



## **CPS 2022 RFP FINAL PROJECT REPORT**

### **Project Title**

Development of an infrared-functionalized microbalance sensor for *Cyclospora cayetanensis* detection and differentiation

### **Project Period**

January 1, 2023 – December 31, 2023

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### **Objectives**

1. *Determine the physiochemical signatures of model organisms using a cantilever-based microbalance sensing system.*
2. *Test the ability of the cantilever-based microbalance sensing system to accurately distinguish between the physiochemical signatures of multiple model organisms.*
3. *Determine the physiochemical signature of *Cyclospora cayetanensis* on the cantilever-based microbalance sensing system.*
4. *Test the ability of the cantilever-based microbalance sensing system to accurately distinguish the physiochemical signatures of *C. cayetanensis* from other protozoan parasites.*

**Funding for this project was provided partly through the CPS Campaign for Research**

## FINAL REPORT

### Abstract

The intestinal protozoan parasite *Cyclospora cayetanensis* is an important human pathogen with a global distribution. Because *C. cayetanensis* is spread through contaminated food and water, testing of food and water is needed to limit transmission, develop control strategies, and improve food safety. The current testing protocols for *C. cayetanensis* detection are time intensive and require extensive sample preparation and highly trained laboratory personnel. Thus, new methods for *C. cayetanensis* detection that are simple, fast, and low-cost with the potential for scalable implementation in the field would greatly enhance food safety. To build toward this goal, this project collected the foundational data needed to develop a sensing system that pairs infrared microscopy with cantilever-based microsensor technology to detect *C. cayetanensis*. Commercially available protozoan parasites, including *Cryptosporidium muris* and *Giardia muris*, were used to assist in the system design. Data from these model organisms demonstrated that the sensing system can be adapted for use with protozoan parasites and that signatures unique to individual parasite species can be used for differentiation. This data can be applied towards developing the sensing platform into a tool that can be used to detect and quantify *C. cayetanensis* quickly and cost-effectively. Such a tool could have a major impact on *C. cayetanensis* transmission and benefit consumers by improving the safety of fresh produce.

### Background

*Cyclospora cayetanensis* has emerged as a public health risk in the United States, and seasonal outbreaks have become a regular occurrence with significant health costs [1]. Additionally, when fresh produce is indicated as a transmission vehicle the resulting recalls have substantive costs to industry. These factors have made controlling *C. cayetanensis* transmission a priority issue for the United States Department of Agriculture (USDA), Food and Drug Administration (FDA), Centers for Disease Control and Prevention (CDC), and the Center for Produce Safety (CPS). However, *C. cayetanensis* control will not be an easy goal to achieve. *C. cayetanensis* is a difficult organism to study because it: 1) has a complicated life cycle; 2) is not possible to conduct *in vitro* studies as it cannot be cultivated; and 3) has no animal models for propagation or *in vivo* studies [2, 3]. These issues have left many aspects of *C. cayetanensis* biology, ecology, and risk contributors unresolved.

A key factor in controlling *C. cayetanensis* transmission is the ability to detect *C. cayetanensis* rapidly and accurately in food and water resources. However, the methods currently used for *C. cayetanensis* detection and identification, such as microscopic and molecular methods, are laborious, time intensive, expensive, and require highly trained laboratory personnel to be performed successfully. Additionally, although molecular methods are more sensitive than microscopy for parasite detection, inhibitors which may be present in samples from irrigation water and produce can impact the sensitivity of PCR, leading to false negatives [4]. PCR positive samples may also require additional sequencing steps to accurately identify *C. cayetanensis* resulting in additional time and costs. Novel research tools which can easily, accurately, and efficiently identify and quantify *C. cayetanensis*, and differentiate between infectious and innocuous forms of *C. cayetanensis* in irrigation water and fresh produce are urgently needed to facilitate effective intervention strategies and mitigate transmission risk. Thus, the purpose of this project is to use multi-modal sensing technology to develop an accurate, rapid, and scalable tool to detect *C. cayetanensis*. We have identified a microsensor platform that would make it possible to analyze samples for the presence of *C.*

*cayetanensis* faster than existing technology. This platform could be made available to industry to test irrigation waters or fresh produce for the presence of *C. cayetanensis*. Testing could be performed in minutes or hours, without the need for specialized laboratories and trained personnel, and would provide the information needed to better address risk factors associated with *C. cayetanensis* transmission.

Sensing technologies have attributes that can address the aforementioned industrial needs and be used to produce an accurate, rapid, and scalable tool for the study of risk factors associated with *C. cayetanensis* transmission. In particular, the field of nanosensors has flourished in recent years [5-7]. Some have been considered for agricultural uses, such as to monitor humidity in soil [8] or nutrients levels [9]. Others have been proposed for pest recognition but require the development of a DNA barcode [10], which retains the need to carry out painstaking PCR analysis of a large number of species. Microcantilever-based resonator sensors have attracted much attention because of their unique advantages such as extremely high sensitivity, miniature size, low-power consumption, label-free detection, and array-based sensing of multiple analytes in real-time [11-18]. Though most developments involving microcantilever-based resonators have been in fields such as explosive and warfare agent detectors or biomedical applications, recent advances have shown that they can be used to determine the mass and chemical fingerprints of microbial systems [19].

The core functioning of a cantilever-based resonator sensing platform consists of monitoring the cantilever resonance frequency (similar to a mass-spring) and its static deflection [12]. Changes in these two quantities can be used to detect the presence of analyte molecules adsorbed on a cantilever surface producing signals suitable to distinguish organisms of interest within a sample, such that it could become possible to develop a simple software interface to indicate the presence of *C. cayetanensis*. Thinking of the cantilever as a mass-spring system (**Fig. 1d**), adsorption of molecules or cells on a cantilever surface results in changing its mass, and thus its resonance frequency. When the adsorption is confined to a single side of the cantilever, cantilever deflection also changes [12] (**Fig. 1c**). Both the resonance frequency shift and the cantilever deflection vary in proportion to the type of molecular interactions and the number of adsorbed entities. From this, the mass of adsorbed analyte can be determined. To prevent false-readings and obtain biological specificity, a common approach has been to immobilize receptor molecules on the cantilever surface [14-17], which has shown partial success, though false-positives remain for heterogeneous samples. To improve this, a new functionality for analyte fingerprinting can be added by combining it with infrared (IR) illumination to detect the fingerprint of molecular vibrations of the analytes. This is possible because the analyte produces localized heat due to non-radiative relaxation processes at the IR wavelength corresponding to the excitation of vibrations relative to the different chemical bonds (C=C, C=O, etc.) comprised in the analyte (**Fig. 1e**). The changes in heat result in bending of the cantilever, which can be extracted to plot the IR fingerprint of the adsorbed material. Recent studies have shown that IR spectra, i.e., plots of heat-induced cantilever deflection as a function of illumination wavelength, of small volumes of analytes can be obtained by this approach [19] (**Fig. 1**).

This new capability addresses the need to 1) detect the presence of an adsorbed substance, 2) determine its composition, and 3) do so, in time, without laborious, time-intensive, expensive steps, and without the need for highly trained laboratory personnel. This platform has been used successfully in the food safety field to detect the mass and chemical fingerprint of food-borne pathogenic bacteria *Listeria monocytogenes* [19] (**Fig. 1**). Generally speaking, the integration of multiple signal generation techniques into a single device is highly desired to enhance the detection sensitivity and selectivity of a microresonator-based platform [20]. The type of signals detected can be adjusted to take advantage of specific traits of the targeted entity.

In this proof-of-concept project, the core sensing capabilities to acquire foundational data needed to combine mass, IR spectroscopy and fluorescence signals with a microresonator-based platform were developed. The sensing system developed can detect the presence of and measure compositional difference between cells, therefore providing the ability to identify the presence of *C. cayetanensis* (**Fig. 2**). IR spectroscopy was selected for this as it will excite molecular vibrations in cells, which is expected to provide a unique fingerprint, as is the case for other biological entities such as bacteria or mammalian cells. The position of the IR bands is directly related to the nature of the chemical bond. Hence, the IR spectrum provides information on the presence of C-H, C=O, C-N, N-H, C-C bonds and more [21]. As a result, changes in the cell wall composition, or denaturation of a cell can be detected from monitoring the IR signature of the cell. In a system using oocysts of *Cryptosporidium parvum*, one of the most important waterborne parasites in industrialized nations, it was reported that IR spectra exhibit similar IR bands to those observed in bacterial cells, which can be seen as combinations of macromolecules in the form of proteins, polysaccharides, nucleic acids, and lipids. For example, *C. parvum* oocysts exhibit IR fingerprints that can be used to monitor small changes in their surface adhesion, and IR spectroscopy has been successfully used to demonstrate the effect of inactivation treatments through measurement of changes to the amide II band signal of oocysts [22]. The literature on parasites remains sparse but IR spectroscopy has extensively been used to study bacteria [23, 24]. Several groups have demonstrated successful discrimination of bacterial strains and live/dead cells in culture from IR fingerprints [25]. It is likely that this approach has not been considered for *C. cayetanensis* because the lack of *in vitro* or animal model-based oocyst propagation systems has hampered protocol development. One important advantage of combining IR spectroscopy with the microcantilever-based microbalance is the significant reduction of cells needed to get a detectable signature – i.e., possibly down to a single cell at a time. The number of cells being detected can be adjusted to find a good balance between high sensitivity and discrimination in the composition of a given water sample while assessing a representative sampling.

Oocysts of *C. cayetanensis* are spherical (~10  $\mu\text{m}$  in diameter) with a ~50 nm bilayered wall with a “wrinkle” coat [3]. Just like other eukaryotic cells or bacteria, they contain a complex mixture of phospholipids, proteins and other organic molecules [26]. As oocysts sporulate, they undergo changes in the nature and organization of these organic components. Hence, we infer that by monitoring the IR signature of one to a few cells, it will be possible to determine the IR signature of *C. cayetanensis* in its various forms. Furthermore, the ability to calibrate the microcantilever-based platform to determine the mass of the substance adsorbed on its surface will lead to a microbalance functionality for the proposed sensor, which can be optimized for characterization of the parasite at the single oocyst/cyst level to increase the sensitivity and specificity of the sensor. As shown in **Fig. 2**, the sensor will be designed to play the role of a rapid screening method for the identification of entities with characteristics (size, mass, chemical signature – potentially others) corresponding to those of *C. cayetanensis*.

This 1-year project was the first step toward developing a sensitive and specific sensing system for *C. cayetanensis*. Initial efforts were focused on pairing IR spectroscopy with microbalance sensor technology to optimize parasite detection. While the main goal was to assess detection of *C. cayetanensis*, initial testing and design of the sensing system utilized model organisms such as *Cryptosporidium* spp. oocysts and *Giardia* spp. cysts. We acknowledge the limitations of model organisms for direct translation to use for *C. cayetanensis* screening. However, these model organisms are also important food safety protozoan parasites and commercially available in the quantities needed for the exploratory experimentation needed in the testing phase of the sensing system design.

Our long-term goal is to design a cost-effective sensing system so that it can be easily used in screening laboratories and by growers and processors to screen food and water samples for the presence of *C. cayetanensis*, providing a much-needed tool in the study and

control of *C. cayetanensis* transmission. However, this year-long proof-of-concept project only sought to obtain the foundational data needed to develop an infrared-functionalized microbalance sensor for *C. cayetanensis* detection and differentiation.

## Research Methods and Results

For the development of a cantilever-based platform which can measure the mass and IR signature of model organisms with high sensitivity and selectivity, we tested a first version of the cantilever-based sensing platform using commercially available cantilevers. Delivery of sample onto the cantilever was performed using a micropipette delivery system for controlled drop-by-drop delivery. Individual droplets containing parasite cysts/oocysts were used to demonstrate the mass determination capability and the IR fingerprinting capability of the system. Measurements on individual cysts/oocysts cannot be done with conventional analytical tools, hence our process involved, in parallel, acquiring signals that could be used as benchmarks to determine the viability of the data acquired with our sensing platform.

This platform was used to collect orthogonal signals: 1) cantilever deflection (bending), 2) shift in resonance frequency due to selective adsorption, and 3) calorimetric infrared spectroscopic signal from the analyte in the channel and cantilever bending fluctuations (**Fig. 3**). Changes in microchannel cantilever deflection were measured using an optical-beam deflection method, as illustrated in **Figs. 1 and 2**. Monitoring the deflection as a function of illuminating wavelength produced the IR spectra of the sample present on the cantilever. This cantilever-based sensor combines the selectivity of IR spectroscopy with thermal sensitivity of a bi-material cantilever to obtain IR spectra of nanoliters to picoliters of samples. The work involved the development of an interfacing scheme to synchronize the parameters of the laser, the recording of the cantilever motion resulting from the presence of cells, and its reaction to infrared illumination.

To assess and calibrate sensor performance several figures of merit of the sensor had to be established. We first determined the limit of detection of the cantilever for mass detection based on 1) static deflection and 2) resonance frequency shift. This was done using sequential dilution of polystyrene (PS) beads. Given the spherical dimensions of the beads, it was possible to get a reliable estimate of their mass to calibrate the resonance shift to the mass of the analytes. These experiments allowed us to develop a computer-assisted platform to monitor the resonance frequency of the cantilever as a function of added mass. With this system in place, we were able to demonstrate that accurate detection of added mass could be achieved with the system using polystyrene beads of a similar size to *C. cayetanensis* oocysts. Such data can be used to calibrate mass and assist in estimating oocyst concentrations present in a sample.

Next, we determined the limit of detection of the IR platform by sequentially decreasing the concentration of beads and carrying IR measurements. We also compared the performance between the IR laser illumination and conventional Fourier-Transform Infrared (FTIR) spectroscopy illumination configurations. Here we first developed the computer-assisted platform that monitors the cantilever resonance frequency and the infrared spectrum of the entities on the cantilever. These data were necessary to confirm that the resonance frequency obtained with the mechanical shaker is identical to the resonance frequency obtained with the pulsed laser. In addition to the resonance shift, PS beads were used to demonstrate that the sensor detects a chemical fingerprint in agreement with the fingerprint obtained using conventional analytical tools (FTIR spectroscopy). We next evaluated the performance of the sensor to detect materials with different fingerprints by assaying polyvinyl acetate (PVA) and graphene-like carbon rods on the platform. Through these experiments, we were able to verify that the platform consistently fingerprints compounds according to expectations (**Fig. 4**). PS and

PVA fingerprints are well known from FTIR spectra databases. However, the carbon rods illustrate that entities with dimensions so small (<50  $\mu\text{m}$ ) that they cannot be detected by FTIR spectroscopy, can clearly be measured with our platform, as shown by the bands corresponding to graphitic and amorphous carbon in **Fig. 4**. The last step in validation of the IR capabilities of the system included testing of mixtures of different materials, with the sensor successfully differentiating mixtures of polystyrene and PVA. In this case, as shown in **Fig. 5**, we first prepared a cantilever with PS beads (~10) and very limited amount of PVA. The fingerprint exhibited a strong signal of both polymers. However, the fingerprint changed significantly in the strength of the PVA signal after adding an additional layer of PVA to the system. Quantification of these signals can be performed to determine more precisely the mixture patterns on cantilever-based sensors.

To take advantage of the autofluorescence properties of *C. cayetanensis* as an added mode of detection in the sensing system, fluorescence sensing was tested on the cantilever for the first time. This is important as it would provide an additional level of screening for cases in which pathogen fingerprints would exhibit strong similarities, with only *C. cayetanensis* emitting an autofluorescence signal. We first developed a system to focus visible excitation laser on a cantilever and collect the resulting scattered light and optimized the cantilever orientation to collect scattered photons. To validate the system, testing with pyranine, a common fluorescent dye, and with Ruthenium and FITC encapsulated in silica ( $\text{SiO}_2$ ) nanoparticles, which is a custom-made pH nanosensor developed at the University of Central Florida (UCF), was performed. Successful excitation and detection in expected wavelengths were achieved for these fluorescent compounds on the sensor and provided emission spectra corresponding to measurements carried out on bulk quantities of the entities. The signature obtained from the Ruthenium and FITC encapsulated in  $\text{SiO}_2$  nanoparticles is shown in **Fig. 6**.

Initial testing of the sensing system and determination of the conditions needed for parasite detection was performed using two model parasites. This strategy was necessary because *C. cayetanensis* is not available in the quantities needed for the initial testing of the cantilever-based microbalance sensing system which required high numbers of parasites. For the initial testing, two parasites that can be propagated in animal hosts were used. These parasites, *Cryptosporidium muris* and *Giardia muris*, are protozoans like *C. cayetanensis* and served as model organisms in the testing and design of the sensing platform. Both *C. muris* and *G. muris* can be propagated in rodents to produce large quantities of oocysts and cysts, are commercially available, and are not human pathogens, making them safe to handle in a BSL-1 laboratory setting.

To determine the physiochemical signatures of individual model organisms, *C. muris* oocysts and *G. muris* cysts were obtained from Waterborne Inc. To prepare samples for analysis with the sensing platform, parasite preparations including additional cleaning, concentration, and quantification steps were performed. Readings of parasite physiochemical signatures were hampered by a lack of data on the IR signatures of these organisms to serve as a basis for exploring their signatures when paired with the cantilever-based detection system. Thus, the first step in this process was obtaining the IR fingerprints of these organisms using conventional IR spectroscopy. Repeated readings were required to obtain the data needed for statistical differentiation of parasite unique signatures. This data was tedious to obtain but can now be used to inform expectations of variability in physiochemical signature of these organisms. Importantly, the IR fingerprint of these organisms has been obtained using the microcantilever sensor, and we were able to demonstrate that unique aspects of the fingerprint of these parasites exist and can be used to differentiate them (**Fig. 7**). These results further confirm that this sensing system can be developed for *C. cayetanensis* detection and differentiation.

## Outcomes and Accomplishments

Several major accomplishments have been achieved through this project that build toward our goal of developing an infrared-functionalized microbalance sensor for *Cyclospora cayetanensis* detection and differentiation. We have demonstrated the suitability of the system for parasite detection based on mass sensing. This achievement builds on Objective 1 where we aimed to demonstrate the ability of the microbalance to sense the presence of cells. The goals of Objective 1 were also met in our achievements in assessing the capacity to perform infrared sensing on the cantilever. Importantly, we demonstrated that the sensor detects a chemical fingerprint that agrees with the fingerprint obtained using conventional analytical tools. We evaluated the performance of the sensor to detect materials with different fingerprints including polystyrene, PVA, and carbon rods and demonstrated that these materials could be identified even when assayed in combination. These observations addressed the goals of Objectives 2 and 4 to test the ability of the cantilever-based microbalance sensing system to accurately distinguish between the physiochemical signatures of multiple model organisms even when present in mixtures. Additionally, we demonstrated that model parasites *Cryptosporidium muris* and *Giardia muris* had IR signatures that can be detected with the cantilever system and be used to differentiate these organisms. Acquiring these spectra with a microcantilever-based platform is a unique demonstration of the capabilities of the platform, which had never been previously achieved. We also demonstrated for the first time that fluorescence sensing can be used as an additional orthogonal channel for *C. cayetanensis* fingerprinting on the cantilever. This achievement addresses Objective 3 as it builds toward a specific set of parameters that can be used for *C. cayetanensis* detection and differentiation.

Taken together these accomplishments represent significant progress in a novel field of study and can be combined with other data in the future development of the multimodal sensor that is the long-term goal of this project.

We experienced several unexpected outcomes throughout the course of the project that affected what could be achieved within the project period. The first major issue was in the delivery of the microdroplet containing sample onto the cantilever. Our goal was for the solution to be delivered to the cantilever drop-by-drop, which was a decision made to adapt to the proof-of-concept period. This design was selected for the initial design phases to bypass the time-consuming nanofabrication recipe development required to design a microfluidic channel integrated on the cantilever. Troubleshooting this delivery system proved much more time consuming than we anticipated, as is often the case when adapting emerging technologies for new applications. More specifically, the size of the droplet produced with the low-cost system developed remains slightly larger than the cantilever dimensions, which led to forces that repelled the droplet away from the free-standing beam toward the larger part of the chip. However, a suitable solution was finally achieved, and we were able to obtain much of the data needed to validate the concepts outlined in our project. We were also challenged by the repeated measurements of the mixtures needed to be made with conventional infrared spectroscopy. Conventional infrared measurements are tedious because they require a lot of replicates for statistical analysis because of the low number of cells, which is at the limit of what the conventional tool can detect. In fact, even more measurements are required for statistical analysis to be satisfactory to present the first fingerprint data in the literature. This challenge is also significant for the cantilever-based IR measurements, as each cell can provide slight variations that are not usually accessible from the conventional infrared measurements. Additionally, we have observed that preparing mixtures of cells on the conventional cantilever is challenging and will not be representative of the concentrations in the solution. However, we were still able to obtain valuable data that can be used for future refinement of the sensing system.

## Summary of Findings and Recommendations

Through this year-long proof-of-concept project, we have developed a new sensing platform for the characterization of single parasite cells, providing information about their mass, their composition, and their fluorescence properties. The data collected to date suggests that the sensor produces signals in agreement with what is measured with conventional tools in cases that were selected to work on both platforms. This provides confidence that the signal recorded with the new platform on systems that are beyond the limit of detection of conventional tools can be trusted. Thus, an infrared-functionalized microbalance sensor seems promising as a potential tool to improve *C. cayetanensis* detection and differentiation.

Delivering the samples to the sensor was the most challenging aspect of the project, indicating that moving toward a microfluidic flow cell solution is necessary for a more comprehensive study on this topic. However, our results showing the different morphology, stiffness, and composition of the single parasite forms suggest that this will be a viable solution. While the best application of the technology may be in the identification of new contaminants, or detection of minute quantities of a sample, rather than in the continuous screening of large amounts of water, the strides made in the project are expected to steer new ideas of applications of these types of sensors for the detection of *C. cayetanensis* and related pathogens.

## APPENDICES

### Publications and Presentations

One poster presentation of this research was made at the annual CPS Research Symposium in Atlanta in 2023, and final results will be presented at the symposium in Denver in 2024.

### Budget Summary

A total of \$100,757 in research funds was awarded to this project. Budget was expended on reagents and supplies needed to complete the experiments described in this report. Because unexpected delays that occurred early in implementation of the sensor design, the entire budget was not spent.

**Figures 1–7** (see below)

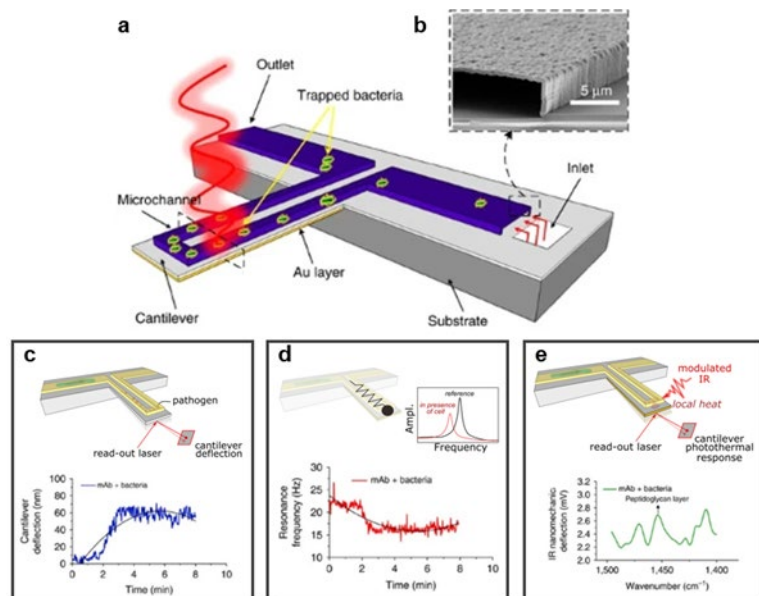


Figure 1. Summary of state-of-the-art multi-functional sensing platform (adapted from Etayash et al [19]). (a) Microfluidic cantilever resonator optimized to detect the presence of bacteria by monitoring the deflection (c), resonance frequency (d), and IR signature (e) as the cells flow through the device. Dimensions of the microfluidic device were optimized for bacteria, as shown in the electron microscopy image of the cross section of the microfluidic channel (b).

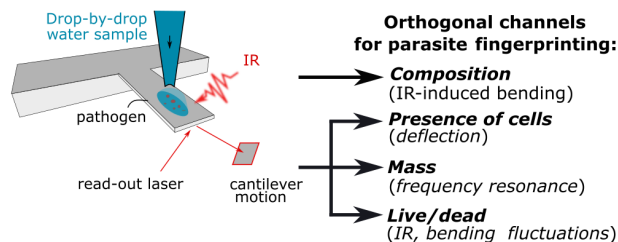


Figure 2. Proposed work for 1-year period: determine the optimal conditions and performance of a cantilever-based platform for multi-modal detection (non-specific detection of presence of a cell, mass, composition, live/dead assessment) of single cell to few cells of parasites, using model systems and *C. cayetanensis*.

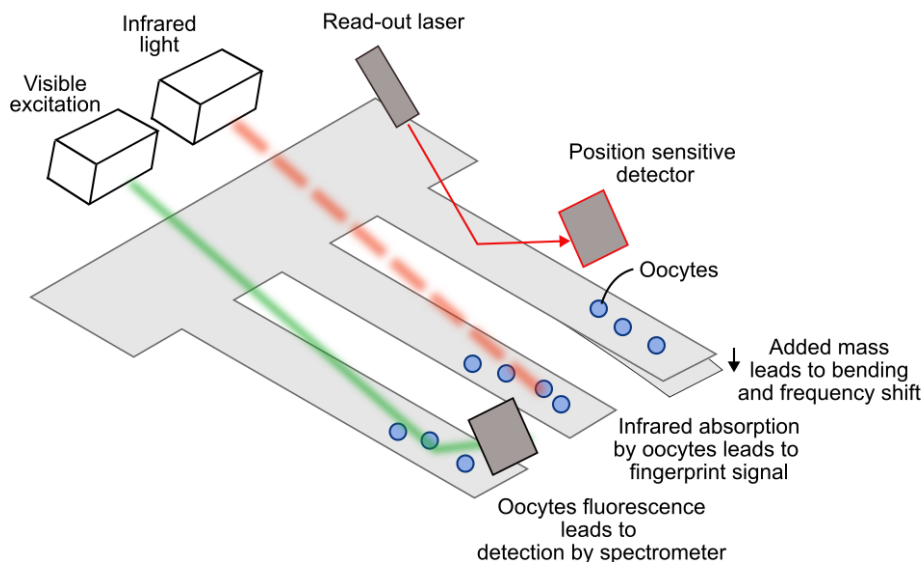


Figure 3. Experimental design of the sensing platform showing multimodal measurements tested in this project.

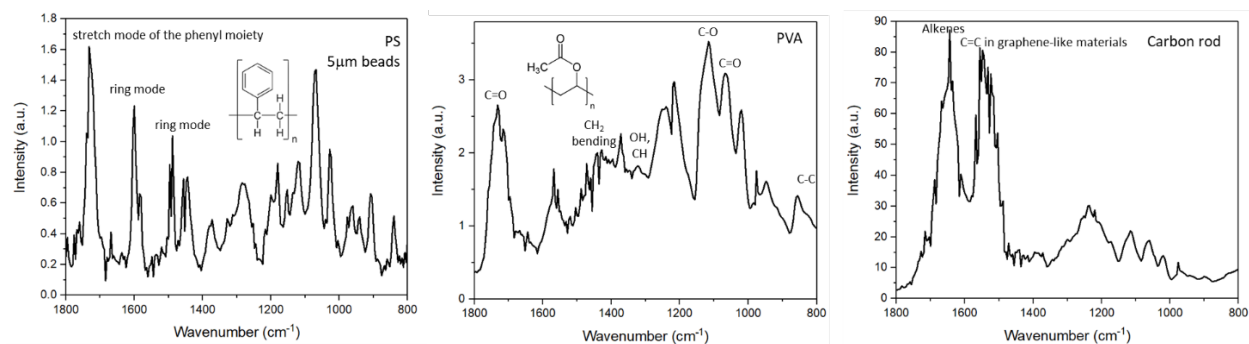


Figure 4. Infrared (IR) spectra collected with the cantilever-based sensing platform decorated with (left) PS beads, (middle) PVA and (right) carbon rods.

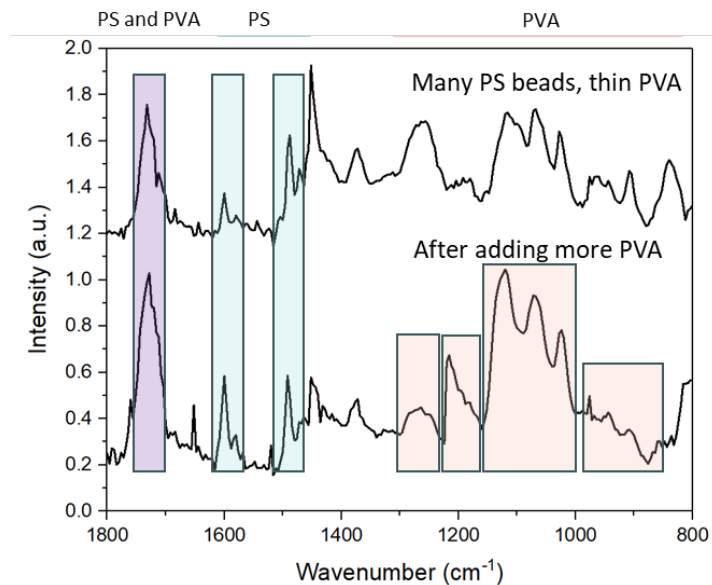


Figure 5. IR spectra collected in presence of (top) ~10 PS beads and a thin layer of PVA, and (bottom) after adding an additional coat of PVA to the cantilever.

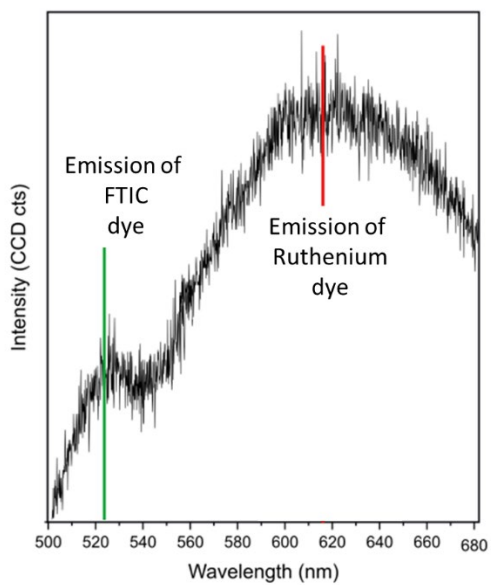


Figure 6. Fluorescence spectrum acquired from the dual-dye silica nanoparticles positioned on the cantilever-based sensor.

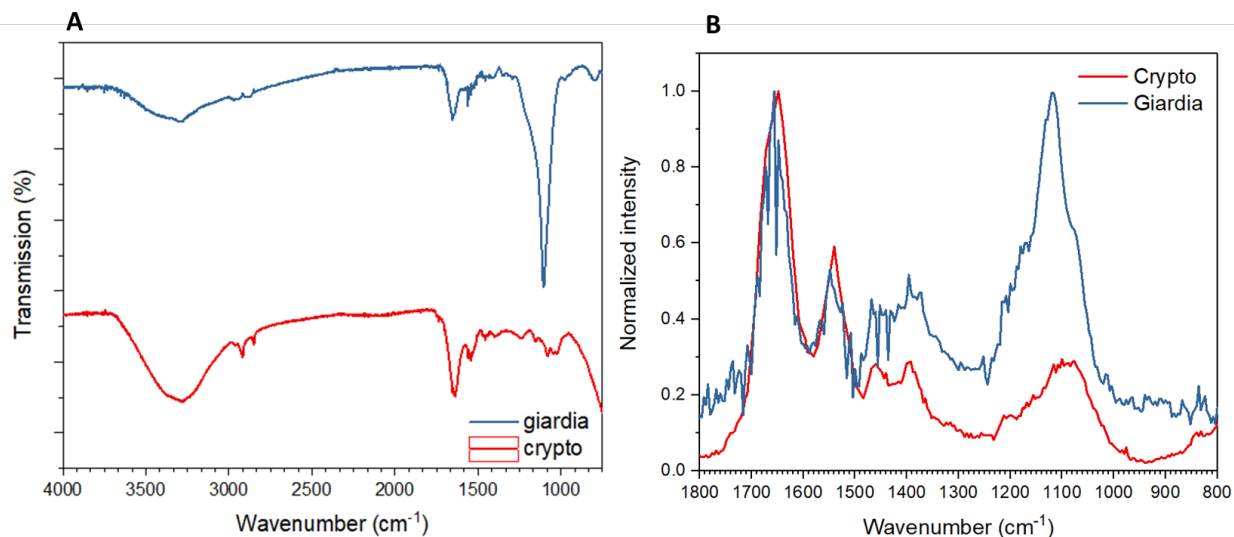


Figure 7. Infrared spectroscopy fingerprinting data for *Cryptosporidium muris* and *Giardia muris*. Panel A shows IR fingerprinting (transmission signal) overlays from conventional spectroscopy. Panel B shows IR fingerprinting data (absorption signal) collected from microcantilever sensor.

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