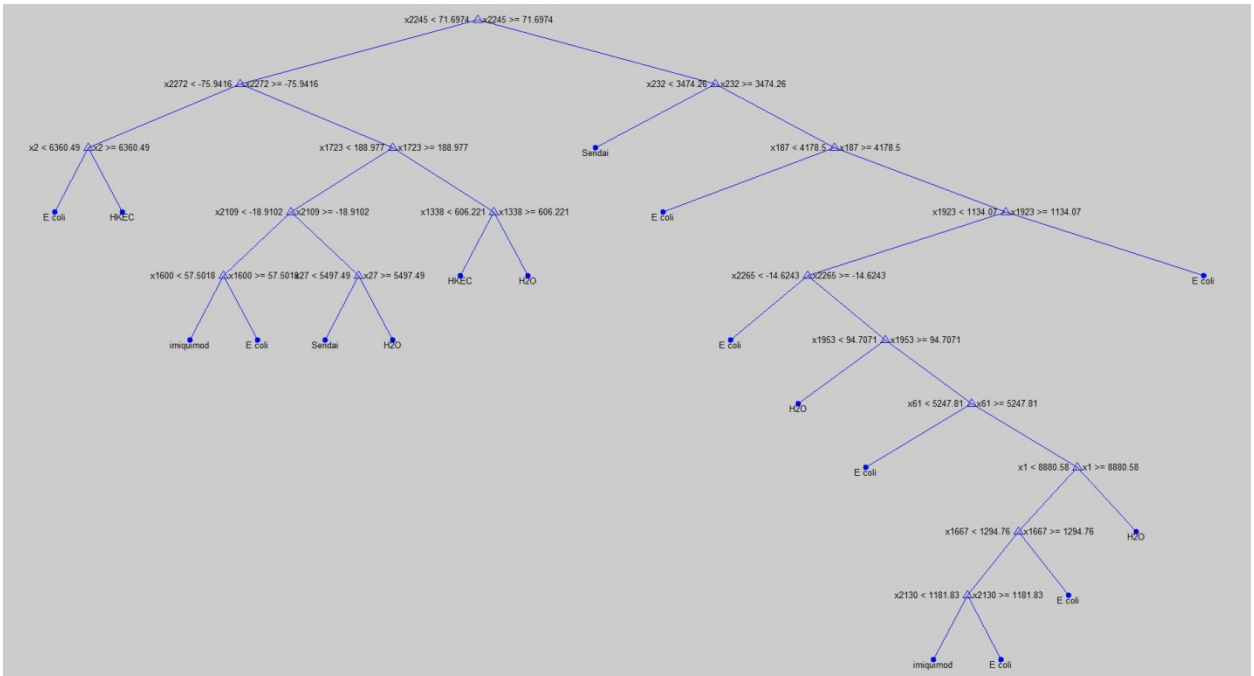

Figure 13. Full Classification & Regression Tree for Stimulation Agents

3.3. DC frequencies in whole blood

In order to determine the approximate numbers of DC subsets in whole blood and extrapolate the sample volume needed for Raman disease signature detection, heparinized whole human blood was purchased from Bioreclamation LLC (New York) and delivered at room temperature within 48 hours of blood draw. Blood from 3 male donors and 3 female donors made up of n = 6 for the DC enumeration study. The enumeration was performed using the Blood Dendritic Cell Enumeration kit, human (no. 130-091-086, Miltenyi) according to the manufacturer's instructions. The results of the DC enumeration experiment are shown in figure 14. There are approximately ~170 paces per 100 µl of whole blood, which translates to ~600ml of whole blood is required for sample preparation of pDCs for diagnostics. The large cell number requirement for label free Raman spectroscopy prompted the quest to find alternative source of more abundant diagnostic cell fraction.

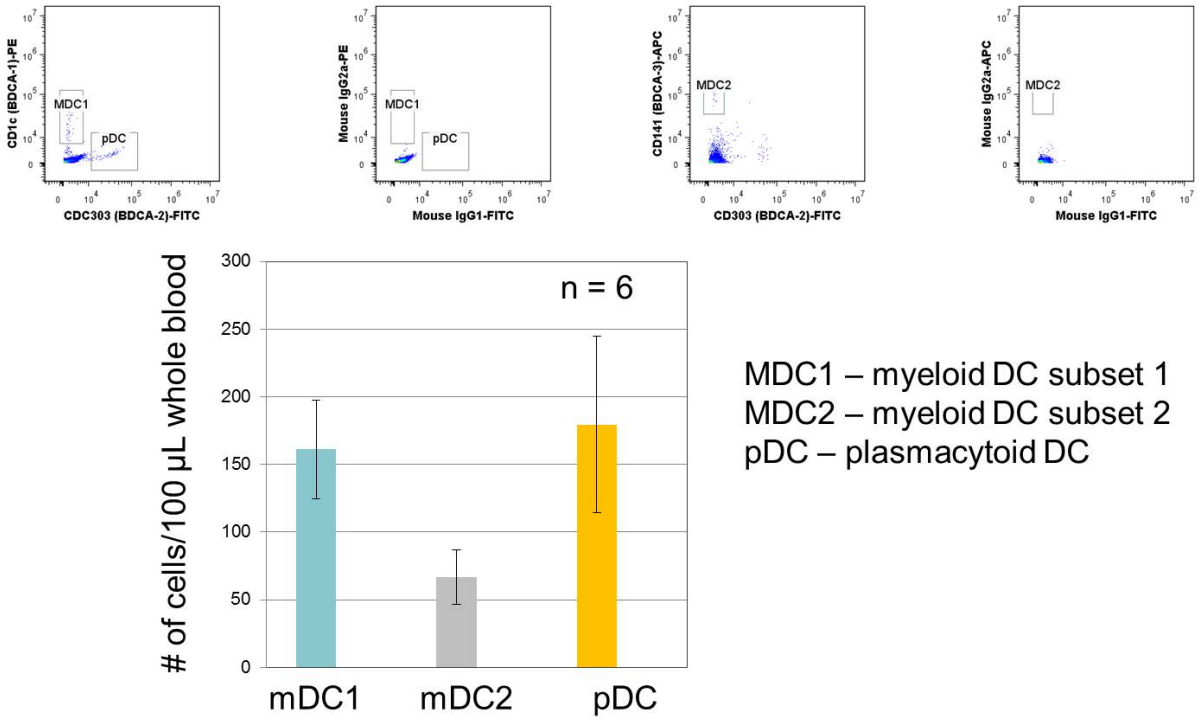

Figure 14. DC subtype frequency in 100 µl of whole blood.

3.2. Bacterial vs. Viral infection biological signatures determined by flow cytometry

To confirm that a diagnostic immune activation profile that can distinguish infected vs. uninfected, bacterial vs. viral infection does indeed exist in peripheral blood, a series of

immunostaining and flow cytometry experiments were carried out to identify the immune subsets that respond differentially to bacterial vs. viral infections. E. coli LPS and the TLR7 agonist Imiquimod were used as simulants of bacterial and viral infections (respectively) in fresh human whole blood. The most likely subsets that respond differentially to different pathogens were identified as the CD68+ population (macrophages and monocytes) and CD303+/CD123+ population (plasmacytoid dendritic cells, pDC). The whole blood (<4h) was stimulated with either LPS, Imiquimod, or vehicle control for 5 hours , with the later 3 hours incubated in the presence of Golgi release inhibitor Brefeldin A (Bfa) to entrap nascent cytokines. TNFα and IFNα were cytokines known to be released in large quantities by macrophages and pDCs upon bacterial or viral infection, and used as Biomarkers for activation. The results of the flow cytometry study are shown in figures 15 and 16. Figure 15 is a density chart showing the exclusive activation of pDC by TLR7 agonist Imiquimod, and not by TLR4 agonist LPS (figure 15, A), and CD68+ cells are highly activated by LPS, but only slightly activated by Imiquimod (Figure 15B). Figure 16A and 16B shows the histograms of intracellular cytokine production by pDC and macrophages in the presence of LPS vs. Imiquimod. 16A clearly shows pDC activation by Imiquimod and not LPS, 16B shows significant activation of monocytes/macrophages by LPS, but minimal activation by Imiquimod. Additionally, fresh blood was stimulated with heat-killed bacterial species L. Monocytogenes, S. Aureus, and E. coli have different stimulatory effects on the monocyte/macrophage population, with E. coli being the most stimulatory, and L. monocytogenes the least activating(figure 16C).

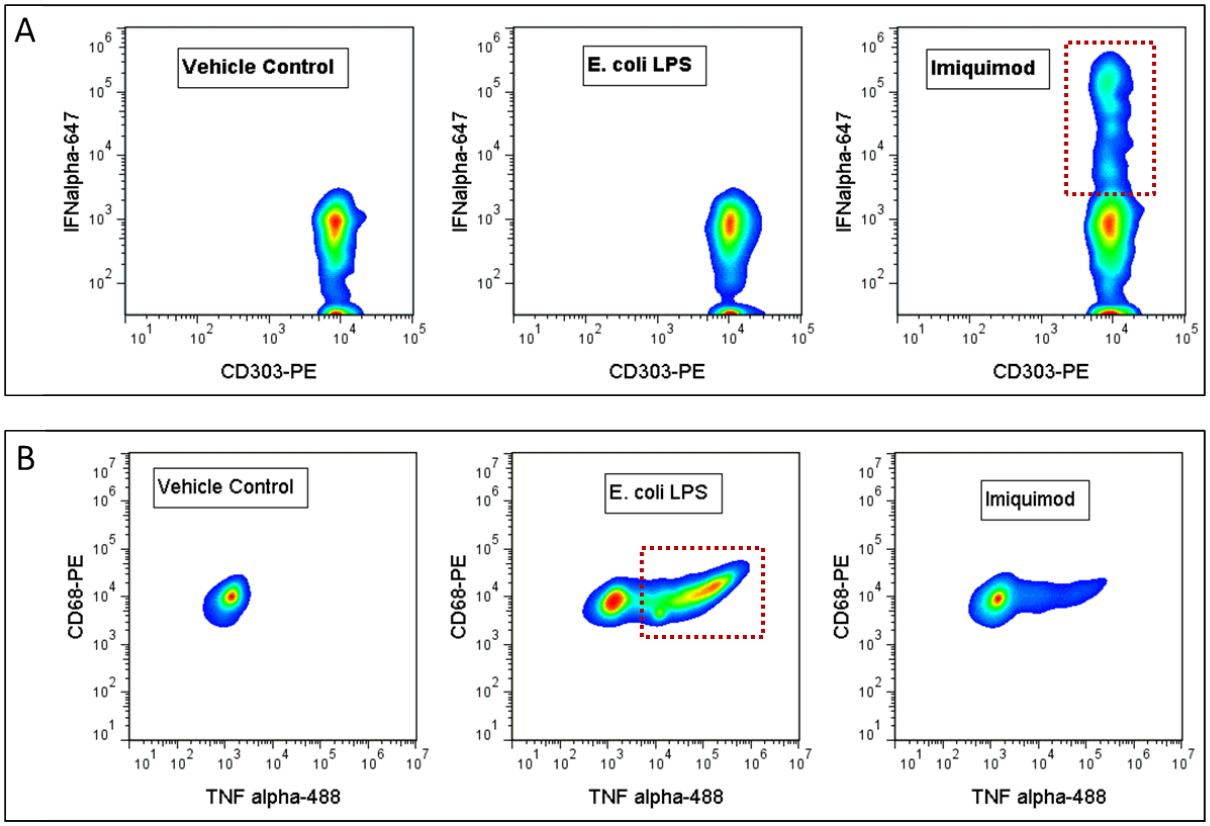

Figure 15. Fresh whole blood displaying infectious disease signatures as detected by immunostaining and flow cytometry. Top panel (A). The CD303 positive cells (paces)

display IFN alpha production when stimulated by Imiquimod(viral simulant) and no IFN alpha production when stimulated with bacterial LPS(bacterial simulant). Bottom panel (B). CD68 positive cells (monocytes/macrophages) display significant TNF alpha production when stimulated by LPS whereas there is only slight TNF alpha production when stimulated by Imiquimod.

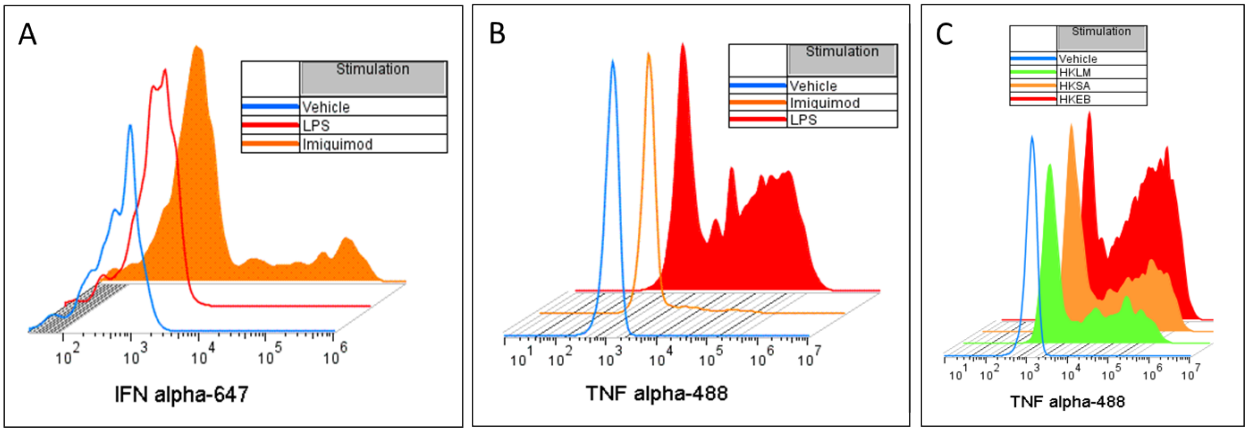

Figure 16. Biological signature of differentiating innate immune responses to bacterial vs. viral agonists using immunostaining and flow cytometry (A). pDC does not produce interferon alpha (IFN) when stimulated with vehicle (blue histogram), or 100ng/ml bacterial LPS (red histogram), whereas pDCs show clear activation by virus simulant Imiquimod (orange filled histogram). (B). Monocyte/macrophage population demonstrating TNF-alpha production in the presence of bacterial LPS (Red filled histogram), but not to vehicle (blue histogram) and Imiquimod (orange histogram). (C) Activation of monocyte/macrophages by HKLM- heat killed *L. Monocytogenes* (green filled histogram), HKSA- heat killed *S. aureus* (orange filled histogram), HKEB- heat killed *E. coli* (red filled histogram). No TNF-alpha production is detected with vehicle only control (blue histogram).

3.4. Label free Raman spectroscopy of infected whole blood

Infection studies with whole blood was performed with live Sendai virus. Heat killed E. coli, and vehicle control. Fresh human whole blood was infected with either 10^7/ml HKEC or 10µg/ml sucrose purified Sendai virus for 6 hours in the cell culture incubator. The peripheral blood mononuclear cell (PBMC) fraction from the infected blood was isolated by density centrifugation and fixed with 1% PFA before spotting onto glass slides and subjected to Raman spectroscopy analysis. There was not enough blood to do sample preparation with pDC. Approximately 25 technical replicates were performed for each condition, and the spectra data subjected to PCA analysis. The no cell control with only PBS and paraformaldehyde was included in the analysis. Figure 17 shows that the no cell controls (red oval) were readily distinguished from the cell samples. The vehicle control PBMCs were labeled as (H2O) were clearly separated from all stimulated samples (figure 18). Within the infected cell data space, the Sendai infected cells and HKEC cells were not clearly distinguished using the 2 component PCA

analysis (figure 19). There appeared to be a bimodal distribution of HKEC cells with unknown cause (figure 19, right).

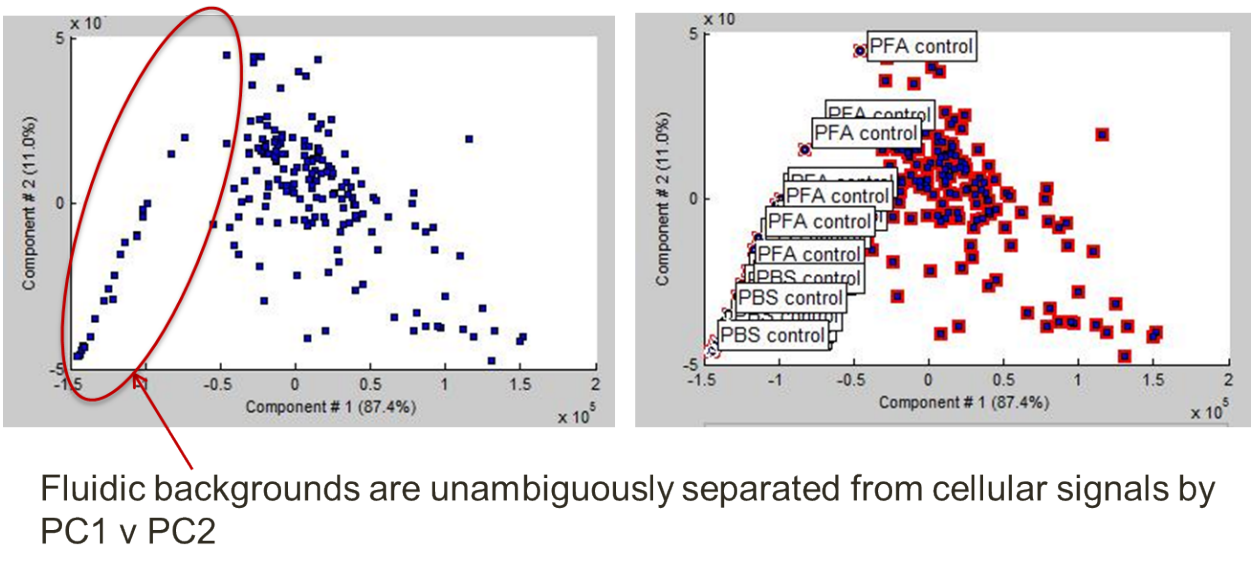

Figure 17. PCA analysis separating no cell controls from all cell samples.

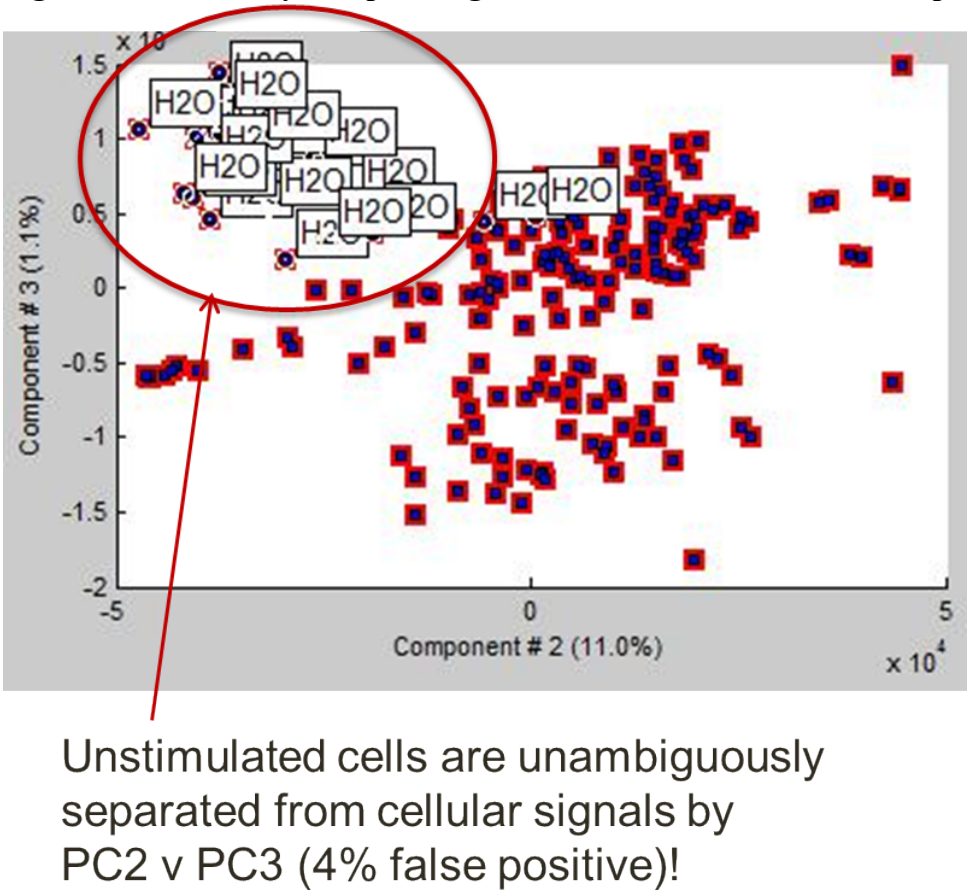

Figure 18. Clear identification of uninfected (H2O data points) PBMCs from infected PBMCs (unlabeled).

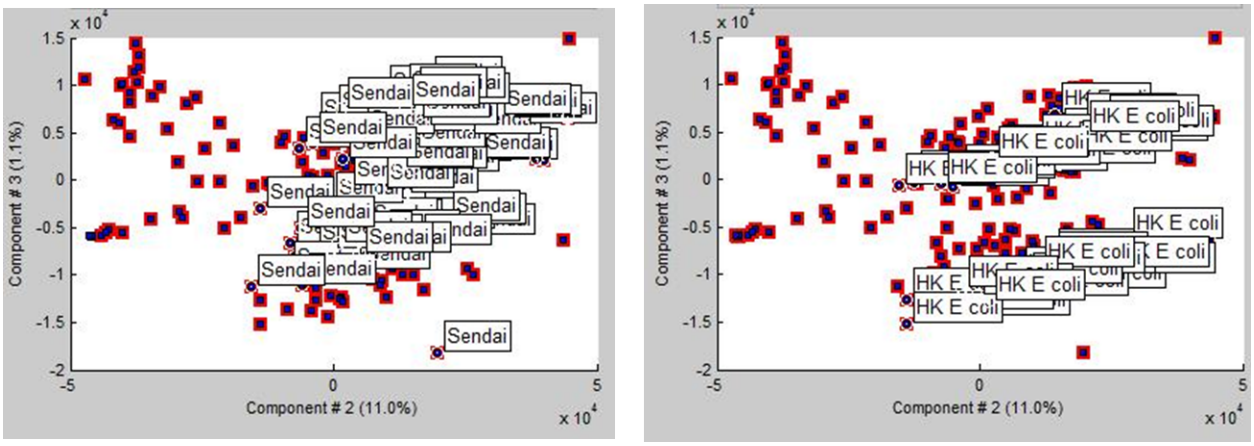

Figure 19. Sendai infected and Heat killed E. coli infected cell data was not clearly distinguished by 2 component PCA.

3.3. SERS enhancement of Raman signal

To determine the feasibility of using nanoparticles to enhance the Raman signal from immune cells, literature research was performed to determine the optimal nanoparticle for signal enhancement. 980nm Resonant gold nanoshells with peak plasmon resonance at 980nm was purchased from Nanocomposix for the feasibility study. The gold nanoparticle was mixed in with fixed RAW cells at 0.125mg/ml and spotted onto glass slides at $2x10^5$/ul PBS. The resultant signal enhancement is shown in figure 20. Even at the low concentration of 0.125mg/ml, there was Raman signal enhancement. Demonstrating that gold nanoshells can be used to enhance immune cell Raman spectroscopy and possibly allow for diagnosis with fewer cells than the $2x10^5$/µl that was used for the course of this project.

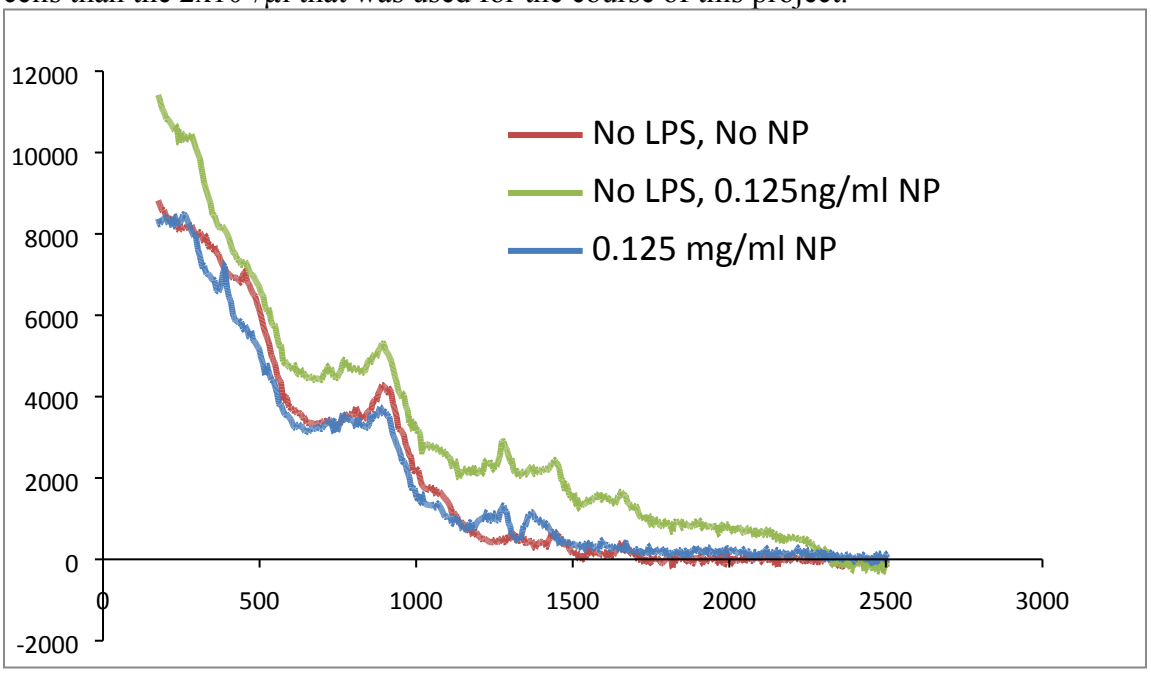

Figure 20. Raman signal enhancement by gold nanoparticle (NP)

4. CONCLUSIONS

4.1 Feasibility of using label free Raman spectroscopy for portable infectious disease diagnostics

Based on the results of this one year LDRD study, we established that a portable Raman detection system for early infectious disease diagnostic is very feasible, albeit some additional simple engineering and customization of sample preparation and detector miniaturization will be necessary for a prototype. A label-free detection system will address the issue of portability and user skill, as well as cold-chain storage issues plaguing all affinity based point-of-care diagnostics. A portable label-free diagnostic system will be more rapid than any PCR or immunoassay based system, and will truly be a point-of-care/point-of-need technology that will be robust and inexpensive to produce.

4.2 Scientifically significant findings

We were able to prove the concept that label-free le detection of Raman signatures in immune cells can distinguish between different states of activation resultant from different pathogens. This finding in itself is novel and scientifically significant as Raman spectroscopy is gaining popularity in Biological sample analysis. State-of-the-art Raman based cell analysis studies are performed on microscope based systems on single cells, often employing surface enhanced Raman micro-spectroscopy (SERS)***(13)***. Though Raman based biological detection is gaining popularity, cellular analysis studies are mostly confined to academia. One major hurdle to translating Raman cell analysis into real world applications is the time-consuming data acquisition step. Single-cell Raman techniques typically employ 1μm laser increment interrogation that must scan through hundreds if not thousands of individual cells in order to gather enough spectral information for reliable statistical analysis in complex cell mixtures such as blood or homogenized tissue. Through this LDRD, we proved that the throughput limitation of current Raman detection systems can be overcome by performing bulk Raman spectroscopy on concentrated cell sample spots with > 50,000 cells/μl so that hundreds of the same cell type can be interrogated at once, to provide averaged reading over the population. The 1064nm Raman detector we used also provided means to eliminate the background fluorescence from many cellular components.

Perhaps the most surprising result from this study is the finding that macrophages contain within them all the necessary Raman signatures to diagnose infected vs. uninfected, bacterial vs. viral infection. The original research plan for this project focused on dendritic cell populations being the subpopulation to provide the diagnostic information to distinguish identify viral infection, but using dendritic cells posed logistic challenges due to the scarcity of dendritic cells in peripheral blood. However, the results from macrophage cell line (figure 10, 11) indicate that the macrophage population can be used to extract actionable diagnostic information indicating whether they have been exposed to bacteria, virus, or no infection. The tight clustering of virus infected macrophage cells in figure 10 provided the highest confidence level. Since monocytes/macrophages are much more abundant than dendritic cells at ~8000 cells/100 μl whole blood, a spectroscopic based focusing on a enriched monocyte/macrophage population can is much lower technical bar than one proposed to examine rare dendritic cell types. In addition, the SERS enhancement by gold nanoshells is another avenue by which to increase the cellular Raman signal, and will allow the assay developer to use less blood for diagnosis.

4.3 What is needed for further develop and mature this technology

To further develop the portable label-free diagnostic technology, we will need to perform additional testing to narrow down the optimal sample preparation method. We were able to distinguish infected vs. uninfected from the PBMC fraction, but further enrichment of the innate immune cell populations in PBMC will make distinguishing bacterial vs. viral infection in whole blood samples much easier to detect. This can be done by using a molecular sieve in combination with CD14 antibody, which will isolate monocyte/macrophages, or use various TLR antibodies to isolate all cells that bare innate immune pattern recognition receptors that make them at least in theory responsive to pathogens in very early infection time points. Engineering to make the sample preparation step safe and easy to use will need to be done, and the Raman detector can be further miniaturized. Raman technology is very mature, and there are a wealth of vendors that provide miniaturized laser and detectors that can be used to build a customized detector that integrates with the sample preparation components for simple, reliable Raman spectroscopy. A simple computer will be integrated into the detector and will contain trained algorithm that allows the user to predict the pathogen based on the Raman signature gathered from the sample.

4.4 Application space for this technology

For a technology to be truly point-of-care, it cannot use any sophisticated methodologies such as RNA extraction or micro-liter scale liquid handling, therefore a compact, simple label-free diagnostic device will be immensely useful in aiding the physician's decision making in regard to infectious disease treatment. The application for a portable early infection diagnostic device will be widely applicable to anyone working in diagnostics. One can easily imagine a portable device being carried into combat or to MASH units, where field acquired infections require immediate triage and correct countermeasure administration. Alternatively, for civilian populations, a rapid, label-free test that a doctor can perform at the office to aid the decision for prescription of antibiotics can stem the emerging threat of antibiotic resistance development in pathogenic bacteria. Another use for this technology is in the field of hematology/immunology. Since the proposed portable Raman device is a peripheral blood diagnostic device, one can easily modify the sample preparation method to expand into diagnosing immune cell pathology and dysfunction in many immune related diseases such as autoimmune disorders, lymphoma, and leukemia.

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