

Review Article

Sub-Terahertz Spectroscopic Signatures from micro-RNA Molecules in Fluid Samples for Ovarian Cancer Analysis

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Abstract

Lack of success in diagnosing cancers at an early stage of disease leads to lethal outcomes in many cancer patients. In this Report, small sample sub-THz resonance spectroscopy technology with high spectral and spatial resolution is introduced as a companion, inexpensive, optical, fast, label-and reagent-free approach to improve molecular and cellular characterization analysis for potential early detection, diagnosis, prognosis, and therapeutic treatment of cancers that are currently difficult to detect.

This work details preliminary results that demonstrate significant differences between cancer and control sample signatures. The unique spectroscopic features support the role of specific micro-RNAs known from medical research to be significantly overexpressed and important for ovarian cancer development. The results present introduction and initial validation of sub-THz resonance spectroscopy technique for fingerprinting and quantification of potential molecular biomarkers in malignant and normal samples with high sensitivity and discriminative capability.

This companion technology to be developed to complement traditional clinical diagnostic methods, promises to open an entirely new field for cancer research and clinical care.

Keywords: ovarian cancer, micro-RNAs, THz spectroscopy

Introduction

Vibrational resonance spectroscopy for molecular bio-sensing in the sub-THz range is an emerging technique that utilizes specific resonance features in absorption spectra of molecules or entire biological cells. Many people have recently become familiar with electromagnetic (EM) waves in the terahertz (THz) range thanks to microwave equipment and the discovery of wireless communications. About the same time, it became clear that THz waves are also extremely important for life science applications because of the unique capability of this radiation to interact with low energy vibrations of atoms within biological molecules by exciting these vibrations to produce specific molecular absorption spectral fingerprints.

The value of sub-THz radiation is its unique ability to excite low-frequency molecular vibrations produced by weakly bonded atoms in molecules. Chemical bonds in the backbone of DNA and in peptides are strong, and they have the same characteristics in all varieties of biological molecules. There are additional, hydrogen bonds like those between base pairs that hold two DNA strands together, and those that help hold proteins in a certain shape to allow them to function. Similar kind of bonds are well studied in water to organize it in tetrahedral clusters [1,2]. Water plays a central role in chemical and biological systems. Several different water model are in use: TIP3P, SPCE (Extended Single Point Charge) and TIP4P water models. Only simulated spectra of water SPCE model correlate with measured water absorption spectra [3].

Although hydrogen bonds are very weak (~20 times) compared to covalent bonds, these weak bonds in biomolecules and cells do essential work. Because there are so many of the same types of hydrogen bonds or other weak interactions within each biological molecule, these bonds determine the structure and many properties of biopolymers, including the structure of double stranded DNA or different secondary structures of proteins. These weak bonds therefore vital for cellular processes and biomolecule interactions relating to DNA, enzymes, proteins, ribosomes, and other functions.

THz spectroscopy is the only technique that can directly probe and detect the weakest hydrogen bonds and other non-bonded interactions within biopolymers and so address properties that IR, Visible, or UV radiation cannot. Absorption of radiation happens when photon energy coincides with the energy of internal molecular vibrations and the radiation interacts with biomolecules by exciting these vibrations. Since each type of biomolecule has its own vibrational absorption signature, each can be identified and characterized. Because sub-THz radiation propagates through an entire biological cell, molecular components, like proteins and genetic material, all contribute to a detailed absorption spectral signature.

Initial theoretical prediction of vibrational modes in polymer DNA in the 1-100 cm^{-1} frequency range have been published more than 30 years ago [4]. For many years, theory predicted resonances in absorption spectra of biological molecules in the submillimeter range of wavelengths or terahertz range of frequencies [5,6]. However, there were no reliable results and there is still widespread skepticism that a large density of overlapping states contributing to the absorption bands might obscure vibrational resonances and yield essentially structureless spectra [7]. This is because the bio-molecules are so complicated, with many different bonds and the vibrations could be damped. In addition, the time-life of most hydrogen bonds in water is very short, ~1-2 ps, and the intensity of vibrational modes are expected to be very low. The same was expected for hydrogen bonds in bio-molecules. Besides, there were no good components, THz sources and detectors, and sensitive spectrometers to take these kinds of measurements. The first results were not reproducible and were close to the sensitivity limit. It was not clear what kind of samples to use, and what kind of signatures to expect. Nevertheless, vibrational bands in the spectra of protein macromolecules are observed and very well studied in the far IR region at much higher densities of states compared to THz. Not only biopolymers but also whole microorganisms can be characterized in the THz range. [8]. Thus, to confirm the reality of observed features, we used theoretical predictions based on computational modeling of absorption spectra from biological molecules including DNA, RNA and Proteins [9-12].

Vibrational resonance spectroscopy in THz range is now an emerging technique for bio-sensing, that can be used to examine bio-molecular structure and dynamics, and to characterize absorption properties of biological materials [13]. The technique is based on specific resonance features of vibrational modes, or group of modes at close frequencies, in absorption (transmission) spectra of biological materials, molecules and entire cells. This optical technique can provide valuable, global structural information, in particular by monitoring structural changes in response to a physiological stimulus. Our early results [14,15] utilized a commercial instrument- Fourier transform

(FT) spectrometer, Bruker IFS-66, that until recently provided the most detailed information on vibrational spectral signatures from biological molecules in the Far IR. This instrument, used at frequencies 10-25 cm^{-1} required, however, three vacuum systems and a liquid Helium cooled Si-bolometer operating at 1.7°K for signal detection.

Sub-Terahertz Spectroscopic Instruments and a Method for Biological Materials Fingerprinting

A new breakthrough in technology development became possible with introduction of a new imaging mechanism for the development of novel spectroscopic and imaging sensors in the sub- THz region with a very strong enhancement of the EM field and increased radiation coupling to biomaterials utilizing strong electric field edge effects in a subwavelength periodic structure [16]. The new technology has been demonstrated using a spectroscopic sensor prototype recently developed and built by Vibratess, LLC (Vibrational THz Spectroscopy) using SBIR funding from the DoD (Figures 1 and 2).

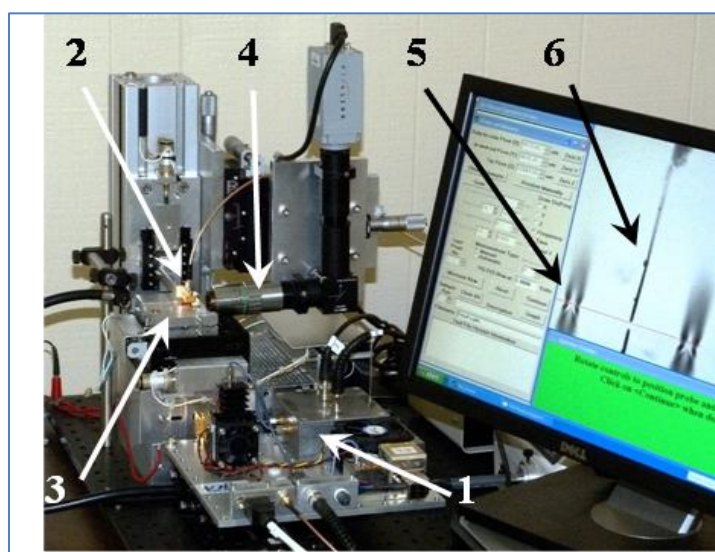


Figure 1: Vibratess' spectrometer-Vibr-2. (1) THz source (2) Detector (3) Positioning stage (4) Objective for visualization (5) Image of a channel with material (6) Probe. A sturdy stage with a sample table movable in 2 directions with the step of ~ 0.13 micron [17].

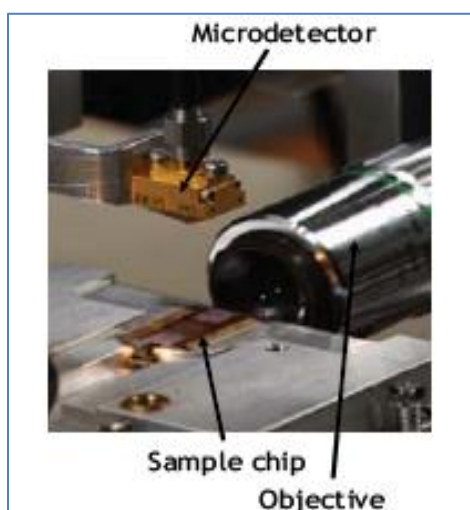


Figure 2: Visualization of disposable chip for sample material and detector in the upper position for loading sample using micropipette or micro-syringe [18].

The prototype features: a tunable source (VDI, LLC), operating between 330-490 GHz with a frequency resolution better than 1 GHz; A Schottky diode detector that operates at room temperature and requires only nanograms of sample materials, liquid or solid. Software for operation of the instrument includes graphical visualization of spectra, data calculation and storage [19,20].

The Vibratess novel, quasi-optical, frequency-domain spectroscopic sensor prototype with imaging capability operating at room temperature in the sub-THz spectral region eliminates the requirement for air evacuation or purging with nitrogen [17,18]. Highly sensitive spectroscopy (Signal/Noise 10^2 - 10^3 depending on frequency) with spectral resolution better than 1 GHz (0.03 cm^{-1}) provides narrow spectral lines (0.07 - 0.1 cm^{-1} or 2-3 GHz) for spectroscopic signatures from biological macromolecules. Spatial resolution ~ 150 microns permits the use of small samples, less than 1 mm^2 , with nanogram of material for characterization. No special sample preparation procedure is required. A drop of solutions/suspension of biomaterial is micropipetted in a spot of the array of microchannels in the sample holder. Due to the high sensitivity, good spectral resolution, and a spatial resolution below the diffraction limit, this new spectroscopic instrument permits us to observe vibrational resonances in transmission/absorption spectra of solid and liquid samples from biological materials. This new method for visualization and characterization of biological molecules is low energy, nondestructive and fast. With a 3D movement of a detector relatively to a sample, this instrument is an ideal for characterizing heterogeneous materials, such, for example, as samples from tissue.

One example of an experimental signature and its comparison with computational modeling can be found in Alijabbari et al. study [21]. The correlation between the theory and experiment validate both and confirm the reality of observed narrow ($\sim 0.1\text{ cm}^{-1}$ or 3 GHz) and intense resonances. To confirm further the existence of vibrational modes in spectra of biological molecules we studied relaxation dynamics of 2 intramolecular H-bonds, O..H-N and O..H-C, in *E. coli* protein thioredoxine using atomic oscillations from MD simulations [22]. Statistical distribution of relaxation times and autocorrelation function of quasi-periodic movements show that the atoms are involved in a number of collective oscillations with relaxation times ranging from 2-3 ps to more than 150 ps. Although there are statistically many short time vibrations, the related absorption is low intensity since the absorption is inverse to the relaxation time. The existence of long lasting relaxation processes that is confirmed by time-resolved luminescence experiments [23,24] permits us to directly observe and study H-bond vibrational modes in sub-THz absorption spectra of biomolecules. We have shown that multiple resonances (vibrational modes) do exist in sub-THz absorption spectra of biological materials and are available for sensitive detection of bio-molecules and species with a high specificity. The emerging, fast and inexpensive optical technique has been introduced that requires less than nanogram of sample material for characterization and analysis and does not require chemical reagents or labels. It combines experimental characterization with computational modeling to predict, verify and confirm the results of measurements and to facilitate their analysis. [25].

Because of the small size of biological cells compared to the wavelength of sub-THz radiation, this radiation propagates through an entire object, allowing all molecular components, DNAs, RNAs and proteins to contribute to the THz signature of a sample. Our experiments, however, have shown significant input contributions from micro-RNA molecules into the entire signature compared to other molecular components, perhaps due to the higher number of the same type of hydrogen bonds in the small volume of a sample. The results of measurements and analysis are leading to many potential applications of a new technology including applications in Oncology, where cancer is studied at molecular level. The initial prototype instrument has now been extensively used for testing and evaluation, as well as for characterizing biological macromolecules, cells and cancer related samples for more than 3 years.

Vibratess has recently developed and built a next generation version of the spectroscopic sensor automated instrument, shown in Figure 3, with fixed positions of a sample microchip and a detector probe [26]. This affordable, compact, simple and easy to operate instrument is ready for many applications in Bio-medical laboratories, clinics and points of care settings with some additional improvements being implemented for use in clinics and points of care settings. In addition to removal of the sample positioning, this new instrument has already been connected with cloud-based analysis, to allow for neural network-based determination of the presence of specific peaks, even at very low absorption by the sample. This prototype is also undergoing incorporation of an embedded processor to greatly increase the scanning speed of the system. This will reduce analysis times from 30 minutes to less than 5 minutes, while at the same time increasing the resolution of the spectrum to better define the peak widths and frequencies.

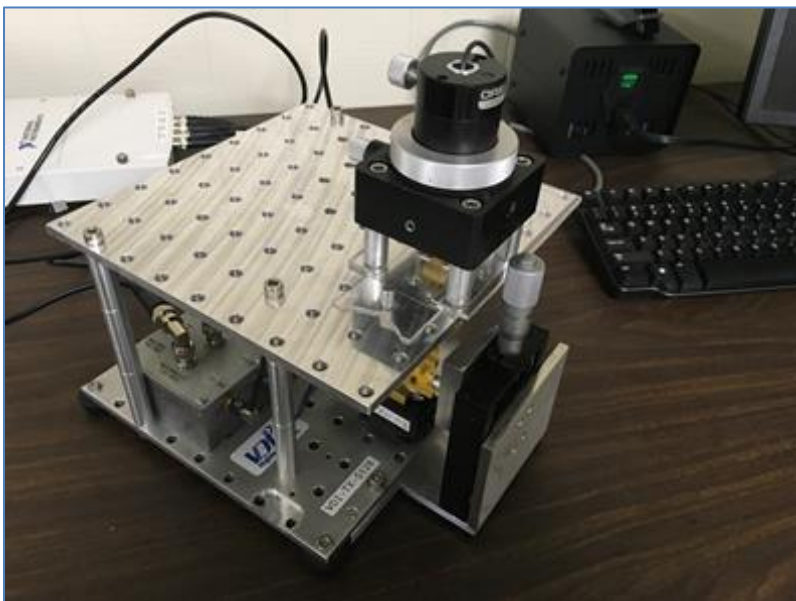


Figure 3: Vibratess' automated spectrometer prototype, Vibr-4, with an electronically tunable sub-THz source.

Cancer and Micro-RNAs (miRNAs)

One of our long-term goals is to use the molecule spectroscopic information generated by sub-THz spectroscopy for visualization and quantification of specific cell free (cf) tumor DNA, RNA and miRNAs molecules in blood, serum or other body liquids. The presence and the profile of these molecules can be used as non-invasive biomarkers for early diagnostic of ovarian and other difficult to detect cancers in potential companion diagnostic methods that are complimentary to traditional, well established diagnostic technologies.

Advances in sequencing technologies have led to an increased focus on blood-derived nucleic acid-based approaches for biomarker discovery [27-29]. If appropriate biomarkers are available, human serum and other body fluids could be analyzed for clinical diagnosis of cancer.

A decade ago, small (~22 base-pair nucleotides) micro-RNAs (miRNAs) were discovered as important regulators of gene expression [27,30,31]. Cell-free miRNAs in body fluids were found to be stable under harsh conditions [32-36] and the miRNA abundance profile of bodily fluids reflects physiological and/or pathological conditions more accurately than the longer and well-studied messenger RNA (mRNA) molecules abundance profile.

Because they are stable and found in biofluids, miRNA biomarkers could be evaluated using less invasive procedures - blood draws rather than biopsy, to potentially detect cancer at an earlier stage in individual tests.

Quantification, however, remains a problem with extracting and analyzing circulating miRNA. These are multi-step, time consuming procedures yielding terabytes of data requiring bio-informatics analysis capabilities.

Thus, there is a crucial need for a fast, simple to perform and interpret screening test based on nucleic acid differences between normal and malignant cells, as well as new tools, to help identify the biology involved in cancer development and progression [37].

In our work we introduce and validate our Sub-THz resonance spectroscopy combined with MD computation as a promising technology for optical analysis and potential quantification of molecular biomarkers for ovarian cancer (OC) that is currently difficult to detect [38].

In the first series of experiments, samples of two ovarian cancer lines SKOV3 (ATCC® HTB-77™) and ES-2 (ATCC® CRL-1978™) were compared with normal control cell line from fallopian tube epithelium [39]. All samples were fixed in alcohol in the laboratory of Dr. Amir Jazaery, (Department of Obstetrics and Gynecology, University of Virginia). Measurements have included experimental characterization of absorption spectra of cancer and normal cells, cell free solution, and molecular components of cells. The measurement procedure has been described in [39]. For each sample, an empty sample holder was placed into the THz spectrometer and the detection probe positioned directly above the array ($\sim 6 \mu\text{m}$) to measure the reference background spectrum. Frequency scans were performed by varying the voltage to the THz source. In the next step, the detection probe was raised, and a dilute solution (suspension) of a sample was added using a micropipette or micro-syringe onto a small spot ($\sim 1 \text{ mm}^2$) on the array, where background scans had been performed, and allowed to dry for ~ 5 min. The detection probe was lowered, and measurements were then taken of the sample material. Transmission (T) was calculated as the ratio of the signal spectrum with material to the background spectrum. Transmission was then recalculated for absorbance using $A = -\log(T)$, which is proportional to absorption coefficient, for analysis of scaling results depending on the amount of sample material deposited, and for further comparison with the computational modeling results. The test results demonstrated high repeatability and sensitivity of absorbance spectra measurements with the error standard deviation less than 4% almost at all points, except two ends of the frequency diapason. The results are reproducible within the limit of several % for the amplitude (transmission or absorbance) and less than 0.05 cm^{-1} on the frequency position for measurements of the same sample.

In a parallel computational study, a set of miRNAs selected from literature data has been chosen based on their importance for ovarian cancer development prediction. Molecular dynamics simulation was conducted, and absorption spectra were calculated for selected miRNA molecules. The predicted sub-THz spectra from miRNAs, including absorption resonance peaks frequencies and intensities, have been compared with experimental results for cancer cells.

All collected data have been used to understand the significant differences between signatures of cancer and normal samples and evaluate the hypothesis that the cancer related sub-THz spectroscopic signature might be attributed to miRNAs. Variability of spectra has been used to determine the utility of these quantified characteristics of miRNA for discovery of new biomarkers as well as for cancer detection, prognosis, and probable therapeutic treatment.

A dramatic difference between the THz absorption spectra of cancer and normal control samples is shown in Figure 4. In all measured spectra of ovarian cancer characterized in [39], a very sharp absorption peak is observed with high reproducibility, centered at frequency 12.95 cm^{-1} (388.5 GHz), with the accuracy within 0.05 cm^{-1} .

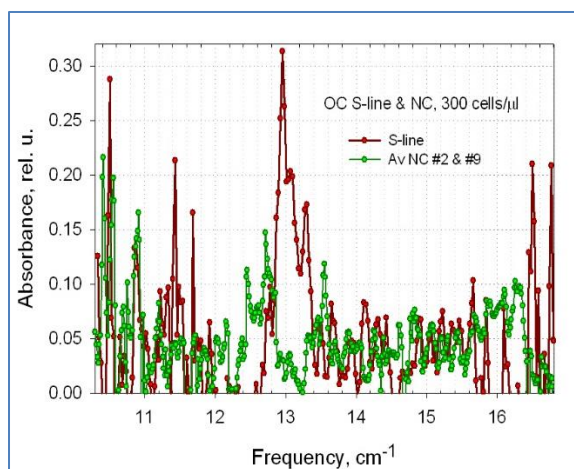


Figure 4: Absorbance from ovarian cancer S-line (OC in brown) & normal control (NC in green) samples. Results for NC are the first demonstration of sub-THz spectroscopic signature from fallopian tube epithelium cells. Signature and specifically the central peak position at 12.95 cm^{-1} is stable over two years [39]. In the signature from control samples (green), the peak at 12.95 cm^{-1} is absent. Modification of this NC signature might be the first sign of cancer [39].

Do miRNA molecules from the 200 family signaling Cancer?

One source of this strong feature in cancer samples could be matured miRNAs (with defects but without the loops). In cancer cells miRNA become deregulated, some of them showing increased concentration in blood, serum and tissue samples. There are ~700 Human miRNAs. The mir200 family is very important and has ~50-600 times higher level in ovarian cancer (OC) cells compared to normal human ovarian cultures [40-43]. The microRNA-200 family sequences of interest are:

miR-200a (22bp) CAUCUUACCGGACAGUGCUGGA

miR-200b (22bp) UAAUACUGCCUGGUAAUGAUGA

miR-200c (22bp) CGUCUUACCCAGCAGUGUUUGG

miR-141 (22bp) UAACACUGUCUGGUAAAGAUGG

To verify if the contribution from miRNAs could explain the observed cancer signature, molecular dynamic (MD) simulations were performed to predict sub-THz absorption signatures from these four miRNAs overexpressed and important in ovarian cancer. The results for miR-200c, and especially for miR-141 correlate better with experimental data for both cancer lines. The presence of the mismatches (not shown above) clearly contributes to the absorption peak at $\sim 13\text{ cm}^{-1}$ [39]. Modeling miR-141 with mismatches not only predicts the central peak at $\sim 13\text{ cm}^{-1}$, but points to a presence of a band consisting of several peaks close on the frequency scale, which is also observed in experimental OC spectra. The simulation reproduces the fine structure of this band (up to 10 individual peaks) with the accuracy within 0.3% on the frequency scale [39].

Samples prepared from biopsy tissue have been tested as well in parallel with control epithelial ovary samples (Prof. Moskaluk, Department of Pathology, UVA). Characterizing tissue samples using their absorption spectra is a much more complicated task compared to characterization of cells in alcohol samples [39]. The intensity of absorbance is lower in the samples with the same amount of material and additional peaks from other molecular components from tissue that are present (probably proteins or different microRNAs). The most serious problem with tissue characterization is due to the heterogeneous nature of tissue. At the same time spectroscopy of tissue might potentially provide much more detailed information compared to samples in liquid, which give the averaged results.

Figure 5 shows an absorption spectrum from a tissue sample that is a high grade serous papillary carcinoma with a tumor stage 3C (more than 2 cm) extended to lymph node and one known metastasis. The absorption band between 12.6-13.2 cm^{-1} with the dominated peak centered at $\sim 12.95 \text{ cm}^{-1}$ is very similar to a signature from a sample fixed in alcohol [39] demonstrated in Figure 4 although some other absorption peaks are unique. However, absorption spectra from the same as well as from different ovarian cancer sample materials demonstrated that the fine structure of this central absorption band can vary significantly as shown by comparing the results in Figures 5 and 6. Figure 6 shows the signature from ovarian tissue sample TG-5 that is also high grade serous papillary carcinoma, 3C, but without extension to lymph node and no known metastases. This spectrum demonstrates the characteristic peaks at frequencies 13.2 cm^{-1} and 13.4 cm^{-1} instead of 12.95 cm^{-1} . This variation can be explained by the different ratios of contributions from individual micro-RNAs in the family 200

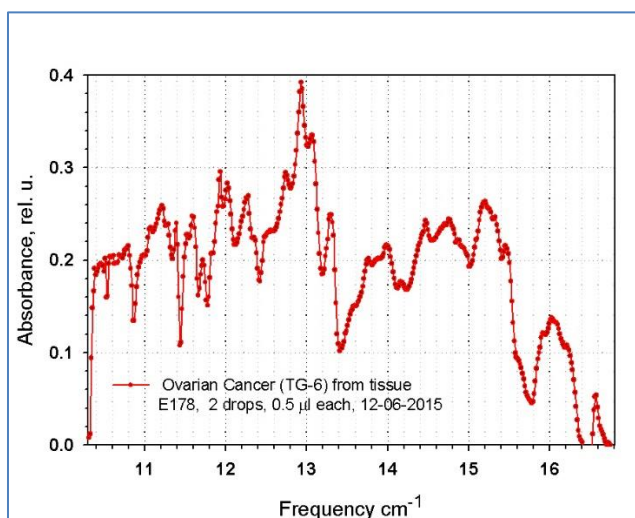


Figure 5: Absorption signature from a high grade Ovarian Cancer tissue sample TG-6.

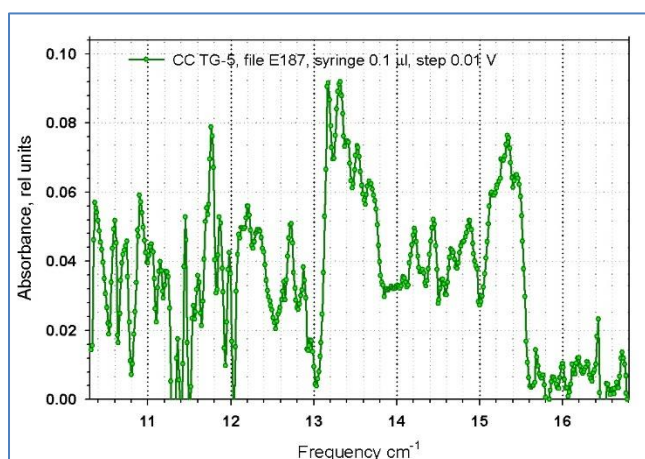


Figure 6: Absorption spectrum of another high grade Ovarian Cancer tissue sample TG-5 with central band shifted to higher frequencies.

Spectroscopic Signatures from Urine and Saliva Samples

The analysis of results of spectroscopic signatures from control samples might be very important since these samples may have features that indicate the molecular variations in the initial stage of cancer. The choice of samples for this analysis is also important. These samples have to be easily accessible and have to reflect the contribution from

essential molecular components. Below we compare the spectroscopic signatures from normal samples of saliva and urine using our Spectroscopic device. Since our most important goal is to find specific discriminative signs of ovarian cancer at an early stage of disease, we below present the results of variations in signatures from control samples in saliva and urine that might indicate the initial stage.

These tests are much easier to take compare to the tissue samples. The character of the spectra is very close to that for the samples in alcohol or tissue samples. This fact indicate that the spectra reflect again the presence of specific molecular components. Figures 7 and 8 show the similarity of absorbance spectra taken from samples of saliva and urine from the same patient in June 2016.

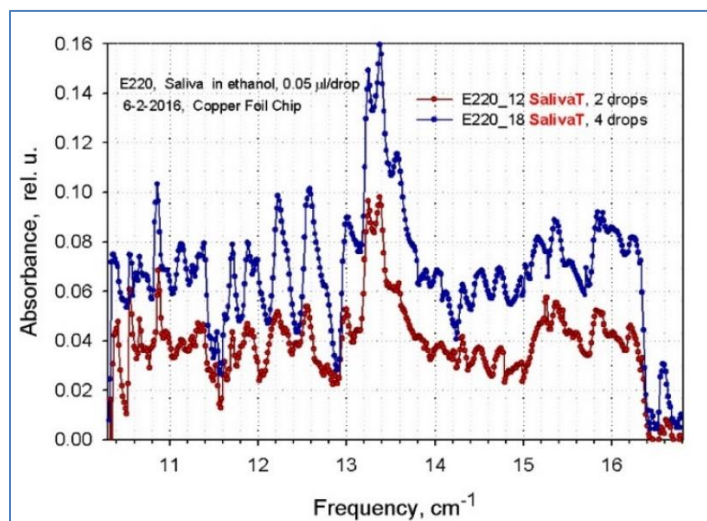


Figure 7: Scaling the results of absorbance with the amount of material in the sample from saliva in ethanol 1:3, June 2016.

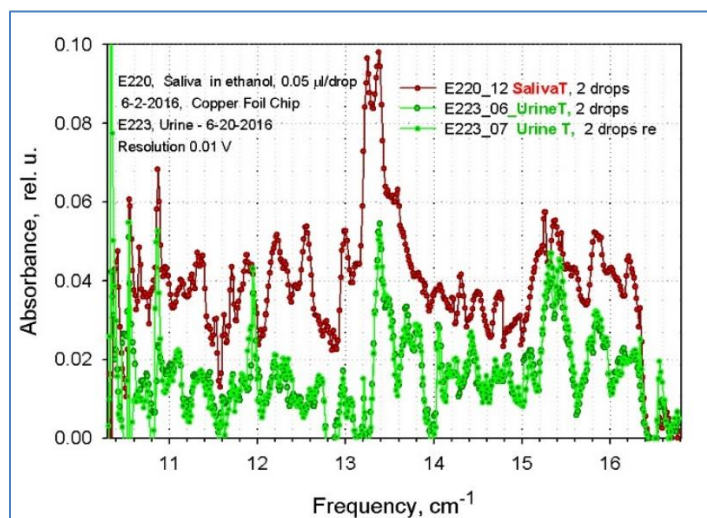


Figure 8: Similarity of absorbance spectra of saliva and urine samples from taken from the same patient at close times, June 2016.

Comparison urine sample spectra in Figure 8 (green line) and in Figure 9 taken with the interval of almost 2 years show significant difference in patient physiological conditions, that is reflected by the changing of molecules contributing to signatures. This patient probably have or had a problem of a Breast cancer as indicated by the similarity of spectroscopic pattern in Figures 8 and 10.

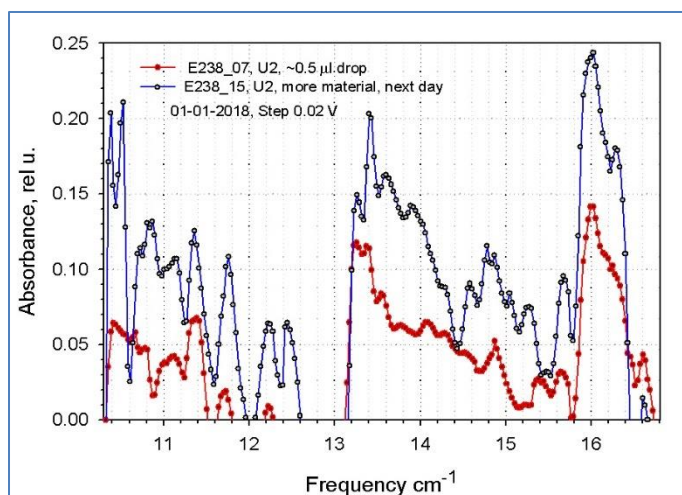


Figure 9: Absorbance spectra of urine sample from the same patient as shown in Figure 8 taken in January 2018 with the time interval of almost 2 years

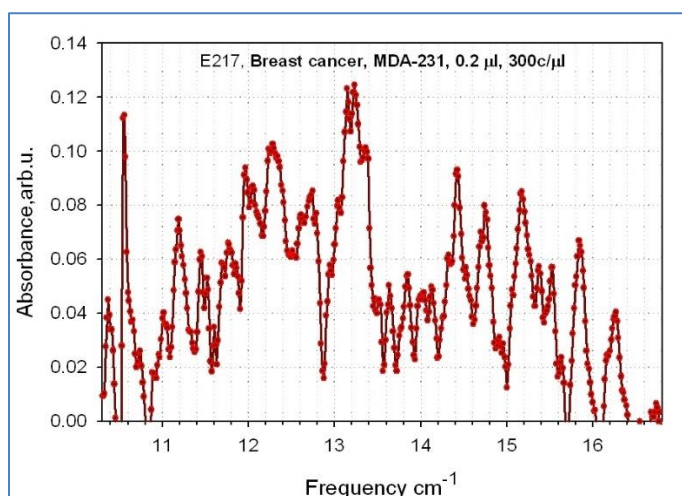


Figure 10: Absorption spectrum of Breast cancer sample MDA-MB-231

Conclusions

In this review we present the very first results demonstrating application of a novel sub-THz spectrometer for analysis of a wide variety of sample types for cancer diagnosis. Differences between cancer cell lines and normal cell lines were easily observed, and these differences were also observed between normal and cancer tissue samples. Tissue samples provided more background peaks due to the presence of additional tissue components and proved to be heterogeneous across the samples. The major differences are shown to be related to the presence of micro-RNA known to be overexpressed in ovarian cancer samples. This work, however, moves us forward towards our goal of earlier cancer detection by evaluating urine and saliva samples to see if the presence of specific microRNA can also be detected in these types of easily obtainable samples. This work is also beginning to define a new instrument that will be able to be utilized in clinical and point of care settings with faster measurements and automated cloud-based analysis. There are no current good blood-based biomarkers that can be analyzed by traditional methods for some difficult to detect cancers, and no traditional methods that can provide the discriminatory power with the speed of analysis possible with sub-THz spectroscopy measurements. The ultimate goal will be testing of urine or saliva from

every patient directly at the doctor's office, to detect the beginning stages of cancer, long before a mass appears, and traditional clinical diagnostic techniques could be applied.

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