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Low-Frequency Raman Spectroscopy as a Diagnostic Tool for COVID-19 and other Coronaviruses

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Complete List of Authors:	Jacobi, Leor; Bar-Ilan University, Damle, Vinayaka Harshothama; Bar-Ilan University Rajeswaran, Bharathi; Bar-Ilan University Tischler, Yaakov R. ; Bar-Ilan University
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Abstract: Amidst the current worldwide COVID-19 pandemic, improved diagnostic tools are of vital importance. Low-Frequency Raman (LFR) Spectroscopy provides a robust theoretical scientific basis for eventual development of a non-invasive Diagnostic Tool for specimens or patients directly. This preliminary research will begin mapping the nanostructure of COVID-19 via LFR and regular Raman Spectroscopy, in comparison with different Coronaviruses and other viral materials, helping to lay a groundwork for future research. In addition to its distinct nanostructure, effects on it by thermal fluctuations or decomposition, decay under laser excitation, and interference from buffers will be examined.

Low-Frequency Raman Spectroscopy as a Diagnostic Tool for COVID-19 and other Coronaviruses

Background and Rationale

Coronaviruses are named for their distinctive outer fringe, crown or “corona” of embedded envelope protein. Members of the family *Coronaviridae* cause a broad spectrum of animal and human diseases. The RNA genome replicates through the generation of a nested set of viral mRNA molecules. In 2003, coronaviruses first began to attract broad interest with the zoonotic SARS-CoV (Peiris & Poon, 2008); later, in 2012 the emergence of MERS-CoV has confirmed the coronaviruses as significant causes of severe respiratory disease (Al-Hazmi, 2016; De Wit et al., 2016; Ramadan & Shaib, 2019).

The current 2019–2020 COVID-19 pandemic is caused by the SARS-CoV-2 viral infection, also a member of the Coronavirus family. Genetically distinct and not descended directly from the strains which caused prior outbreaks, the infection is apparently the result of a separate animal/human viral transfer incident (Sohrabi et al., 2020; Y. Wang et al., 2020).

SARS-CoV-2 is currently diagnosed directly primarily via laboratory tests which include Real-Time Polymerase Chain Reaction (RT-PCR) with smears from the upper and lower respiratory tract (Sheridan, 2020). The test is used for primary laboratory diagnostic examination of patients with symptoms clinically suspected to stem from SARS-CoV-2 infection. Additionally, serological tests are capable of detecting antibodies against SARS-CoV-2 in the blood (*CDC Tests for COVID-19 | CDC*, 2020; Li et al., 2020). These can supplement other forms of detection, support a diagnosis of SARS-CoV-2 infection and/or help confirm RT-PCR results. CT Scans of the chest can help identify and characterize lung pathology, including COVID-19 infection. However, findings are nonspecific and found as well in other types of pneumonia. No study to date has demonstrated that CT scans can distinguish COVID from other viral pneumonia (Bai et al., 2020).

In the absence of either a vaccine or widely effective and applicable treatments, development of improved diagnostics is the primary means of controlling the spread of the SARS-CoV-2 infection. Only maintenance of strict segregation between infected and healthy members of the population through strict quarantine can slow the spread of the pandemic. Experts say South Korea's broad availability of testing helped reduce the spread of the novel coronavirus. Testing capacity, largely in private sector labs, had been built up over several years by the South Korean government (Kuhn, 2020).

Existing diagnostic methods suffer from drawbacks of speed, reliability, and cost. They do not adequately meet the needs of large populations where communal infection has set in and large-scale rapid testing is required. Intensive efforts have gone into development of immunoassays for improved testing (Amanat et al., 2020; Trivedi et al., 2019; Xiang et al., 2020) by detection of specific levels of antibodies generated by the immune system to fight off the SARS-CoV-2 infection. Although these advances are highly promising, the tests have not been approved to date by many government agencies and are considered experimental. Development of alternative diagnostics to work independently of or in tandem with existing methods remains a primary objective.

Raman Spectroscopy is a powerful technique for identifying chemicals and characterizing materials based on their vibrational modes. It has been applied to a broad range of scientific fields and a broad range of material phases, ranging from single organic molecules to bulk inorganic semiconductor

crystals. Raman spectra provide insight into numerous properties, including morphology, stress/strain, crystallinity, doping level, conductivity, local temperature, and polarizability, whether in bulk, thin film, monolayer or nanostructure form. Raman spectroscopy finds applications in the physical sciences, life sciences, medicine, drug discovery, and semiconductor metrology. In chemistry, Raman Spectroscopy is often employed to produce structural fingerprints to identify molecules. Raman's scientific potential continues to develop (Barron, 2015; Schmitt & Popp, 2006). Given both its proven and theoretical diagnostic potential, Raman Spectroscopy remains a relatively unexplored method for Coronavirus diagnostics (*Expert suggests a new way to study COVID-19 - The Hindu*, 2020).

Near-infrared Raman Spectroscopy has demonstrated enormous potential for non-invasive detection of blood Glucose levels (Berger et al., 1997; Pandey et al., 2017). Intensive research on this issue hopes to eventually revolutionize the standard invasive maintenance procedure for patients suffering from Type 1 Diabetes. It also informs the current study on methodologies for developing non-invasive diagnostics as a future objective.

Surface-Enhanced Raman Spectroscopy (SERS) can rapidly detect and discriminate among different food and waterborne viruses, to provide “rapid, sensitive, and reproducible detection results with minimum sample preparation for virus detection” (Dardir et al., 2020; Fan et al., 2010; Moor et al., 2013; H. N. Wang et al., 2013). Raman Spectroscopy can be applied to either individual viruses or entire classes (Blanch et al., 2002; Cialla et al., 2010), especially when employing Raman Optical Activity (ROA), invented and developed by Laurence Barron, et al (Barron, 2015; Barron et al., 2004).

Various forms of Raman Spectroscopy have been demonstrated to be valuable methods for investigating structures of viral proteins and nucleic acids, as well as the assembly pathways and architectures of native viruses (Němeček & Thomas, 2009a, 2009b). SERS can be finely attuned to the molecular level of identification of virus mutations (Dardir et al., 2020) and classification of virus strains (Hermann et al., 2011) which other diagnostic tools do not promise to provide in the near future.

Analysis of viral cells can be performed either on specimens (Moor et al., 2013; Salman et al., 2013) or developed for non-invasive use on animals and humans as an important tool for virologists (Lambert et al., 2006). Experimental trials of Raman Spectral analysis as a tool for rapid screening of populations suffering from viral infections have proven successful (Mahmood et al., 2018) and has even been applied to detection of cancer in individuals (Salman et al., 2013).

In Raman Spectroscopy, instrumental limitations (associated with filtering out the incident laser to prevent saturation of the spectrometer's CCD camera) dictate that the Raman spectrum is obtained for spectral shifts of about 100 cm^{-1} away from the laser up to about 3500 cm^{-1}, a more than sufficient range to capture the whole "chemical fingerprint region". Modern laser filters, based on volume holographic grating-based filters (VHF), amongst other approaches, now make it relatively straightforward to obtain Raman spectra from 100 cm^{-1} down to 5 cm^{-1} without the need for triple-monochromator instrumentation. In this spectral range, referred to as Low-Frequency Raman (LFR), the Raman scattering is sensitive to the phonon dispersion relation and vibrational modes associated with the nanostructure of the material. LFR has been applied to characterize chiral purity of organic crystals and formulations, biomolecular assemblies, hybrid organo-metallic perovskites, and metal-organic frameworks (Nemtsov et al., 2018). The structural and morphological signatures of a material are often distinctly “expressed” in the LFR Spectrum (Colaianni & Nielsen, 1995b; Uliel et al., 2019a). This spectrum evolves from acoustic modes, lattice interactions, shear vibrations, hydrogen bonds, π - π stacking, etc. Nanostructures are known to have a pronounced Raman spectrum in the LFR region. The COVIDCOVID-19 Coronavirus, as depicted in Figure 1(A) below, also can be presumed to have a

distinct nanostructure. Whereas other nanostructures have been employed to detect Coronaviruses (Ahmed et al., 2018; Chen et al., 2016), the nanostructure of the virus itself is poorly characterized. Hence, LFR has the potential to serve as a powerful spectroscopic tool for diagnosing COVID-19. The ultimate long-term goal of this preliminary research is to deliver a hand-held Raman detector to diagnose the COVID-19 Coronavirus non-invasively as depicted schematically in Figure 1(B).

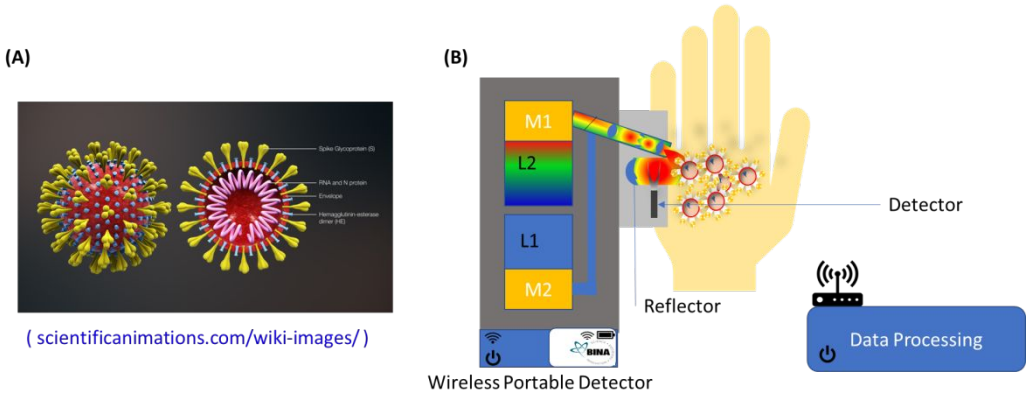

Figure 1. (A) Structure of COVID-19 virus and (B) schematic of portable detector with a remote data processing unit.

Various challenges are anticipated in attaining the goal of adapting Raman spectroscopy to tissue. These include fluorescence, background signals, and laser intensity below ANSI limit. It is our hope that our work will serve as a preliminary study for an in-vivo point-of-care diagnostic device. Certainly, such a device will have to address many issues that are beyond the scope of the present study and are beyond our current experience. LFR filters based on VHF technology are commercially available for wavelengths as short as 405 nm and longer than 1550 nm. Hence, these experiments can be performed in spectral regions that are at wavelengths which are shorter or longer than the typical fluorescence spectra of tissue. An advantage of using near-UV wavelengths would be a near resonant increase in LFR scattering. Advantages of using 785 nm would be transparency and increased penetration into body tissue as well as lower-cost solutions for high quality single mode laser sources. Moving to 1550 nm would allow for higher laser power while still maintaining ANSI safety regulations since the naked eye does not detect such long wavelengths (*ANSI Z136.1 - Safe Use of Lasers | The Laser Institute*, 2014). It may not be possible to detect the LFR contribution of COVID-19 in an in-vivo setting or the applicability of the method if it only works on very high concentrations of COVID-19. As of now, we simply do not know our limit of detection, or the concentration of virus in a real in-vivo setting.

The present study represents a preliminary developmental foray into building a scientific foundation for a technological device which could supplement existing diagnostic tools during the current pandemic. Despite the great promise of Raman Spectroscopy as an outstanding diagnostic for Coronavirus which could eventually result in cheap, quick, and noninvasive virus detection, much groundwork remains to be done. Even if the full potential of Raman Spectroscopy as a diagnostic tool may not be realized until a future epidemic of another strain of Coronavirus or another viral agent, research in this field remains of vital importance.

Hypotheses

Hypothesis 1: the nanostructure of COVID-19 does not change during the measurement due to thermal fluctuations or decomposition. The duration of the measurement period for obtaining LFR spectra

could be up to 5 minutes, while the lifetime of COVID-19 on surfaces has been reported to be more than a week. Hence, the nanostructure of the COVID-19 virus should remain stable enough to detect.

Hypothesis 2: the distinct nanostructure of COVID-19 will cause distinguishable differences in the LFR spectrum, i.e. a unique spectral signature. Often a single change in the hydrogen bonding network, if repeated throughout the nanostructure being measured, can result in pronounced spectral shifts, as has been reported in comparing the LFR spectrum of chiral amino-acids nano-crystallites vs. racemic nano-crystallites (Aviv et al., 2017; Damle et al., 2018).

Hypothesis 3: the nanostructure of the COVID-19 will not decay due to laser excitation, rendering it difficult to detect.

Materials and Methods

Materials:

Samples for this study will be obtained by a certified virologist following all the required ethics protocols. These samples may be derived from blood, saliva, or swabs from the throat of consenting patients infected by Coronaviruses and other viruses or viral materials may be grown in laboratories (*CDC Grows SARS-CoV-2, the virus that causes COVID-19, in Cell Culture | CDC*, 2020). Although it will not be attempted during the proposed preliminary research, samples may eventually be "taken" non-invasively by illuminating the patient's body with a laser directed at the lungs, or nasal cavity, etc., and then collecting spectra (Pandey et al., 2017), which should be present in cases of viral infection whether or not the patient displays symptoms.

The obtained samples will likely be dispersed in an appropriate buffer solution or host-matrix to maintain their freshness. If dispersed in liquid, then this liquid will be supplied between a microscope slide and glass cover slip to prevent evaporation of the buffer and denaturing of the viruses. If dispersed in a host-matrix, then the host/virus-guest dispersion will be drop-casted or spin-casted on to a glass microscope slide. Sample preparation and extraction has been proven to be of high importance for reproducible results in the field of bacteria identification (Němeček & Thomas, 2009b; Pahlow et al., 2015).

Methods:

For this study, all the Raman Spectroscopy measurements will be performed in a micro-Raman configuration, consisting of microscope combined with laser light excitation, appropriate collection optics, and spectrometer. In particular, the microscope is based on the upright microscope module BXFM (Olympus, Shinjuku, Japan). The optical set up for regular Raman spectroscopic measurements is presented in Figure 2(A). The set up presented corresponds to a reflection geometry that consists of an excitation laser at wavelength λ_{exc} = 532 nm, which is focused on the analyte using a microscope objective lens. The Raman scattered light is collected using the same objective lens; however, the collection geometry is decoupled from the excitation geometry using a 50:50 beam-splitter. The decoupled Raman signal beam is passed through a Razor Edge Long Pass Filter (Semrock, Rochester, NY) in order to block out the laser excitation from passing into and saturating the detector. The signal beam is then coupled into an imaging spectrometer (SP-2500i, Princeton Instruments, Trenton, NJ, USA) with an EM-CCD camera (Pro-EM 16002, Princeton Instruments, Trenton, NJ, USA); where, an optical grating resolves the spectrum and projects it onto a CCD. For the regular Raman measurements, the laser optical power will be set to a maximum of 30 mW, with acquisition times of 5 seconds and a grating groove density of 600 g/mm.

For the LFR measurements, here we will use an optical setup that employs the special VHF type of laser blocking filters) that enable the probing of spectral shifts as low as 5 cm^{-1}. The schematic is presented in Figure 2B. In particular, the LFR measurements will be taken using an integrated laser and VHF system (ONDAX, XLF-MICRO 532 nm) with 50 mW of optical power at an excitation wavelength of λ_{exc} = 532 nm. In this case, the laser output is again focused into the sample via a lab-built microscope based on the upright microscope module BXFM (Olympus). The LFR signal after passing through 2 VHF elements is clean of the laser excitation to about 9 orders of magnitude suppression and is then routed into the imaging spectrometer system described above. For LFR measurements, we will use a longer acquisition time of 25 seconds, and the groove density of the selected grating will be set to 1800 g/mm in order to obtain higher spectral resolution data of the LFR Raman peaks.

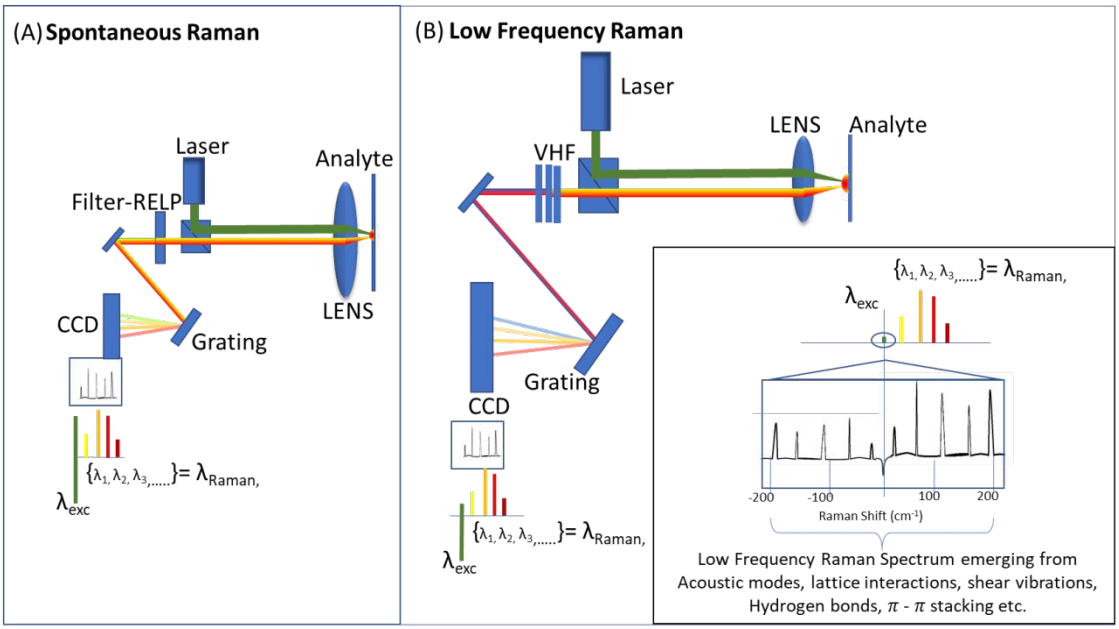

Figure 2 Schematics of optical set up: (A) Regular Spontaneous Raman Spectroscopy (B) Low-Frequency Raman Spectroscopy. Here, λ_{exc} is the excitation wavelength and $\{\lambda_1, \lambda_2, \lambda_3, ...\} = \lambda_{Raman}$ is the set of Raman bands. In the inset id the schematic representation of Low-Frequency Raman bands and the source of their origin.

We will examine the regular Raman results of the samples. If the regular Raman spectrum is inconsistent with the generally anticipated spectral features of viruses, then we will exclude the LFR spectrum of the sample from the overall analysis and statistical modelling. Such discrepancies in the regular Raman spectrum will be indicative of samples that have undergone changes in their constituency. Thus, specimens which have been corrupted or altered will be excluded from the population of samples. The COVID-19 nanostructure indicated by the results will be compared with that of other Coronaviruses and other viruses which have already been researched (Blanch et al., 2002; Dardir et al., 2020; Mahmood et al., 2018; Mori et al., 2018), using both LFR spectroscopy and regular Raman spectroscopy. We will also compare the various viruses to their host-matrices and buffers.

It will be very important to address background scattering from the solvent or host matrix that the virions are situated in, whether these compounds generate light scattering in the same spectral range as the COVID-19. Although water does not generally exhibit significant background in Raman

Spectroscopy, it poses a problem in the LFR region (Nielsen, 1993), where it exhibits a very strong scattering due to "tail" scattering from the Rayleigh line, thus obscuring the LFR effect of the solute (Colaianni & Nielsen, 1995a). If this scattering spectrum is much stronger than the scattering from the virus, then it will be necessary to perform careful background subtraction and it will thus be more difficult to detect the COVID-19. One work-around would be to use a solution with a higher concentration of virus. In solid form, it will be necessary to characterize the LFR contribution of the host-matrix and to choose a matrix that exhibits minimal scattering. If the intensity of this contribution, which mainly originates from the solvent/matrix, is much greater than that of the target, advanced techniques, such as a dry sample, without liquids from the solvent in the background, Stimulated Raman or Microcavity-Enhanced LFR, might be necessary to recover the spectral information of the target (Uliel et al., 2019b). If a significant fraction of a solid sample consists of the virus, then it is reasonable to assume that LFR scattering from the host will not be a major source of background.

Since we are not virologists, we intend to follow the lead of these experts and their protocols will serve as a starting point for our study. Our main requirement is that whatever extraction methods are used should maintain the nanostructure of the COVID-19 virus, and preferably also lead to a high concentration of the virus, with a relatively low concentration of water and/or host matrix material.

We often see that high laser light intensity can degrade biological and even inorganic material. Hence, there is little doubt that the virus will degrade upon prolonged laser excitation. We are committed to characterizing this process of the degradation of the virus during laser excitation in both LFR and Regular Raman spectral regions and characterized spectral changes exhibited, if they present themselves. This can be an important channel of information that results from our study. Although it is certainly speculative to think that this information will help to make diagnoses soon, we are optimistic. We have previously employed similar degradation channels to characterize the stoichiometry and material phase of different copper oxide hybrid films.

The intent of this paper is not to provide a stand-alone alternative to existing SERS based Raman methods of detecting viruses. Rather, our goal here is to introduce a second channel of data, namely LFR, that we hypothesize can provide important spectral information to aid in developing more accurate methods of diagnosis. Is examination of the LFR spectrum alone enough to enable accurate diagnoses? It is certainly too early to conclude, but even if not, together with SERS it should result in a powerful diagnostic tool. Furthermore, we also appreciate that the EAR spectrum can provide valuable data as to the precise size of the virus (Burkhartsmeyer et al., 2020), and this information can also constitute a significant contribution to the diagnosis picture. After conclusion of the proposed research, the research community will be in a more informed position to evaluate how various Raman Spectroscopy methods can be employed to diagnose the COVID-19 and other Coronaviruses.

Question	Hypothesis	Sampling plan	Analysis Plan	Interpretation given different outcomes
Will Low-Frequency Raman scattering spectroscopy be useful for detecting and diagnosing COVID-19?	The nanostructure of COVID-19 does not change during the measurement due to thermal	Measure 30 samples using regular Raman and LFR.	Perform time series measurements, wherein each sample is measured multiple times to identify any	If the spectrum changes, either in the regular Raman spectral range or the LFR range, then this may constrain the optical power

	fluctuations or decomposition.		changes in spectrum over time. Check for changes in intensity and spectral peak positions as well as their peak widths.	levels that we use when obtaining data. Such changes may turn out to be a useful diagnostic if COVID-19 decays differently than other viruses.
	The distinct nanostructure of COVID-19 will present distinguishable differences in the LFR spectrum.	We will measure 30 samples of each virus that is being compared.	From the multitude of samples, for each virus, we will be able to determine the spectral widths or variation in spectrum from sample-to-sample.	Depending on the spreads that we obtain for the spectral peaks for each virus, we will be able to determine whether COVID-19 has distinguishable differences in its LFR spectrum.
	The laser excitation will not destroy the nanostructure of the COVID-19, which would make it difficult to detect.	We will investigate regular Raman and LFR for COVID-19 under different laser excitation power levels and times	We will determine what power levels and/or illumination times are detrimental to the virus, and thus cause it to break down.	This may constrain how we may eventually be able to implement a POC diagnostic or it may present a new way to diagnose if COVID-19 decomposes under a specific set of conditions and/or presents a unique decomposition product.

Ethics

Research will be carried under the supervision of the Bar-Ilan University Research Authority.

Data accessibility

Study data and the preregistered protocol will be made publicly available and a link provided in the Stage 2 manuscript.

Authors' contributions

To be completed at Stage 2.

Competing interests

The authors declare no other competing interests.

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