JoVE61007 Manuscript: Terahertz Imaging and Characterization Protocol for Freshly Excised Breast Cancer Tumors

The authors would like to thank the Editor, Associate Editor, and Reviewers for their consideration and comments on this manuscript. We have responded to all comments/suggestions provided by the editor and the Reviewers. Changes to the manuscript have been highlighted both in this letter and in a revised file for the manuscript.

Note: all revisions are highlighted in yellow in the revised manuscript and revised table of materials. The 2.75 pages for filmable content are marked in red text in the revised manuscript.

Editorial Comments:

Comment 1: Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues. The JoVE editor will not copy-edit your manuscript and any errors in the submitted revision may be present in the published version.

Response: The manuscript has been proofread thoroughly for the spelling and grammatical errors and revised accordingly. Since these edits are relatively minor the full list of changes is not detailed here, but have been highlighted in the manuscript.

Comment 2: Please provide an email address for each author.

Response: The email addresses corresponding to each author are added in the revised manuscript as requested.

The following email addresses has been added to page 1:

Nagma Vohra: nvohra@email.uark.edu

Tyler Bowman: tcbowman@email.uark.edu

Keith Bailey: kbailey1@illinois.edu

Magda El-Shenawee: magda@uark.edu

Comment 3: Please provide at least 6 keywords or phrases.

Response: The sixth keyword is added to the KEYWORDS section in the revised manuscript.

The following keyword has been added to page 1:

"Cancer research"

Comment 4: JoVE cannot publish manuscripts containing commercial language. JoVE cannot publish manuscripts containing commercial language. For example TPS Spectra 3000 THz, TeraView, Ltd, Kim wipes, Davidson tissue dye, ScanAcquire software, etc.

Response: The manuscript has been revised thoroughly and the commercial language names of the system, materials, and software have been removed. The full list of changes is not detailed

here, but have been highlighted in the manuscript. The names and description of the two software used in the protocol are added and highlighted in the Table of Materials file.

The following two rows are added in the Table of Materials file:

Name of Material/ Equipment	Company	Catalog Number	Comments/Description
ScanAcquire Software	TeraView, Ltd	N/A	System Software for THz reflection imaging measurements
TPS Spectra Software	TeraView, Ltd	N/A	System Software for THz transmission spectroscopy measurements

Comment 5: Please ensure that all text in the protocol section is written in the imperative tense as if telling someone how to do the technique (e.g., "Do this," "Ensure that," etc.). The actions should be described in the imperative tense in complete sentences wherever possible. Avoid usage of phrases such as "could be," "should be," and "would be" throughout the Protocol. Any text that cannot be written in the imperative tense may be added as a "Note."

Response: We have made sure that the manuscript is written in the imperative tense and the phrases such as "could be," "should be," and "would be" are not used anywhere in the protocol. The additional information that could not be written in the imperative tense has been added as a "NOTE" wherever needed.

The following sentence has been revised on page 3:

"Keep any used tissues, wipes, and gloves on the biohazard material surface to dispose of at the end of the protocol."

The following sentence has been added as a NOTE to page 6:

"NOTE: After adjusting the optical delay axis, two pulses should appear on the window, as shown in Figure 6, one from the lower interface of the polystyrene plate (primary reflection) and one from the upper interface of the polystyrene plate (secondary reflection)."

The following sentence has been added to page 6:

"4.10. Window out the primary reflection from the polystyrene plate and keep the secondary reflection in the window which will contribute to the reflections from the tissue during the imaging procedure."

The following sentence has been added as a NOTE to page 6:

"NOTE: For a 1.2 mm thick polystyrene plate, the primary reflection is windowed out when the secondary reflection minimum peak is approximately -0.3 mm on the optical delay axis of the time domain window."

The following sentence has been added as a NOTE to page 8:

"NOTE: By default, Axis1 is A-axis and Axis2 is B-axis."

The following sentence has been added as a NOTE to page 8:

"NOTE: Setting the **Axis1step** and **Axis2step** will set the stepper motors to increment at every 200 µm step size during scanning process. The total scan time can be estimated in the Image acquisition parameters window."

The following sentence has been added to page 9:

"This tumor staining process is conducted for the pathologist's reference to provide the information about the tumor's imaging side and its orientation."

The following sentence has been added as a NOTE to page 10:

"NOTE: A typical frequency domain spectrum should provide data ranging from 0.1 THz to 4 THz."

The following sentence has been added twice as a NOTE to page 11:

"NOTE: The solution for the refractive index and absorption coefficient is the pair of values giving the lowest error."

Additionally, all definitions of terms following equations in the protocol have been converted to Notes.

Comment 6: Please ensure you answer the "how" question, i.e., how is the step performed?

Response: It is ensured that every step mentioned in the protocol is detailed on how it is to be performed.

Comment 7: Please make the spectroscopy measurements as a separate section.

Response: As requested, the spectroscopy measurement details are introduced as a separate Section #3 in the protocol. The alterations to other section numbers and the corresponding step numbers following new Section #3 are made as well. The full list of changes in the section numbers and step numbers in not provided in this letter, but are highlighted in the revised manuscript.

The following new Section has been introduced in the protocol to page 4:

"3. THz transmission spectroscopy measurements."

Comment 8: 2.3.1, 2.3.5: How is this done?

Response: The setting of the module mentioned in step 2.3.1, which is now renumbered as 3.1, is performed by mounting it inside the core THz chamber and locking it down by tightening the two mounting screws. The mounting screws are depicted in the modified Figure. 3A.

To record the air reference signal in step 2.3.5 (renumbered as 3.5), steps 2.3.6 and 2.3.7 (renumbered as 3.5.1 and 3.5.2, respectively) are followed. To clarify that these steps are performed to fulfill the prior step, steps 2.3.6 and 2.3.7 (renumbered as 3.5.1 and 3.5.2, respectively) are made as the sub-steps of the step 2.3.5 (renumbered as 3.5).

In response to the step 2.3.1, the renumbered step 3.1, the following edits have been added to page page 4:

"Set the transmission spectroscopy module inside the THz core chamber by aligning the module handles over the mounting posts in the core system and sliding the stage down into the system. Tighten the two mounting screws in the upper right and lower left corners of the module as shown in Figure 3A."

In response to the step 2.3.5, the renumbered step 3.5, the following edits have been added to page 4:

"3.5. After 30 minutes of purging, record an air reference signal by following the steps below. [Place **Figure 3** here].

3.5.1. Under the **Scan settings** tab in the spectra scan setup window, input an appropriate **Name** for the reference, set **Num Scans** to 1800, and set the **Start Delay (s)** to 0. Leave the other settings as their default values.

3.5.2. Click on **Measure Reference** in the scan setup window to take the air reference measurement. Then click on **Measure Sample** to measure the transmission signal through air as a sample average of 1800 signals over ~1 minute."

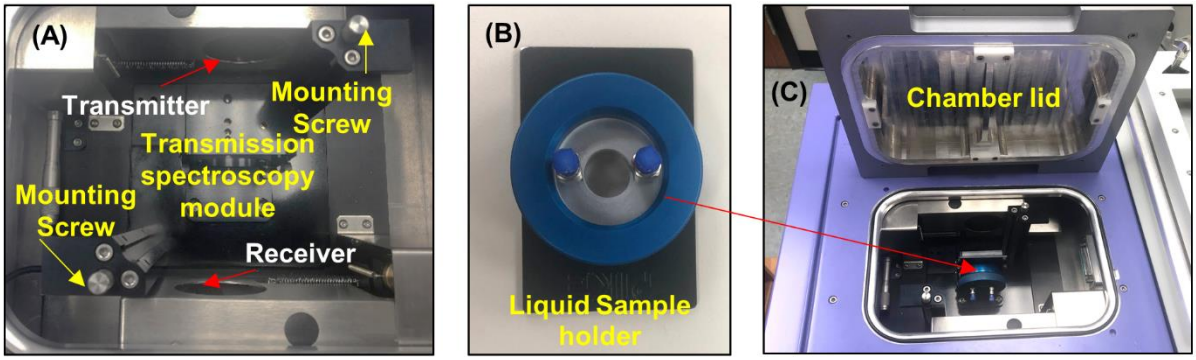


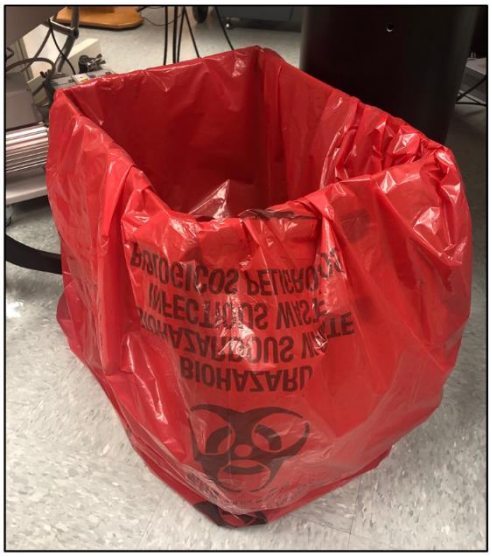
Figure 3: THz transmission spectroscopy module set-up. (A) THz core chamber with the transmission module mounted on it. **(B)** The photograph of the liquid sample holder. **(C)** The sample holder placed inside the core chamber for the measurements.

Comment 9: There is a 10-page limit for the Protocol, but there is a 2.75-page limit for filmable content. Please highlight 2.75 pages or less of the Protocol (including headings and spacing) that identifies the essential steps of the protocol for the video, i.e., the steps that should be visualized to tell the most cohesive story of the Protocol.

Response: The 2.75 pages for filmable content are marked in red text in the revised manuscript.

Comment 10: Please ensure that you have a result for each section of the protocol.

Response: We have presented and discussed the results for both the methods described in the protocol, THz transmission spectroscopy (Sections #3 and #8) and THz reflection imaging (Sections #4 and #7), for the characterization and imaging of breast cancer tumors. It is to be noted here, that Sections 1 and 2 in the protocol are for tissue handling which are necessary to be done to get to the Sections 3 and 4 in the protocol which provide detail on the experimental methods. Similarly, Section 5 and Section 6 are the post processing steps upon completion of the experimental methods. The figures are added in these sections itself to make it easy for the readers instead of presenting them in the results section. A new figure (**Figure 8**) and the corresponding legend has been added for Section 6 in the manuscript. It is to be noted, that with the addition of Figure 8, the numbering of all the figures following Figure 8 are altered and highlighted in the manuscript accordingly.



The following sentence is added to page 9:

"[Place **Figure 8** here]."

The following legend corresponding to Figure 8 is added to the FIGURE LEGENDS Section to page 13**:**

"Figure 8: Photograph of the biohazardous waste bag."

Comment 11: As we are a methods journal, please revise the Discussion to explicitly cover the following in detail in 3-6 paragraphs with citations:
a) Critical steps within the protocol
b) Any modifications and troubleshooting of the technique
c) Any limitations of the technique
d) The significance with respect to existing methods
e) Any future applications of the technique

Response: (a) The steps most critical for effective THz imaging are the tissue handling (specifically tissue wetness and the tissue-polystyrene interface) and the stage setup and balancing. To more explicitly call out these steps, the Discussion session on page 14 has been revised to specifically indicate the relevant sections of the protocol.

The following sentence has also been added on page 14:

"Extra effort should be taken to ensure good tissue contact with the imaging window, including repositioning the tissue to obtain a better interface."

(b) The modifications of the technique primarily involve the materials being used to mount the tissue. While this protocol specifically uses quartz (for transmission spectroscopy) and polystyrene (for reflection imaging), the important properties of these materials is their low THz absorption and uniform thickness. Any equivalent material can serve as a substitute. Alternatively, non-uniform thickness can be offset by establishing the phase shift of the fixed window if the window can be set up in such a way that it is positioned the same way with and without tissue. The primary troubleshooting portions of the work involve ensuring that a sufficient THz signal exists for measurement, which is highly dependent on the exact system being used and cannot be concisely defined within the scope of this protocol.

The following paragraph was added on page 15:

"The primary area where the protocol can be modified is in the dielectric materials used to mount the tissue such as quartz (in sections 3.6-3.7) and polystyrene (section 4.5 forward). As long as the selected window materials are uniformly thick and of low enough absorption to have good signal interaction with the tumor, other materials can be substituted. Materials should be evaluated ahead of time to determine the one that provides an adequate phase plane. Alternatively, for systems where the imaging window will be fixed, a nonuniform window thickness can be addressed by characterizing the phase shift calculated from an empty window scan. There is also some room for modification in how the tissue is mounted for shipment to the pathologist. While tissue marking dyes are used here out of convention, the important aspect is to have a method in place with the pathologist that enables comparison between THz imaging and pathology. The primary troubleshooting concerns for the protocol will involve obtaining a good THz signal and establishing proper windowing, which will depend on the specific system being used."

(c) The primary limitation of the technique is the amount of time that the tissue is exposed to air. At the suggestion of pathologist collaborator, the protocol was designed such that the tumor samples were immersed in formalin for shipping no more than one hour after being removed from the DMEM solution. This consideration also affects protocol steps such as the selection of step size, as is described in the discussion already. The secondary limitation of the protocol is due to the spectral frequency limit of the THz pulsed signal, which sets a maximum spatial resolution of around 80 μm.

The following sentence has been revised on page 15 to more clearly introduce the limitations:

"A primary limitation of any fresh tissue handling technique is the time that the tissue is exposed to air."

The following note was added on page 15:

"…though the maximum spatial resolution of the system is around 80 μm due to the spectral content of the THz signal."

(d) The main significance of the developed technique is a relatively fast imaging process that can be incorporated into a surgical setting without excess degradation of the surgical tissue or the increase use of the operation room. While some other methods have been proposed for similar applications, the only comparable technology currently in use intraoperatively is specimen radiography. Radiography has some limitations in that two orientations of transmission X-ray images of the excised tissue must be interpreted to estimate what edges of the tissue, if any, have cancer. THz has an advantage that it can be applied to look directly at the surface of the excised tissue for cancer detection.

The following sentences have been added to page 15:

"Using this protocol intraoperatively represents a potential significant decrease in the time to assess the surgical margins of the tumor from several days or weeks to few minutes. This will be accomplished when the hardware of the THz system is advanced to use THz cameras instead of stepper motor scanners in the future. At present the most similar method employed intraoperatively is specimen radiography, which takes transmission X-ray images of excised tumors that must be interpreted by a radiologist to estimate whether there is cancer on the tissue surface. The described imaging protocol provides a means of direct imaging of the tissue surface."

(e) Beyond efforts to move this technique into intraoperative applications, the same principals for the technique should be applicable to most surgical treatment of solid tumors. Additionally, the concepts described in this protocol have already been proven effective of paraffin-embedded tissues and may be useful for more detailed pathology support in the future.

The following sentence has been added to page 15:

"The protocol for the freshly excised breast cancer tumors can also be used for the characterization and imaging of any other type of freshly excised solid tumor[8-11]."

The following sentence has been added to page 15:

"Similar imaging protocols to the one proposed here could be developed for pathology support in analyzing embedded tissues as well."

Reviewers' comments:

Reviewer #1:

Comment 1: The figures should be improved, particularly, the resolution and appearance of the text in them are rather low. Most of them are hardly readable.

Response: We have improved the resolution and the text on all the figures, as suggested by the reviewer. The improved figures are submitted as separate files during the revised submission process.

Comment 2: It is essential to highlight the drying process (time and so on) of the tissue and the impact of tissue drying during the experiment and measurements, since the drying of tissue significantly change the dielectric contrast. It is not clear from the manuscript, whether authors use some methods in order to prevent drying. See [Gavdush et al., JBO 24 (2019)] and [Fan et al., Phys. Med. Biol., 60 (2015)].

Response: The tissue is received immersed in DMEM solution with antibiotics to maintain the water content of the specimen. During the ~30 minutes of imaging procedure, tissue surface does get little dried but we did not observe an impact on the electrical properties of the tissue. For example, although the experiment was done for a different purpose, we observed that the tomographic imaging results (where the tissue was dried using special paper filters for ~5 minutes) in Figures 9D-9G correlate well with the transmission spectroscopy results in Figures 12C and 12D (where the tissue section was not dried at all). Additionally, the assessment of the tissue by our consulting pathologist did not report any damage to the tissue at the cellular level for the duration of imaging used in this protocol.

In the suggested literature by the Reviewer, [Gavdush et al., JBO 24 (2019)] and [Fan et al., Phys. Med. Biol., 60 (2015)] the tissue is embedded in the gelatin which isolates the tissue from hydration/dehydration effects and help sustain their THz response unaltered for several hours. Similar techniques are currently being investigated as addition to the protocol that would remove any potential effects on tissue hydration levels.

The following sentence has been added to page 15:

"Assessment of the tumor samples by our consulting pathologist has determined that this amount of air exposure does not cause damage to the tissue in an observable way at the cellular level. However, there are some materials such as gelatin that can be used to provide clear THz imaging without excessive drying that may be investigated for future updates to the protocol[29]."

This includes a new reference number [29]:

29. Gavdush, A. A., Chernomyrdin, N. V., Malakhov, K. M., Beshplav, S. T., Dolganova, I. N., Kosyrkova, A. V., Nikitin, P. V., Musina, G. R., Katyba, G. M., Reshetov, I. V., Cherkasova, O. P., Komandin, G. A., Karasik, V. E., Potapov, A. A., Tuchin, V. V., & Zaytsev, K. I. Terahertz spectroscopy of gelatin-embedded human brain gliomas of different grades: a road toward intraoperative THz diagnosis. *J. Biomed. Opt.* **24** (2), 027001 (2019).

Comment 3: Is it possible to estimate the spatial resolution of the described approach? Which minimal size of tumor tissue region could be analyzed by this protocol?

Response: The spatial resolution of the described protocol for the THz reflection imaging is limited by the upper frequency limit of the spectrum associated with the incident time domain signal. For an upper frequency approaching 4 THz, generally the resolvable spatial resolution is

around 80 µm. In all our experiments we have maintained the step size of 200 µm and thus would not be reaching the spatial resolution limit due to step size. However, the system offers the range of step size from 50 µm to 500 µm in 50 µm increments. Thus, the representative pixel dimensions can conceivably fall below the spatial resolution of the system, but this is not done in this work due to the potential increase in the time needed for the scanner when use smaller step size than 200 µm.

The following sentence has been added to page 15:

"…though the maximum spatial resolution of the system is around 80 µm due to the spectral content of the THz signal."