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## Terahertz Imaging and Characterization Protocol for Freshly Excised Breast Cancer Tumors --Manuscript Draft--

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Corresponding Author:	Nagma Vohra University of Arkansas Fayetteville, Arkansas UNITED STATES
Corresponding Author's Institution:	University of Arkansas
Corresponding Author E-Mail:	nvohra@uark.edu
Order of Authors:	Nagma Vohra Tyler Bowman Keith Bailey Magda El-Shenawee
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Elizabeth Heppenheimer  
Science Editor  
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Dear Dr. Heppenheimer,

Based on your invitation, we are here submitting the manuscript entitled “**Terahertz Imaging and Characterization Protocol for Freshly Excised Breast Cancer Tumors**” co-authored by Nagma Vohra, Tyler Bowman, Keith Bailey, and Magda El-Shenawee, to be considered for publication and later on video production in the Journal of Visualized Experiments (JoVE).

The manuscript focuses on the protocol to handle, characterize, and image freshly excised human breast cancer tumors using terahertz spectroscopy and imaging techniques. We believe this manuscript will be of interest to the readers of your journal.

We declare that this manuscript is original, has not been published before and is not currently being considered for publication elsewhere. The permissions for re-publishing figures in this manuscript are granted from the publishers and uploaded as supplements in your website.

As the corresponding Author, I confirm that the manuscript has been read, edited, and approved for submission by all co-authors.

Respectively,  
Nagma Vohra  
PhD Student  
Terahertz Laboratory-ENRC 4906  
Department of Electrical Engineering  
University of Arkansas  
Fayetteville, AR 72701  
Lab: 479 575 7757

**TITLE:****Terahertz Imaging and Characterization Protocol for Freshly Excised Breast Cancer Tumors****AUTHORS AND AFFILIATIONS:**Nagma Vohra<sup>1,\*</sup>, Tyler Bowman<sup>1,\*</sup>, Keith Bailey<sup>2</sup>, Magda El-Shenawee<sup>1</sup><sup>1</sup>Department of Electrical Engineering, University of Arkansas, Fayetteville, Arkansas, USA<sup>2</sup>Oklahoma Animal Disease Diagnostic Laboratory, Oklahoma State University, Stillwater, Oklahoma, USA

\*These authors contributed equally.

**Corresponding Author:**

Nagma Vohra (nvohra@email.uark.edu)

**Email Addresses of Co-authors:**

Tyler Bowman (tcbowman@email.uark.edu)

Keith Bailey (kbailey1@illinois.edu)

Magda El-Shenawee (magda@uark.edu)

**KEYWORDS:**

cancer research, terahertz reflection imaging, terahertz transmission spectroscopy, human breast cancer tumors, refractive index, absorption coefficient

**SUMMARY:**

Freshly excised human breast cancer tumors are characterized with terahertz spectroscopy and imaging following fresh tissue handling protocols. Tissue positioning is taken into consideration to enable effective characterization while providing analysis in a timely manner for future intraoperative applications.

**ABSTRACT:**

This manuscript presents a protocol to handle, characterize, and image freshly excised human breast tumors using pulsed terahertz imaging and spectroscopy techniques. The protocol involves terahertz transmission mode at normal incidence and terahertz reflection mode at an oblique angle of 30°. The collected experimental data represent time domain pulses of the electric field. The terahertz electric field signal transmitted through a fixed point on the excised tissue is processed, through an analytical model, to extract the refractive index and absorption coefficient of the tissue. Utilizing a stepper motor scanner, the terahertz emitted pulse is reflected from each pixel on the tumor providing a planar image of different tissue regions. The image can be presented in time or frequency domain. Furthermore, the extracted data of the refractive index and absorption coefficient at each pixel are utilized to provide a tomographic terahertz image of the tumor. The protocol demonstrates clear differentiation between cancerous and healthy tissues. On the other hand, not adhering to the protocol can result in noisy or inaccurate images due to the presence of air bubbles and fluid remains on the tumor surface.

The protocol provides a method for surgical margins assessment of breast tumors.

## **INTRODUCTION:**

Terahertz (THz) imaging and spectroscopy has been a rapidly growing area of research in the past decade. The continued development of more efficient and consistent THz emitters in the range of 0.1 THz–4 THz has made their applications grow significantly<sup>1</sup>. One area where THz has shown promise and significant growth is the biomedical field<sup>2</sup>. THz radiation has been shown to be nonionizing and biologically safe at the power levels generally used to analyze fixed tissues<sup>3</sup>. As a result, THz imaging and spectroscopy has been used to classify and differentiate various tissue features such as water content to indicate burn damage and healing<sup>4</sup>, liver cirrhosis<sup>5</sup>, and cancer in excised tissues<sup>6,7</sup>. Cancer assessment in particular covers a broad range of potential clinical and surgical applications, and has been investigated for cancers of the brain<sup>8</sup>, liver<sup>9</sup>, ovaries<sup>10</sup>, gastrointestinal tract<sup>11</sup>, and breast<sup>7,12–19</sup>.

THz applications for breast cancer are primarily focused on supporting breast conserving surgery, or lumpectomy, via margin assessment. The objective of a lumpectomy is to remove the tumor and a small layer of surrounding healthy tissue, in contrast to full mastectomy, which removes the entire breast. The surgical margin of the excised tissue is then assessed via pathology once the sample has been fixed in formalin, sectioned, embedded in paraffin, and mounted in 4 µm–5 µm slices on microscope slides. This process can be time-consuming and requires a secondary surgical procedure at a later time if a positive margin is observed<sup>20</sup>. Current guidelines by the American Society of Radiation Oncology define this positive margin as having cancer cells contacting the surface-level margin ink<sup>21</sup>. THz imaging for high-absorption hydrated tissue is primarily limited to surface imaging with some varying penetration based on tissue type, which is sufficient for meeting the surgical needs of rapid margin assessment. A quick analysis of margin conditions during the surgical setting would greatly decrease surgical costs and follow-up procedure rate. To date, THz has proven effective in differentiating between cancer and healthy tissue in formalin-fixed, paraffin-embedded (FFPE) tissues, but additional investigation is needed to provide reliable detection of cancer in freshly excised tissues<sup>7</sup>.

This protocol details the steps for performing THz imaging and spectroscopy on freshly excised human tissue samples obtained from a biobank. THz applications built on freshly excised human breast cancer tissues have seldom been used in published research<sup>7,18,22,23</sup>, especially by research groups not integrated with a hospital. The use of freshly excised tissues is likewise rare for other cancer applications, with most non-breast human cancer examples being reported for colonic cancer<sup>24,25</sup>. One reason for this is that FFPE tissue blocks are far easier to access and handle than freshly excised tissue unless the THz system being used for the study is part of the surgical workflow. Similarly, most commercial laboratory THz systems are not prepared to handle fresh tissue, and those that do are still in the stages of using cell line growth or have only started to look at excised tissue from animal models. To apply THz to an intraoperative setting requires that imaging and characterization steps be developed for fresh tissue in advance so that the analysis does not interfere with the ability to perform standard pathology. For applications that are not inherently meant to be intraoperative, the characterization of fresh tissue is still a challenging step that must be addressed to work towards in vivo applications and differentiation.

The objective of this work is to provide a guideline for THz application for freshly excised tissue using a commercial THz system. The protocol was developed on a THz imaging and spectroscopy system<sup>26</sup> for murine breast cancer tumors<sup>13,17,19</sup> and was extended to human surgical tissue obtained from biobanks<sup>7,18</sup>. While the protocol was generated for breast cancer, the same concepts can be applied to similar THz imaging systems and other types of solid-tumor cancers that are treated with surgery where success depends on margin assessment<sup>27</sup>. Due to a fairly small amount of published THz results on freshly excised tissues, this is the first work to the authors' knowledge to focus on the protocol of fresh tissue handling for THz imaging and characterization.

## **PROTOCOL:**

This protocol follows all the requirements set by the Environmental Health and Safety department at the University of Arkansas.

### **1. Set up the tissue handling area**

1.1. Take a stainless-steel metal tray and cover it with the biohazard bag as shown in **Figure 1**. Any handling of the biological tissues will be performed within the tray area (i.e., the tissue handling area).

1.2. Prepare laboratory tweezers, tissue wipes, paper towels, filter paper pack, tissue dye bottles, bleach bottle, and ethanol bottle around the tray for easy access when required. Keep any used tissues, wipes, and gloves on the biohazard material surface to dispose of at the end of the protocol.

1.3. Fill a 50 mL centrifuge tube with up to 45 mL of 10% neutral buffered formalin and place it in the centrifuge storage tray near the tissue handling tray.

[Place **Figure 1** here].

### **2. Handling fresh breast cancer tumor for THz transmission spectroscopy**

**CAUTION:** Before handling any live tissues, put on nitrile hand gloves, eye protection goggles, a face mask, and a lab coat. Always use laboratory tweezers to handle tissues and avoid touching them directly with the hands. All work with fresh tissue outside of a sealed container or the scanning stage should be conducted at the tissue handling area established in step 1.1.

**NOTE:** All tissues handled in this work were shipped in Dulbecco's Modified Eagle's medium (DMEM) and antibiotic solution from the biobank.

2.1. Remove the bulk tumor from the DMEM solution and place it in a Petri dish on the tissue handling area (see **Figure 2A**).

2.2. From gross inspection, identify distinct tumor regions from which to slice small pieces for transmission characterization. Cut a 0.5 mm thick segment of tumor from the identified points using a stainless steel low profile blade, as shown in **Figure 2B**. Place this sliced section between two quartz windows with a spacer of 0.1 mm thickness in a liquid sample holder, as shown in **Figure 2C**.

[Place **Figure 2** here].

### 3. THz transmission spectroscopy measurements

3.1. 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**.

3.2. Purge the system with dry nitrogen gas at 5 L/min (LPM) during the entire spectroscopy procedure to remove water vapor from the sample space.

3.3. Open the THz transmission spectroscopy measurement software from the desktop connected to the THz system. It will open up the main window.

3.4. Click on the **Scan** tab on the top of the window. A **Spectra Scan Setup** window will appear. From the drop-down menu of the **Measurement Mode** tab on the top right of the window, select **Transmission** to set up transmission spectroscopy. If the peak is not automatically visible, check the **Enable** option under the **Manual Peak Search** tab and manually step the optical delay to bring the peak into view.

3.5. After 30 min 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 file, set **Num Scans** to 1,800, 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 1,800 signals over ~1 min.

3.6. Measure the two quartz windows in the liquid sample holder as shown in **Figure 3B**.

3.6.1. Place the two quartz windows in the liquid sample holder without a spacer in between.

3.6.2. Open the THz core chamber. Mount the liquid sample holder on the transmission spectroscopy module, as shown in **Figure 3C**. Close the chamber.

3.6.3. Click on the **Scan** tab on the main window. Repeat steps 3.5.1–3.5.2 for the quartz sample, but update **Start Delay (s)** to 900. This allows time to purge any water vapor before measurement.

3.6.4. If the quartz is desired as a reference for additional samples, click on the **Clear Reference** tab under the **Scan Settings**. This clears the air reference. Then click on the **Measure Reference** tab to record the quartz measurements as a new reference.

3.7. Place the sliced tumor section between the two quartz windows inside the liquid sample holder and position the holder inside the chamber for a single point transmission measurement of the tissue. To record the measurement, repeat step 3.6.3.

3.8. Take the liquid sample holder out of the chamber when the measurements are completed and bring it to the area designated for tissue handling. Disassemble the liquid sample holder, wipe the tumor section from the quartz windows with the tissue wipes, and place the used tissue wipes in the same tray to dispose in the biohazard bag along with the other biohazard waste.

3.9. Repeat steps 2.2, 3.7, and 3.8 as necessary to characterize additional tumor slices. When the measurements are completed, go to the main window and click on the **File** tab to save the measurement data. Close the software window.

#### **4. Handling fresh breast cancer tumor for THz reflection mode imaging**

4.1. Remove the fresh tumor sample from the DMEM and antibiotics solution and place it on a Petri dish. Using gross inspection, select a side of the tumor to be imaged that is sufficiently flat and has little blood and few blood vessels. Avoid imaging tissue with blood or blood vessels if possible.

4.2. Place the tumor with the side to be imaged on grade 1 filter paper to dry the excess DMEM and clear the tissue of fluid or secretions from the tumor, as shown in **Figure 4A**. Reposition the tumor on the filter paper to a dry spot as the paper saturates. Dry the tumor for ~5 min.

[Place **Figure 4** here].

4.3. Unmount the transmission spectroscopy module and set the reflection imaging module (RIM) mirror base on the THz core system as shown in **Figure 5A**. Upon setting the mirrors, mount the RIM scanning stage above the mirror base and screw it into the core system (see **Figure 5B**).

4.4. Purge the system with dry nitrogen gas at 5 LPM for 30 min prior to the imaging procedure to remove water vapor from the sample compartment. After 30 min, reduce the amount of dry nitrogen gas to 3 LPM for the rest of time the system is in use.

4.5. Place a polystyrene plate of thickness  $\sim 1.2$  mm on the scanning window of diameter  $\sim 37$  mm. Center the scanning window along with the polystyrene plate on the sample stage.

[Place **Figure 5** here].

NOTE: Other thicknesses and plate materials are suitable for step 4.5 but should have a uniform thickness and be of low enough absorption to not impede the THz signal.

4.6. Open the THz reflection imaging measurement software from the desktop connected to the THz system. A window will pop up showing several dialog icons for specific functions and two subwindows for THz field plots, (Au) against the time and frequency, respectively.

4.7. To set the parameters for the RIM set-up, click on the **Image Parameter Dialog** icon at the top of the window. An **Image Acquisition Parameters** window will pop up. Select **RIM** from the drop-down menu of the **Template** tab for reflection imaging set up. Hit **OK** and go back to the main window of the software.

4.8. On the main window, click on the **Fixed-point Scan** icon. This will activate the THz antennas to start sending the incident THz signal and receiving the reflected THz signal from a single point on the polystyrene plate.

4.9. Click on the **Motor Stage Dialog** icon on the top of the main window. The motor control window will open up. Adjust the optical delay axis by clicking on the **forward/reverse direction arrows** to center the reflected pulse from the polystyrene in the main window.

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).

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. This is done in two steps.

4.10.1. First, click on the **DAQ Settings** button at the top of the main window to open the DAQ settings dialog window. Change the optical delay value from 5 V (default) to 4 V.

4.10.2. Second, adjust the scanning stage's vertical position with the micrometer scale on the scanning stage until the minima of the secondary pulse is the strongest. Adjust the optical delay of the axis in the **Motor Control Window** to put the primary reflection outside of the range of the reflected signal being measured.

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.

[Place **Figure 6** here].

4.11. Level the sample stage and record the reference signal.

4.11.1. Select two points on each axis (A-axis and B-axis) that denote locations on the polystyrene plate near the edge of the sample window. For example, for the A-axis ranging from -15 mm–15 mm, the two position points can be -10 mm and 10 mm; and for the B-axis ranging from -15 mm–15 mm, the two position points can be -10 mm and 10 mm.

4.11.2. Click on the **Motor Control Dialog** button to open the motor control window. Reposition the motor control window and the main software window so that the time domain signal is visible while adjusting the motor positions. Set both the A-axis and B-axis to 0 mm.

4.11.3. Level the A-axis using following steps. A -10 mm–10 mm range is used as an example.

4.11.4. In the **Motor Control Window**, change the value of the A-axis from 0 to -10 and hit **Enter**. The stage moves to the -10 mm position on the A-axis and a shift in the signal position on the main window is observed.

4.11.5. Use the adjustable micrometer scale on the scanning stage shown in **Figure 5B** to move the minimum peak of the signal back to the position set in step 4.10.2.

4.11.6. Change the A-axis value to +10 and hit enter. The stage will now move from the -10 mm position to the +10 mm position on the A-axis and a shift in the signal is observed again. Note the direction and the distance that the signal shifted from its previous position and change the A-axis value again to -10. The signal will go back to the position set in step 4.11.5.

4.11.7. Rotate the leveling screw on the A-axis of the scanning stage, as shown in **Figure 5B** and shift the signal to double the distance in the same direction it moved from the original position. Use the micrometer on the scanning stage to shift the signal back to the original position (-0.3 mm for 1.2 mm of polystyrene).

4.11.8. Repeat steps 4.11.6–4.11.7 until the signal at +10 and -10 are equal and the peak for both positions is focused at the original position (-0.3 mm on the optical axis).

4.12. Once the leveling of the A-axis is achieved, change the A-axis value to 0 and repeat the same procedure for the B-axis. Start by changing the value of the B-axis on the motor control window from 0 to the most positive value (for example +10 mm). Also, while leveling, use the leveling screw on the B-axis of the scanning stage, which is shown in **Figure 5B**.

4.13. Once both axes are leveled, return both the A-axis and the B-axis to 0 mm. Close the **Motor Control Window** and verify that the signal is in its original position in case it is shifted a little.

4.14. Record this signal as the reference.

4.14.1. Go to the set **DAQ Properties** window. Change the averaging value to 5 and keep all other parameters as default.

4.14.2. Click on **New Reference**. The averaging counter in the top right of the window will count from 0–20. Once the counter reaches 20, change the averaging value to 1 and click **OK**. The reflected signal from the polystyrene will be saved as the reference for any scans taken later.

NOTE: If only the THz imaging procedure has to be performed, then it is best to perform steps 4.3–4.14 before taking the tumor tissue out of the DMEM solution.

4.15. Mount the tumor on the polystyrene plate covering the scanning stage window.

4.15.1. Remove the imaging window from the scanning stage and bring it to the tissue handling area. Place the tumor on a polystyrene plate, as shown in **Figure 4B**.

4.15.2. Ensure that there are no significant air bubbles between the plate and the tumor. If air bubbles are observed, press the tumor with tweezers or lift the tumor and gently roll it onto the polystyrene until the air gaps are minimized.

4.15.3. Place absorptive spacers at regular intervals around the test sample as shown in **Figure 4B**. Place another polystyrene plate above the tumor and press gently in order to make the tumor surface as flat as possible. Tape down this polystyrene-tumor-polystyrene arrangement on the sample window.

4.16. Flip the sample window as shown in **Figure 4C**, and take photos of the tumor to keep a record of its orientation. Return the sample window with the tumor to the scanning stage.

4.17. Click on the **Image Parameter Dialog** button to open the **Image Acquisition Parameters** window. Set the values of **Axis1min**, **Axis1max**, **Axis2min**, and **Axis2max** to fully enclose the position of the tumor in the imaging window

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

4.18. Set **Axis1step** and **Axis2step** to 0.2 mm for the imaging scan.

NOTE: Setting the **Axis1step** and **Axis2step** will set the stepper motors' step size to 200  $\mu\text{m}$  increments during the scanning process. The total scan time can be estimated in the **Image Acquisition Parameters** window.

4.19. Click on the **Measure** tab on the main window and select the **Flyback 2D Scan** option. In the window that pops up, indicate the directory and file name under which to save the scan data.

## 5. Postprocessing the fresh tissue in preparation for histopathology procedure

5.1. Upon completion of the scanning process, remove the sample window, polystyrene plates, and sample from the core THz system and move them to the area designated for hazardous waste. Remove the tumor from the polystyrene plate and place it on a flat piece of cardboard of a size comparable to that of the tumor. Make sure the orientation of the tumor is the same as it was on the polystyrene, with the imaging face touching the cardboard.

5.2. Dip a cotton swab in red tissue dye and stain the left side of the tumor down to where the edge of the tumor contacts the cardboard. Similarly, stain the right side of the tumor with blue tissue dye. Stain the exposed surface of the tumor with a line of yellow tissue dye connecting the red stain to the blue stain to denote the back of the sample, as shown in **Figure 7A**.

NOTE: To prevent the ink from staining the formalin solution, apply only a thin layer to the tissue. This can be accomplished by dabbing the cotton swab on a different surface before staining the tissue or using a clean cotton swab to wipe off any excess dye. Avoid letting the dye contact the skin or clothing. This tumor-staining process is conducted as a reference to provide information about the tumor's imaging side and its orientation to the pathologist.

[Place **Figure 7** here].

5.3. Let the ink dry for around 3 min–4 min. Cut a piece of filter paper with the same approximate dimensions as the cardboard. Place it on the tumor and wrap a piece of tape completely around the filter paper and cardboard as shown in **Figure 7B**. The tape and filter paper should secure the tumor against the cardboard without applying any significant pressure.

5.4. Immerse the stained tissue affixed to the cardboard in 10% neutral buffered formalin solution and seal the centrifuge tube using a paraffin film, as shown in **Figure 7C**. Designate the sample number, date, tissue type, and tumor number for the sample on the tube label. Send the tumor to the pathologist for further histopathology processing.

## 6. Hazardous waste disposal

6.1. Collect all the waste from the tissue handling tray along with the biohazard bag used to cover the tray and put it in a new biohazard bag, as shown in **Figure 8**. Bring the bag to the designated biohazardous waste area in the building and set an appointment with the Environmental Health and Safety (EH&S) department for the waste pickup. Clean the tissue handling tray and the surrounding area on the table with 10% bleach solution and ethanol.

[Place **Figure 8** here].

6.2. Take the liquid sample holder with the spacers and quartz windows, sampling window on which tumor was mounted, polystyrene plates, and laboratory tweezers to the washing area.

Rinse all materials with water and then 10% bleach solution, wiping with paper towels as necessary to remove tissue debris. Rinse again with water, scrub with alconox solution, and rinse thoroughly. For glass and plasticware, rinse in 70% isopropyl alcohol and set aside to dry.

NOTE: Once the tumor is in formalin and the sample space is clean, data processing can be handled at the same time as imaging or a later time.

## 7. Data processing to construct THz images

7.1. Export the saved .tvl data files from the THz system. The raw data files obtained from the system are written in Python and are best read in Python before saving as MATLAB data files.

7.2. To construct the THz image of the scanned fresh tissue, convert the raw time domain reflection imaging data into the frequency domain using Fourier transform on the third dimension of the raw data matrix (i.e., the time dimension). Also take the Fourier transform of the reference data.

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

7.3. Normalize the sample data with the reference data and perform the power spectra based on the integration of the normalized data over the frequency range from  $f_1 = 0.5$  THz to  $f_2 = 1.0$  THz using the following equation<sup>19</sup>:

$$Power\ Spectra = \int_{f_1}^{f_2} |E_{sample}|^2 / |E_{reference}|^2 df_{THz} \quad (1)$$

NOTE: Here  $E_{sample}$  is the frequency domain reflection imaging data of the tissue sample and  $E_{reference}$  is the frequency domain of a single point reflection data of the reference signal.

7.4. Construct the two-dimensional image by plotting the calculated power spectra data at each point in the matrix defined by the A-axis and B-axis. This is known as the power spectra THz image.

NOTE: The method to obtain a tomographic THz image instead is detailed in steps 7.5–7.7.

7.5. For characterization, calculate the theoretical frequency-dependent reflection for a range of potential tissue properties using the following equation<sup>18</sup>:

$$\left( \frac{E_{samp}}{E_{ref}} \right)_T = \frac{\tilde{\Gamma}_{T,2,samp}}{\tilde{\Gamma}_{T,2,ref}} = \left( \frac{\rho_{T,23} - \rho_{T,31} e^{-j2\tilde{k}_3 \cos \theta_3 d_3}}{1 + \rho_{T,23} \rho_{T,31} e^{-j2\tilde{k}_3 \cos \theta_3 d_3}} \right) / \rho_{T,21} \quad (2)$$

NOTE: Here  $\rho_{T,ij}$  is the complex Fresnel reflection coefficient between region  $i$  and region  $j$ ;  $d_j$  is the thickness of region  $j$ ; and  $\theta_j$  is the angle of propagation in region  $j$  related to the angle of

incidence by Snell's Law.  $\tilde{k}_j = \frac{\omega}{c} n_i - j \frac{\alpha_{abs,i}}{2}$  is the complex propagation coefficient in region  $j$ , where  $\omega$  is the angular frequency,  $c$  is the speed of light in vacuum,  $n_j$  is the real part of the refractive index, and  $\alpha_{abs,j}$  is the absorption coefficient<sup>18</sup>. Region 1 is air, Region 2 is the polystyrene plate, and Region 3 is the tissue.

7.6. Calculate the reflection in equation (2) for a range of user-defined refractive indexes and absorption coefficients for Region 3 ( $n_3$  and  $\alpha_{abs,3}$ ) and compare with the measured signal at each point to calculate the combined mean squared error for the magnitude and phase.

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

7.7. Construct the tomographic THz image from the extracted refractive index and absorption coefficient data ( $n_3$  and  $\alpha_{abs,3}$ ) at each pixel. Analyze the tumor regions by comparing with the pathology slide image obtained from the pathologist. Representative results are shown in **Figure 9**, with examples of insufficient adherence to the protocol in **Figure 10** and **Figure 11**.

## 8. Extraction of electrical properties of the tissue using transmission spectroscopy data

8.1. On the main window of the THz transmission spectroscopy measurement software, go to the **File** tab and click on the **Export** option. A window will pop up to select the **Data Type** and **Sample** to export. Choose **Transmittance** and **Transmittance Phase** data types for the quartz and tissue sample measurements.

8.2. Calculate the theoretical frequency-dependent transmission for a range of potential tissue properties using the following equation<sup>15</sup>:

$$\frac{E_{samp}}{E_{ref}} = \tilde{\tau} e^{(\gamma_1 - \gamma_3)d} \quad (3)$$

NOTE: Here  $\tilde{\tau}$  is the ratio between Fresnel transmission coefficients for the sample and reference setups;  $\gamma_1$  and  $\gamma_3$  are the complex propagation constants of air and tissue, respectively; and  $d$  is the thickness of the tissue. The propagation constant in general is defined as  $\gamma = j \frac{\omega}{c} \tilde{n}$ .  $\tilde{n}$  is the complex refractive index defined as  $\tilde{n} = n - j \frac{c}{\omega} \frac{\alpha_{abs}}{2}$ , where  $n$  is the real part of the refractive index;  $c$  is the speed of light;  $\omega$  is the angular frequency; and  $\alpha_{abs}$  is the absorption coefficient<sup>15</sup>.

8.3. Calculate the combined mean squared error between the magnitude and phase of the transmission in equation (3) and the measurement data from the system for a range of user-defined  $n$  and  $\alpha_{abs}$  values.

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

8.4. Plot the extracted refractive index and absorption coefficient data against the frequency range from 0.15 THz–3.5 THz. Representative results are shown in **Figure 12**.

#### **REPRESENTATIVE RESULTS:**

The THz imaging results<sup>18</sup> obtained following the abovementioned protocol of human breast cancer tumor specimen #ND14139 received from the biobank are presented in **Figure 9**. According to the pathology report, the #ND14139 tumor was a I/II grade infiltrating ductal carcinoma (IDC) obtained from a 49-year-old woman via a left breast lumpectomy surgery procedure. The photograph of the tumor is shown in **Figure 9A**, the pathology image in **Figure 9B**, and the THz power spectra image obtained using equation (1) in the protocol is shown in **Figure 9C**. The assessment of the pathology image was done by our consulting pathologist at Oklahoma State University. Upon correlating the THz image with the pathology image, it was clear that the cancer region (i.e., the red color region in **Figure 9C**) showed higher reflection than the fat region (i.e., the blue color region in **Figure 9C**). The blue circle close to the center of the cancer region in **Figure 9C** was due to the presence of an air bubble beneath the tumor during the imaging process.

Tomographic images based on the electrical properties of the tumor obtained using the above discussed model for each pixel (2,477 pixels in total) are also presented. The tomographic images based on the absorption coefficient ( $\text{cm}^{-1}$ ) data ( $\alpha$ - images) and refractive index ( $n$ - image) data of the tumor obtained at frequency 0.5 THz and 1.0 THz are shown in **Figure 9D**, **9E**, **9F**, and **9G**, respectively. As the frequency increased, the calculated absorption coefficient ( $\text{cm}^{-1}$ ) values for the cancer and fat pixels increased, with cancer pixels showing higher values than fat at both frequencies. In contrast, the refractive index of both tissues decreased as the frequency increased. It should be noted that the measured phase became subject to micrometer-scale variations in the imaging stage leveling, polystyrene plate thickness, and stepper motor jitter as the frequency increased. For example, the horizontal lines observed in **Figure 9E** and **9G** were due to the small phase shift introduced by the stepper motors during the scanning process, which was not observed at lower frequencies.

[Place **Figure 9** here].

The THz results discussed in **Figure 9** were obtained by successfully following the described protocol. Insufficient handling of the tissue can lead to misleading imaging results. For example, the THz imaging results in **Figure 10** for human breast cancer tumor #ND10405 show the effects of insufficient drying. Excess DMEM solution in the tissue dominated the THz power spectra image of the tumor in **Figure 10B**<sup>28</sup> with high reflection that did not correlate to the pathology image shown in **Figure 10A**<sup>28</sup>. This led to a false positive result, suggesting a larger presence of cancer in the tumor. DMEM showed a similarly high refractive index and absorption coefficient to water, as seen in **Figure 10C**<sup>19</sup> and **10D**<sup>19</sup>, so it is highly recommended to dry the tumor properly before imaging.

[Place **Figure 10** here].

Another example of insufficient adherence to the protocol is shown for tumor #ND11713 in **Figure 11**. In this case, the air bubbles between the polystyrene plate and the tumor were not removed when the tumor was placed on the plate for the imaging procedure. This resulted in several spots of low reflection across the THz image in **Figure 11B**, which prevented accurate comparison to the pathology in **Figure 11A**. Thus, if any air bubbles are observed after placing the tumor on the plate, press it with the tweezers or lift the tumor and gently roll it onto the polystyrene until air gaps are removed.

[Place **Figure 11** here].

Transmission spectroscopy results<sup>18</sup> for the same sample (# ND14139) are presented in **Figure 12**. Tumor sections were taken from points ① and ② in **Figure 12A** and characterized following the protocol. Both selected points were taken from the cancer tissue region in the tumor according to the pathology image in **Figure 12B**. The extracted absorption coefficient and refractive index for both tumor sections are presented in **Figures 12C** and **12D**. Both points showed good agreement for the whole frequency range. The black curve from 0.15 THz–2 THz in **Figures 12C** and **12D** represents data obtained from the literature<sup>23</sup> to compare the results obtained in our work.

[Place **Figure 12** here].

#### **FIGURE LEGENDS:**

**Figure 1: Setup of tissue handling area.**

**Figure 2: Tumor sectioning for the THz transmission spectroscopy measurements.** (A) Photograph of the bulk tumor. (B) Photograph of the small sections (0.5 mm) of the tumor cut from the bulk tumor. (C) The sliced tumor section placed in the liquid sample holder between the two quartz windows with a 0.1 mm polytetrafluoroethylene spacer for spectroscopy measurement. Figure republished from T. Bowman et al.<sup>18</sup> with permission from SPIE.

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

**Figure 4: Fresh tumor sample preparation for THz imaging.** (A) Tumor placed on filter paper to dry. (B) Tumor placed on polystyrene plate over the imaging window with tissue wipe pads to absorb excess fluids. (C) Tumor viewed from below to track orientation and check for air bubbles.

**Figure 5: System setup for reflection imaging.** (A) Reflection imaging module mirror base. (B) Scanning stage.

**Figure 6: THz reflections from the lower and upper interfaces of the polystyrene plate.** (A) THz signal incident to and reflected from a 1.2 mm thick polystyrene plate. (B) Measured primary and secondary THz time domain signals from the polystyrene.

**Figure 7: Post processing on the tumor after THz imaging.** (A) Tumor placed face down on cardboard holder and dyed with tissue marking dye. (B) Filter paper placed over tumor and taped to maintain contact. (C) Stained tumor fixed on the cardboard immersed in 10% neutral buffered formalin solution and sealed with parafilm.

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

**Figure 9: Analysis of breast cancer tumor #ND14139 using THz imaging technique.** (A) Photograph of the tumor. (B) Low power pathology image of the tumor. (C) THz power spectra image over the frequency range 0.5 THz–1.0 THz. (D) THz tomographic absorption coefficient image obtained at 0.5 THz. This image was constructed using the extracted absorption coefficient data at each pixel from the raw reflection imaging data of the tumor. (E) Absorption coefficient image obtained at 1.0 THz. (F) Refractive index image ( $n$ - image) obtained at 0.5 THz. This image was constructed using the extracted refractive index data at each pixel from the raw reflection imaging data of the tumor. (G) Refractive index image ( $n$ - image) obtained at 1.0 THz. Figure republished from T. Bowman et al.<sup>18</sup> with permission from SPIE.

**Figure 10: The effect on tumor imaging taken out of the DMEM solution without drying using filter paper.** (A) Low power pathology image of the tumor #ND10405. (B) THz power spectra image of tumor #ND10405 over the frequency range 0.5 THz–1.0 THz. (C) The transmission refractive index plot for DMEM, PBS, and water ranging from 0.15 THz–3.5 THz. (D) The transmission absorption coefficient ( $\text{cm}^{-1}$ ) plot for DMEM, PBS, and water ranging from 0.15 THz–3.5 THz. **Figure 10A, 10B** are republished from T. Bowman et al.<sup>28</sup> with permission from IEEE and **Figure 10C, 10D** are republished from N. Vohra et al.<sup>19</sup> with permission from IOP Publishing, Ltd.

**Figure 11: The artifacts in the THz image caused by the presence of air bubbles between the polystyrene plate and tumor.** (A) Low power pathology image of tumor #ND11713. (B) THz power spectra image of tumor #ND11713 over the frequency range from 0.5 THz–1.0 THz.

**Figure 12: The characterization of breast cancer tumor #ND14139 using THz transmission spectroscopy.** (A) The photograph of the tumor with two selected points marked ① and ② from where the 0.5 mm thick sections of the tumor were cut for the transmission spectroscopy measurements. (B) Low power pathology image of the tumor. (C) The transmission absorption coefficient ( $\text{cm}^{-1}$ ) plot ranging from 0.15 THz–3.5 THz at points ① and ②. (D) The transmission refractive index plot ranging from 0.15 THz–3.5 THz at points ① and ②. Figure republished from T. Bowman et al.<sup>18</sup> with permission from SPIE.

## DISCUSSION:

Effective THz reflection imaging of fresh tissue is primarily dependent on two critical aspects: 1) the proper consideration of tissue handling (sections 2 and 4.15); and 2) the stage setup (primarily section 4.11). Insufficient drying of the tissue can result in increased reflection and inability to visualize regions due to high reflections of DMEM and other fluids. Meanwhile, poor



tissue contact with the imaging window creates rings or spots of low reflection in the THz reflection image that obscure the results. Extra effort should be taken to ensure good tissue contact with the imaging window, including repositioning the tissue to obtain a better interface. For tissue characterization, additional considerations for the stage setup must be carefully implemented. Improper balancing of the stage by even a few microns can cause significant shifts in the calculated refractive index and absorption coefficient of the tissue. This can also be a result of applying too much pressure to the tissue when mounting it on the imaging window, which can cause bowing of the polystyrene plate. For accurate calculations, the reference signal selected for characterization must also be obtained from the same phase plane of the image to avoid artificial phase shift.

The primary area where the protocol can be modified is in the dielectric materials used to mount the tissue, such as quartz (sections 3.6–3.7) and polystyrene (starting in section 4.5). 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 whether they provide 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 that enables comparison between the THz imaging and the 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.

A primary limitation of any fresh tissue handling technique is the time that the tissue is exposed to air. This protocol was designed such that the tissue could remain exposed for no more than 1 h to avoid decomposition prior to the pathology assessment. This is also reflected in the selection of the step size of the image. The THz system in this protocol can reach any step size from 50  $\mu\text{m}$ –500  $\mu\text{m}$  in 50  $\mu\text{m}$  increments, though the maximum spatial resolution of the system is around 80  $\mu\text{m}$  due to the spectral content of the THz signal. The 200  $\mu\text{m}$  step in the protocol provided sufficient detail while maintaining a reasonable scan time of  $\sim 30$  min. Assessment of the tumor samples by our consulting pathologist determined that this amount of air exposure does not cause damage to the tissue in an observable way at the cellular level. However, materials such as gelatin can be used to provide clear THz imaging without excessive drying, and may be investigated for future updates to the protocol<sup>29</sup>. For efficient use of time, steps like purging the system with dry nitrogen and setting up the imaging or spectroscopy can be performed before the tissue is removed from the DMEM. This is also important for future intraoperative applications where the time taken for imaging is a key factor in implementing the THz imaging into the surgical workflow.

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 a minutes. This will be accomplished when the hardware of the THz system is improved 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 for interpretation by a radiologist to determine whether there is cancer on the tissue surface. The described imaging protocol provides a means of direct imaging of the tissue surface. 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<sup>8-11</sup>. While this manuscript focuses on imaging freshly excised breast tumors following the described protocol, THz imaging of the associated formalin-fixed paraffin-embedded tissue blocks has also been successfully validated with pathology<sup>14-17, 19</sup>. Imaging protocols similar to the one proposed here could be developed for pathology support in analyzing embedded tissues as well.

#### ACKNOWLEDGMENTS:

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#### DISCLOSURES:

The authors declare that they have no conflict of interest.

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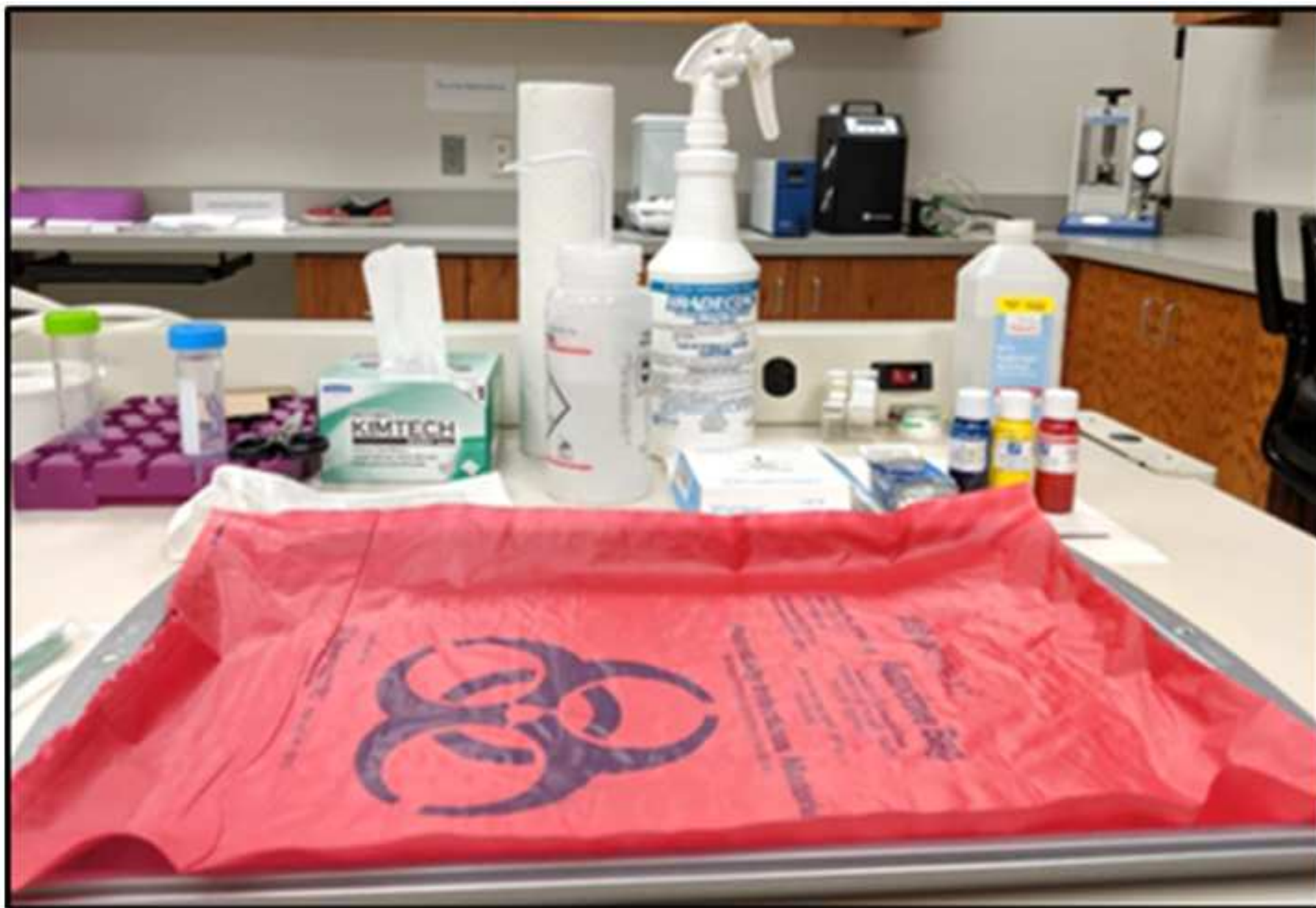
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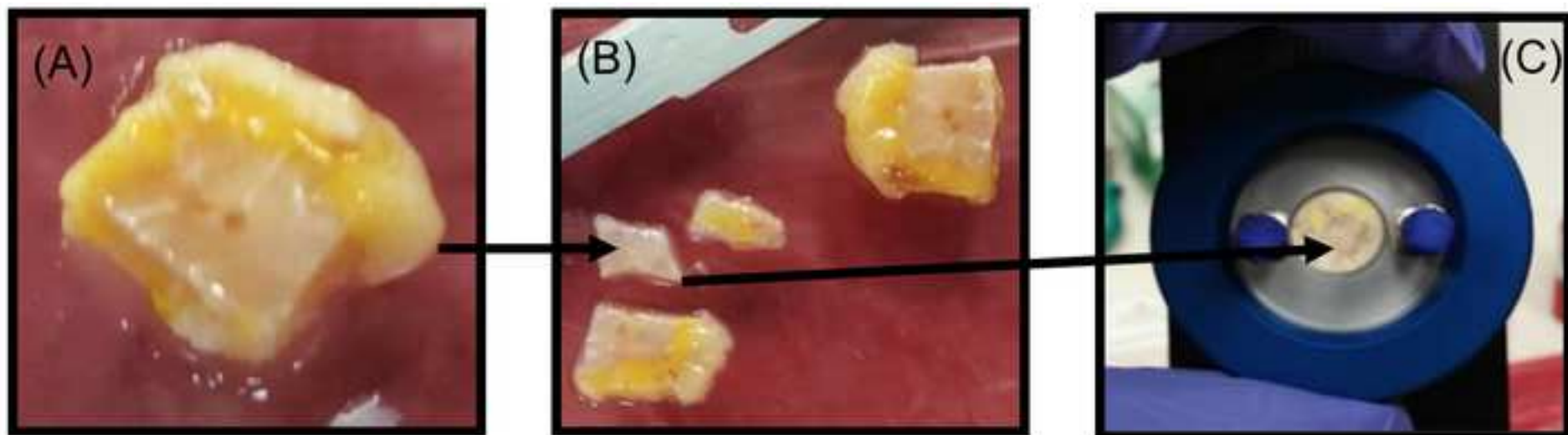
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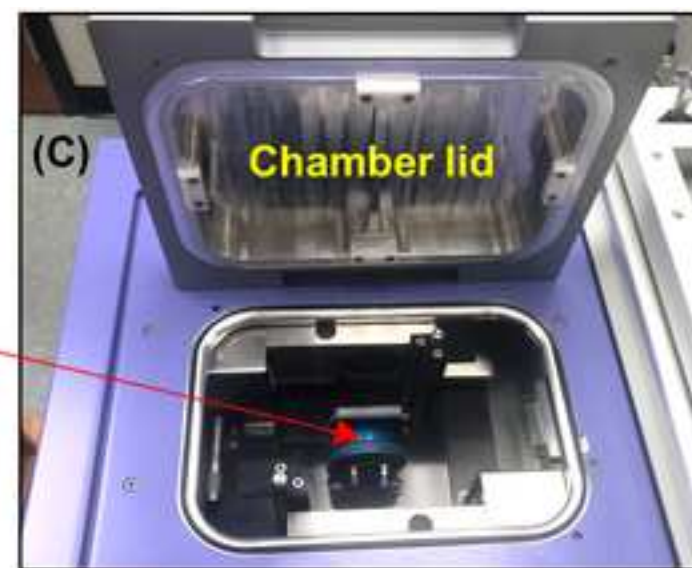
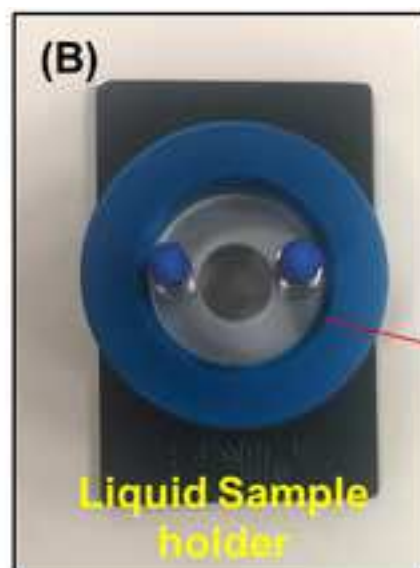
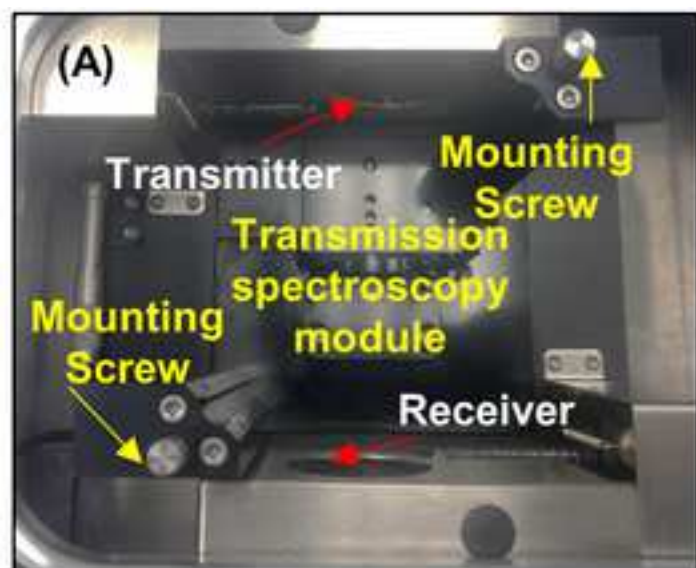
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Figure 1

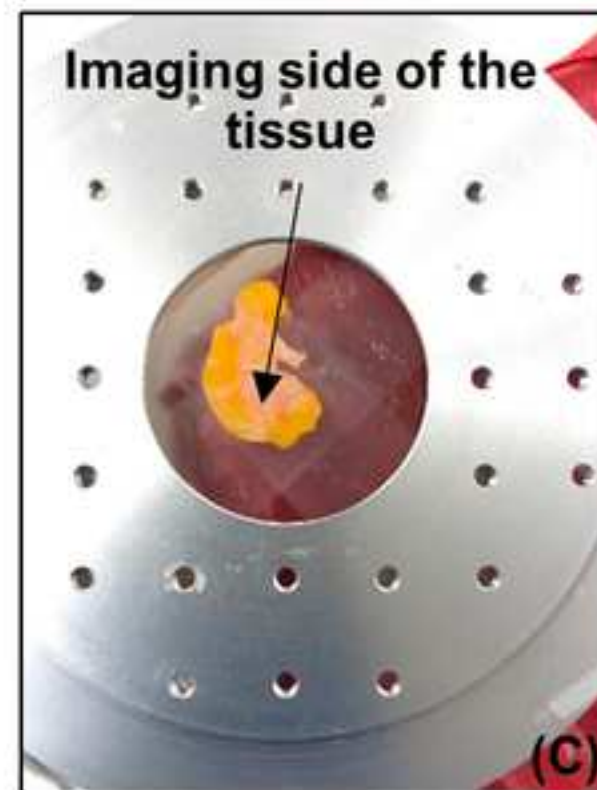
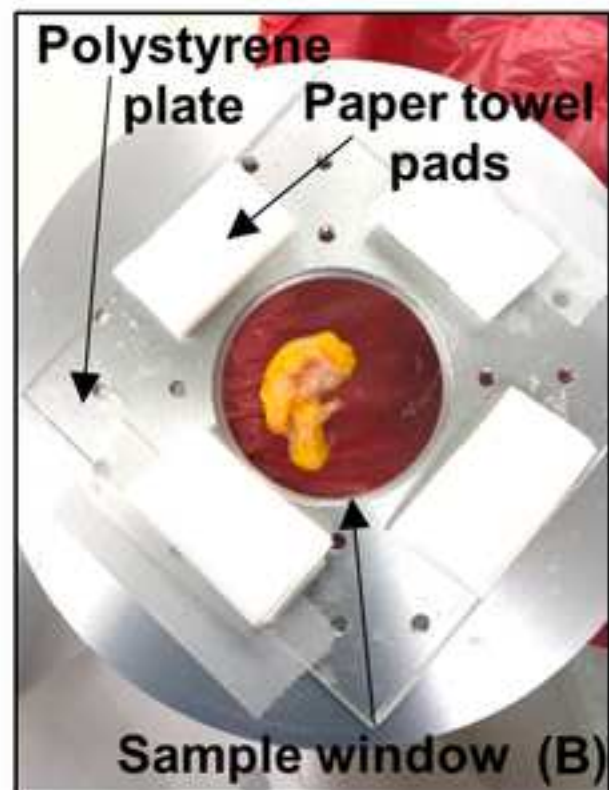
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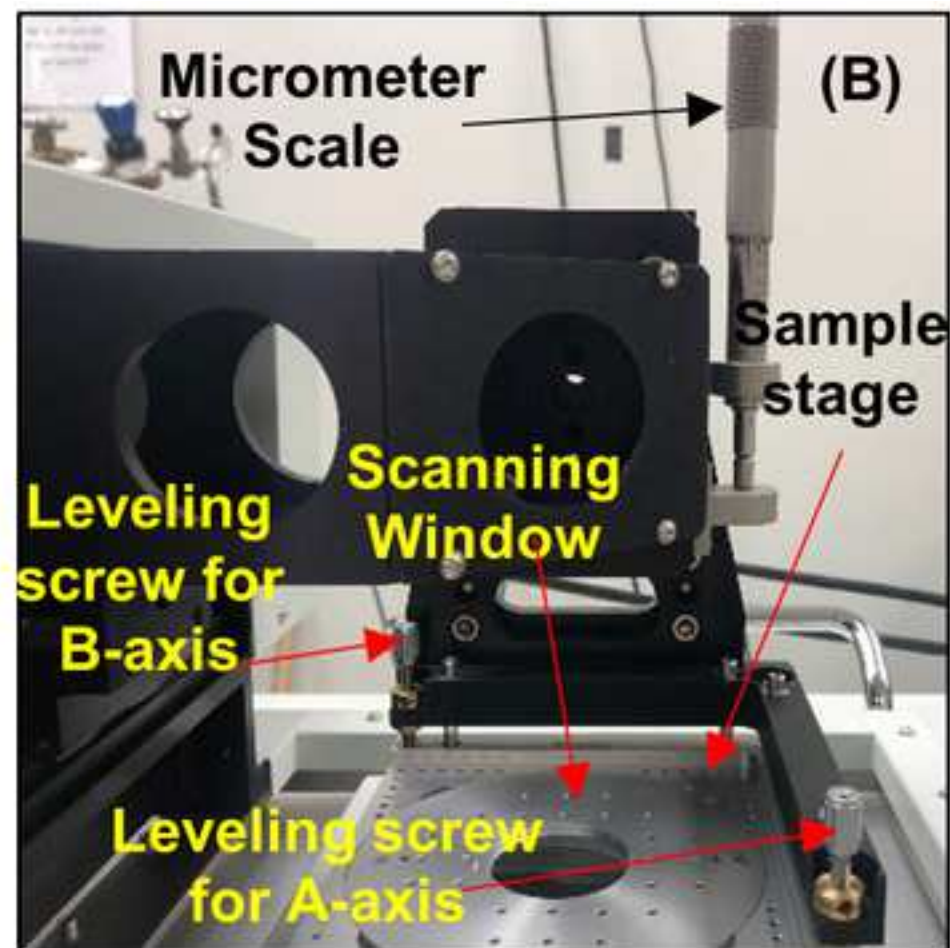
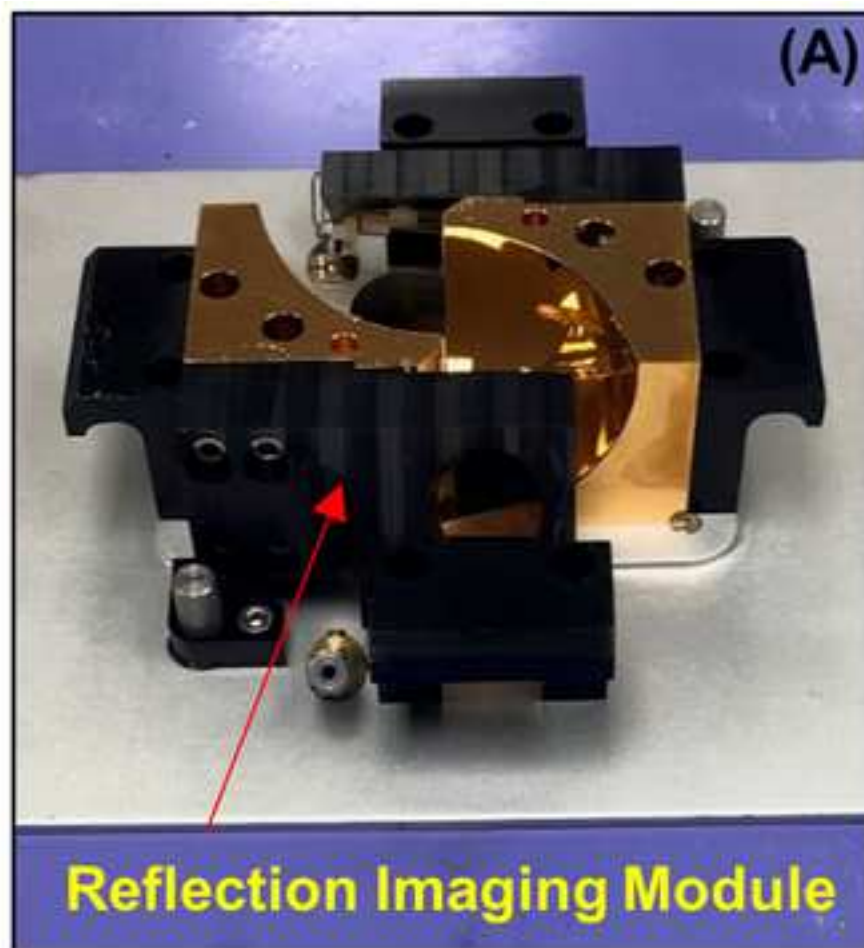


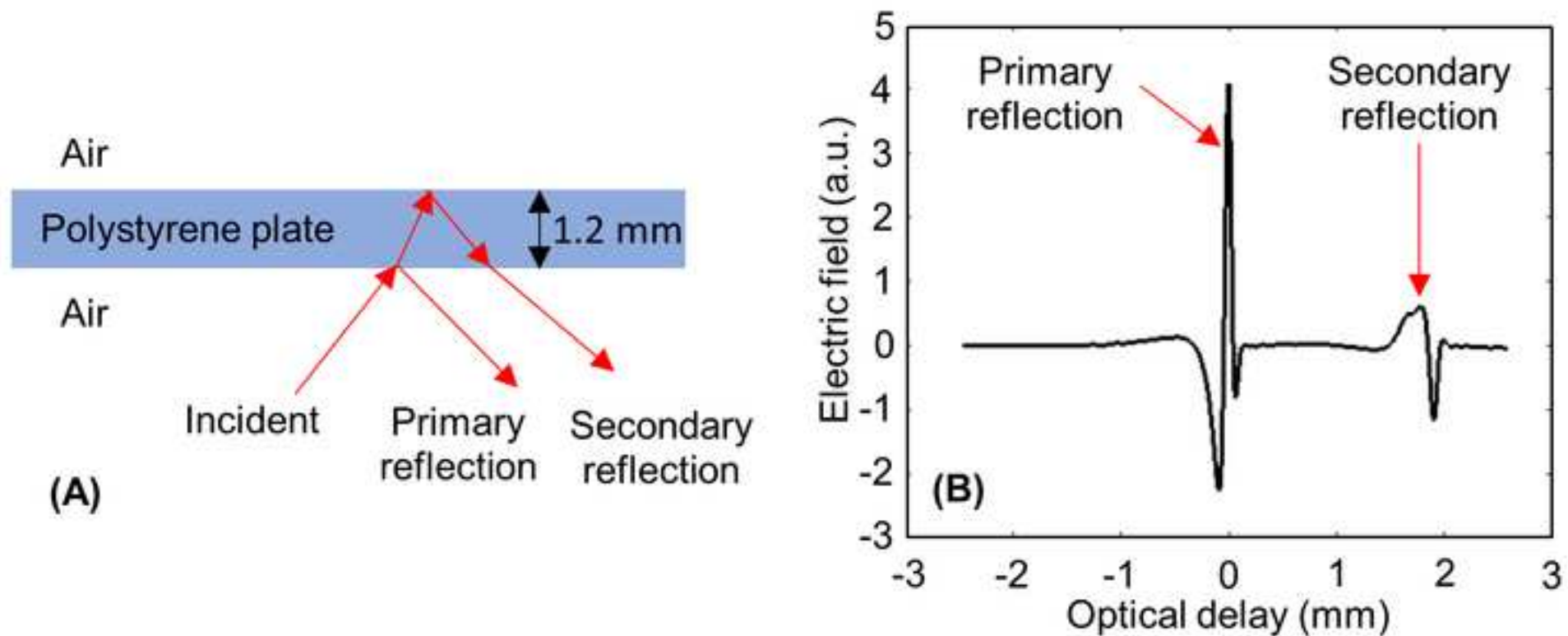












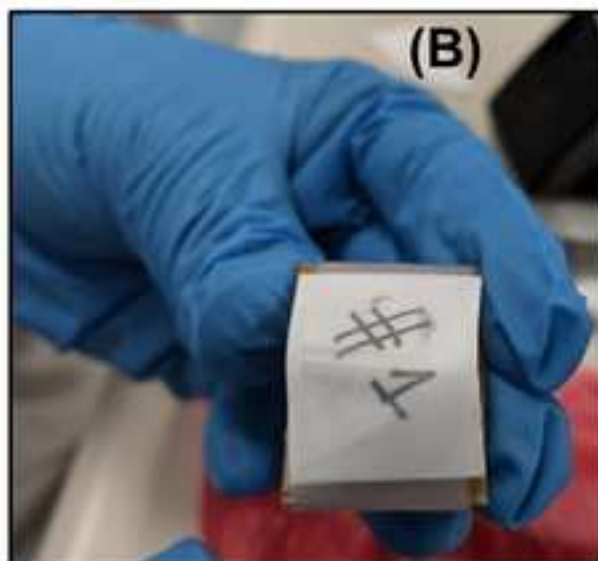
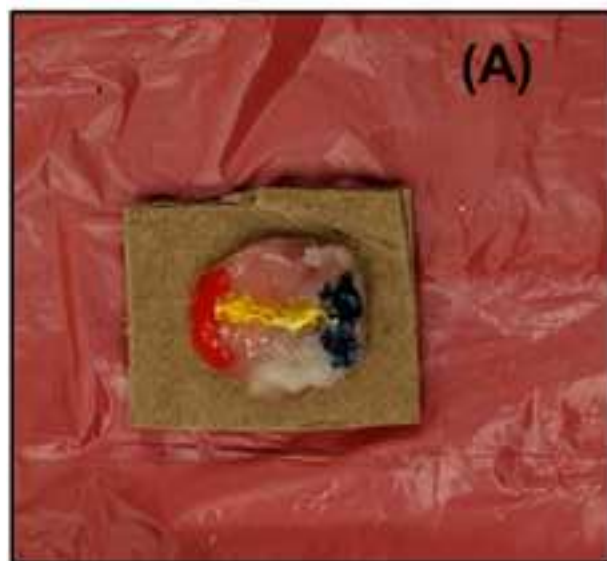




Figure 8

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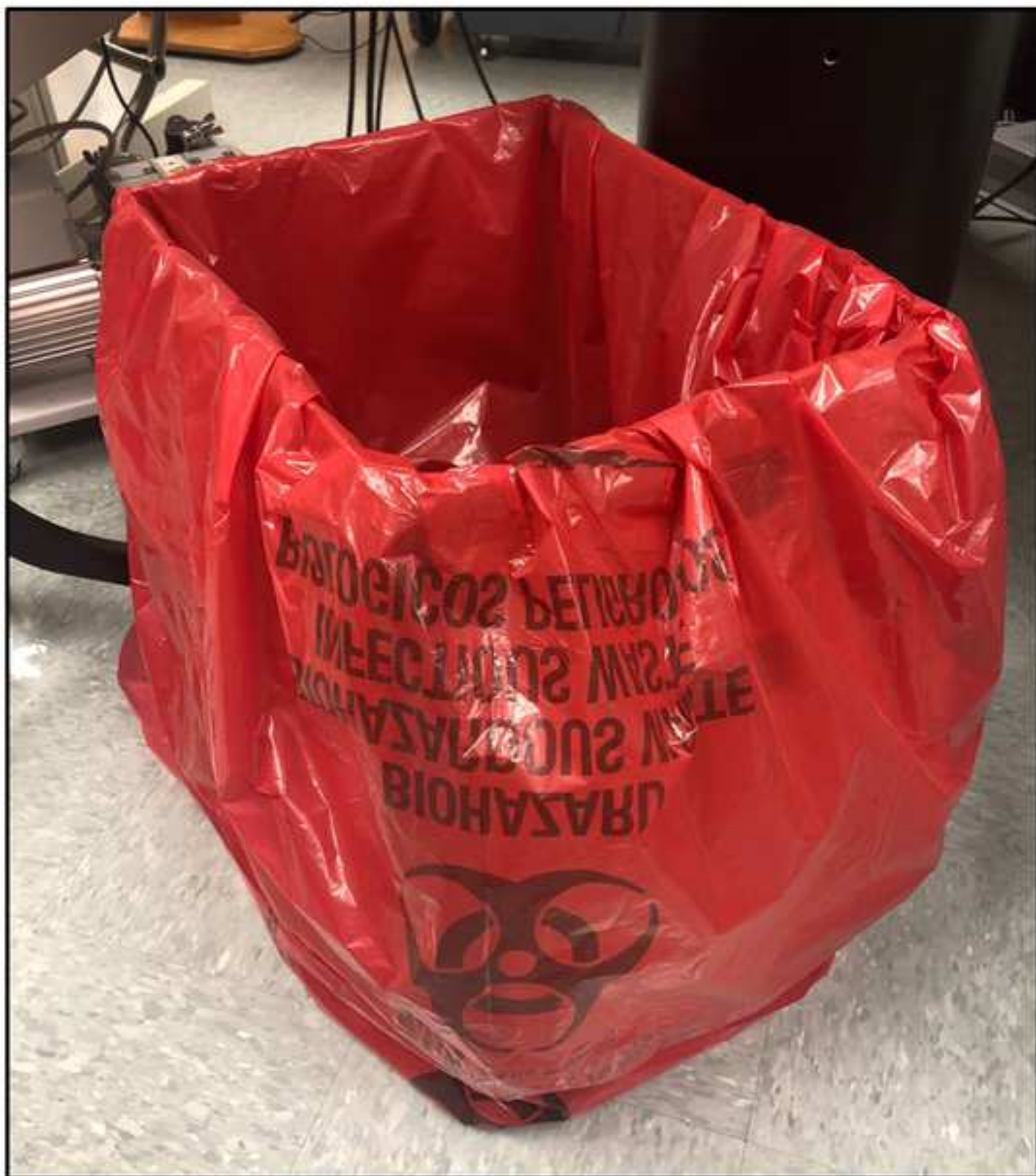
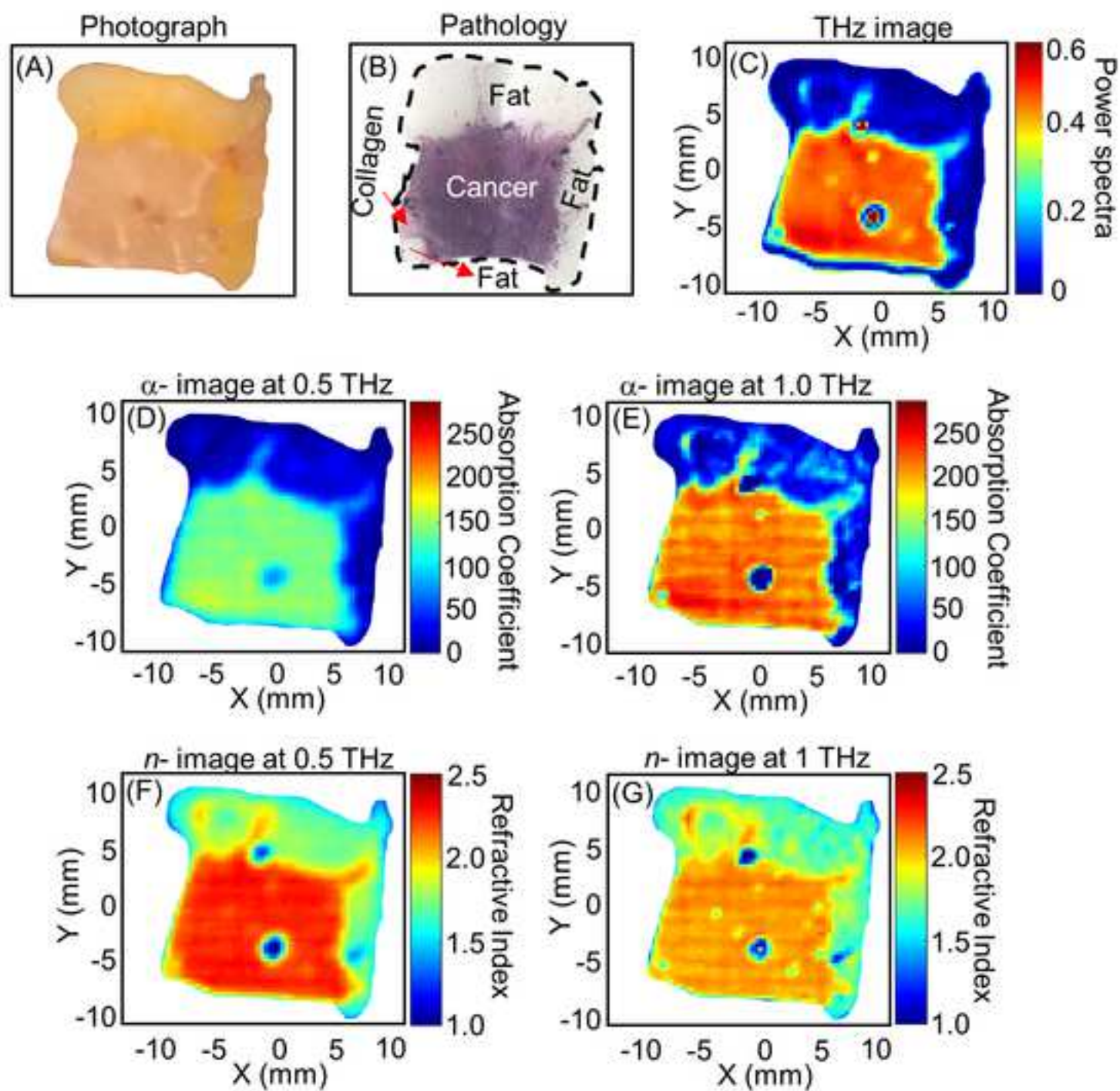
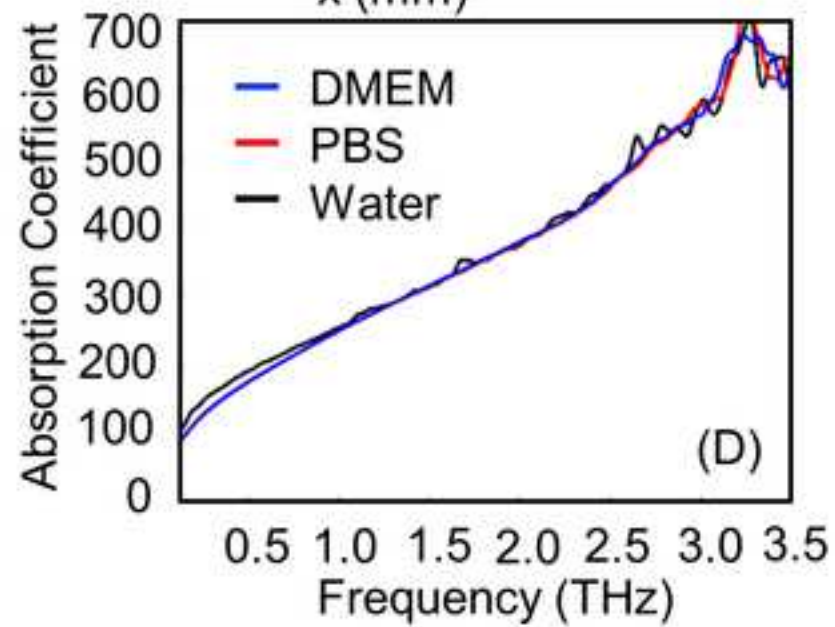
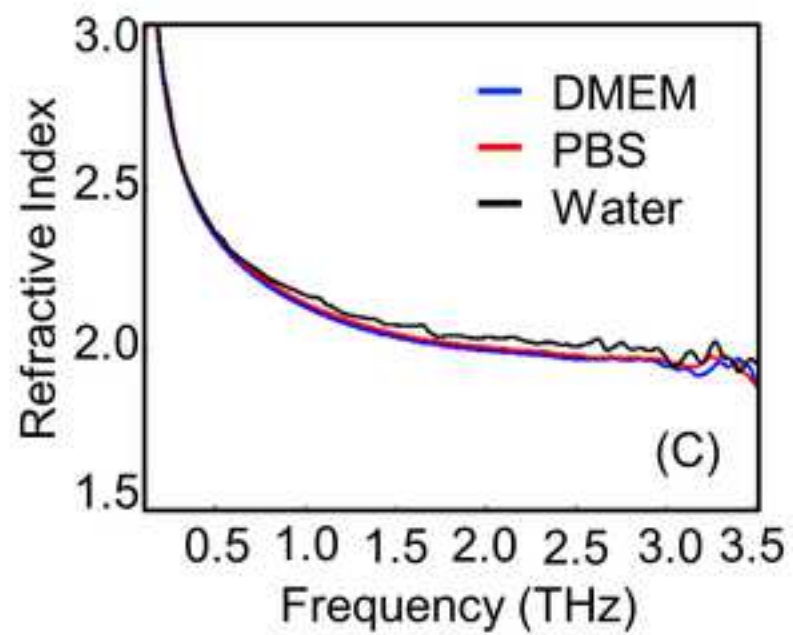
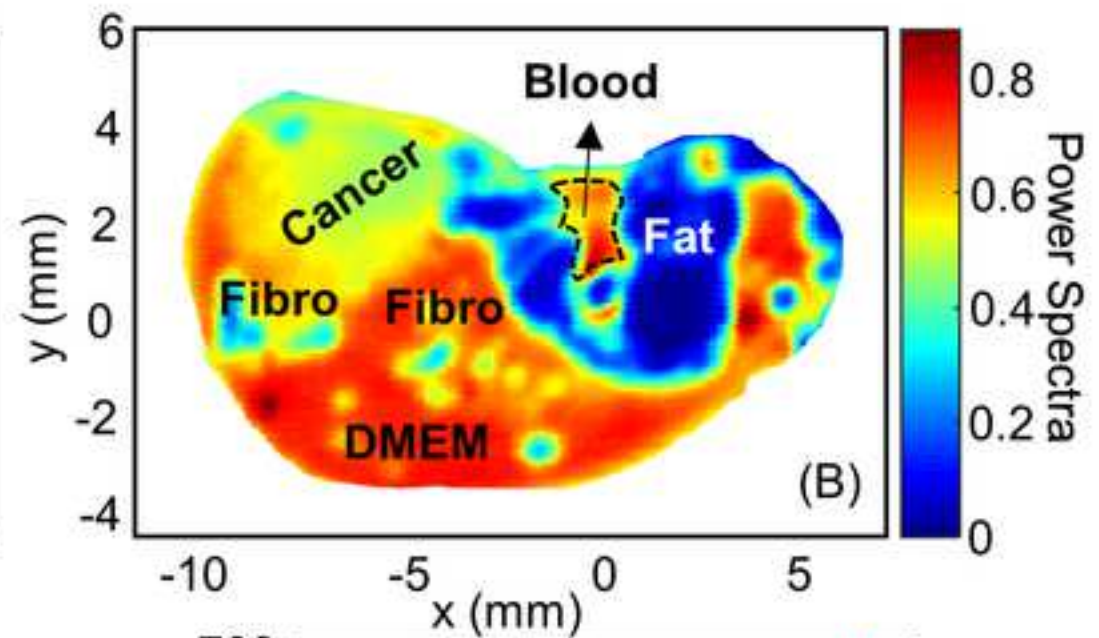
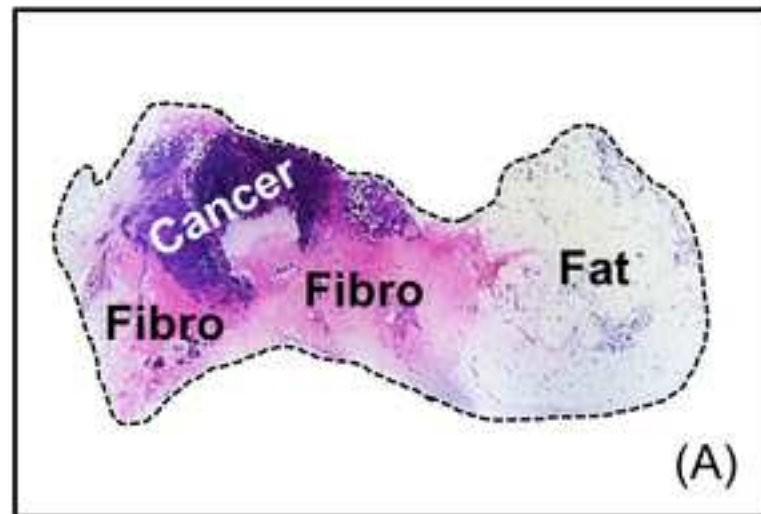
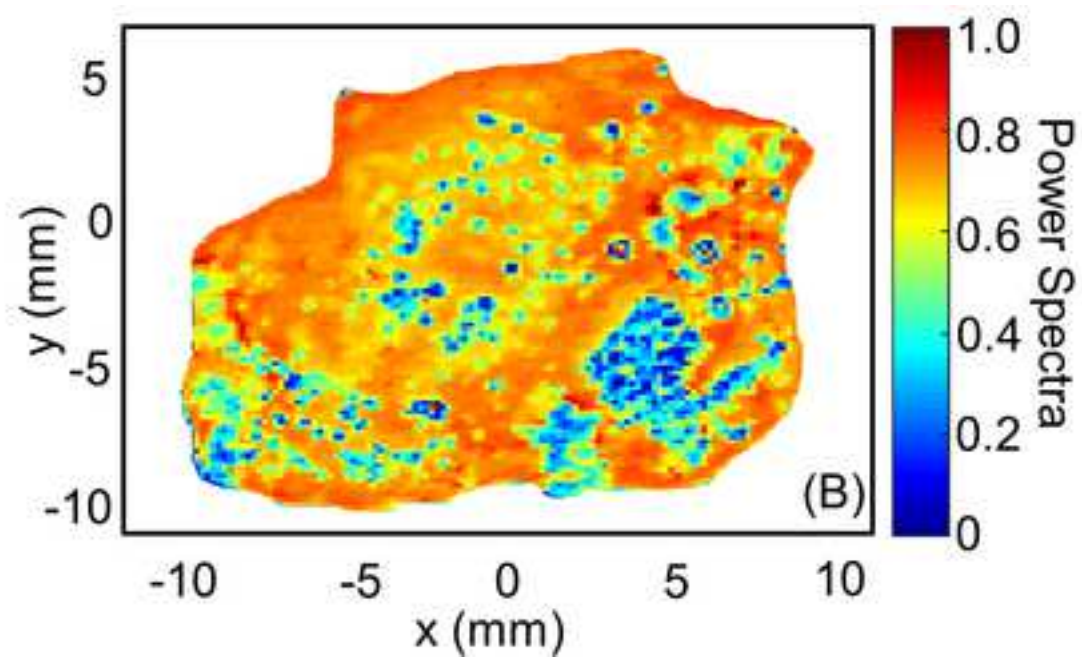
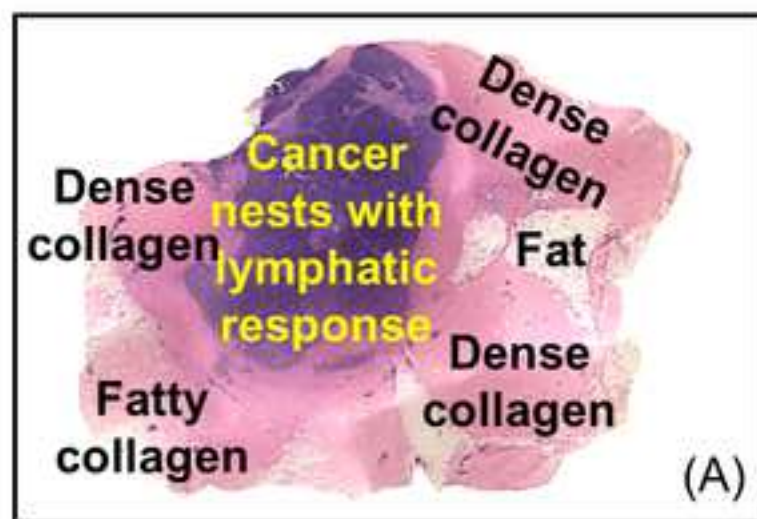


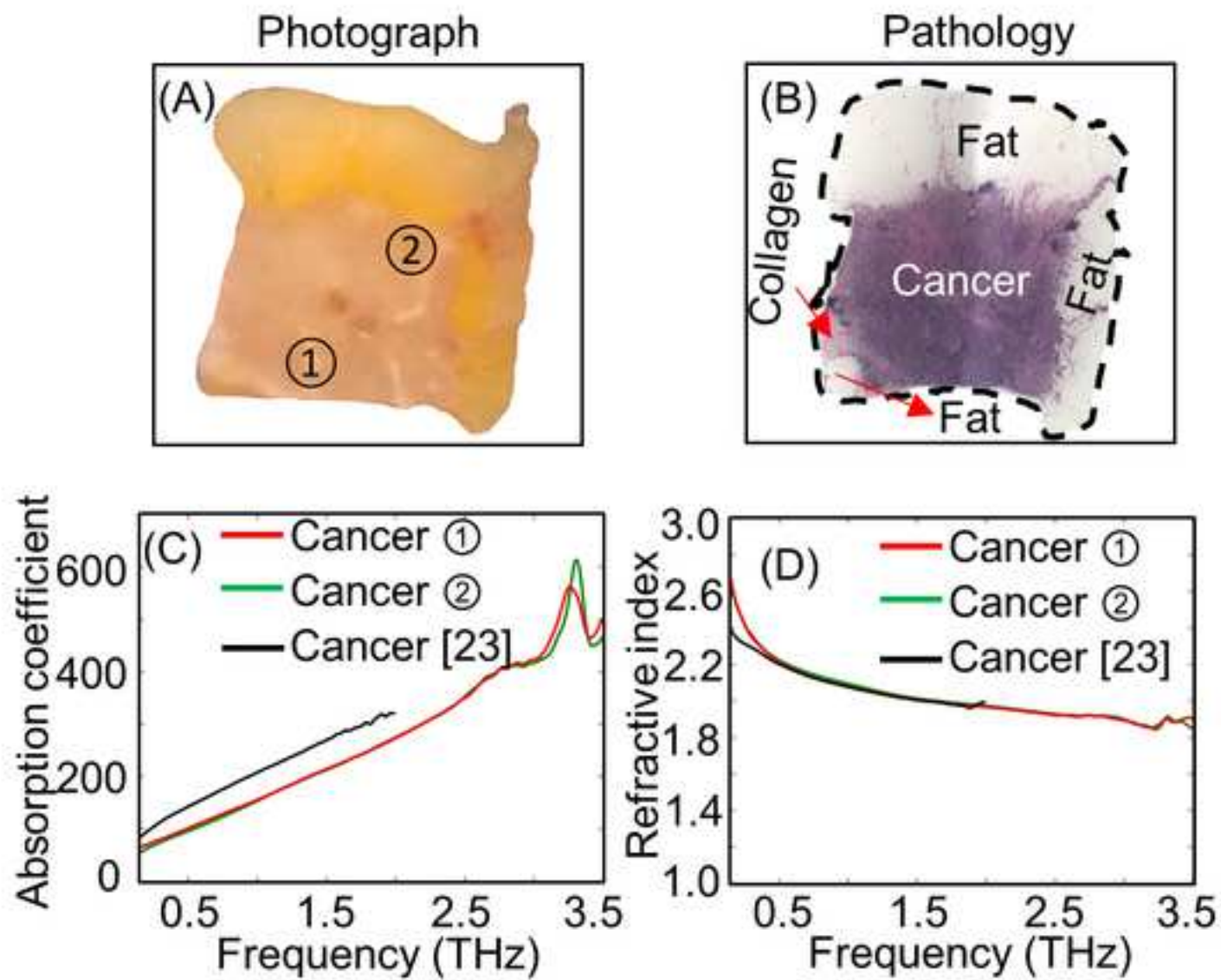
Figure 9













Name of Material/ Equipment	Company	Catalog Number
70% isopropyl alcohol	VWR	89108-162
Alconox powder detergent	VWR	21835-032
Bio Hazard Bags	Fisher Scientific	19-033-712
Cardboard holder	N/A	N/A
Centrifuge Tubes	VWR	10026-078
Cotton Swabs	Walmart	551398298
Ethyl Alcohol	VWR	71002-426
Eye protection goggles	VWR	89130-918
Face Mask	VWR	95041-774
Filter paper	Sigma Aldrich	Z240087
Formalin solution	Sigma Aldrich	HT501128-4L
Human freshly excised tumors (Infiltrating Ductal Carcinoma (IDC))	National Disease Research Interchange (NDRI biobank)	N/A
IRADECON Bleach solution	VWR	89234-816
KIMTECH SCIENCE wipes	VWR	21905-026
Laboratory Coat	VWR	10141-342
Laboratory tweezers/Forceps	VWR	82027-388
Liquid sample holder (two quartz windows with a 0.1 mm teflon spacer)	TeraView, Ltd	N/A

Nitrile hand gloves	VWR	82026-426
Nitrogen cylinder	Airgas	NI UHP300
Paper towel	VWR	14222-321
Parafilm	VWR	52858-076
Petri Dish	VWR	470210-568
Polystyrene Plate	Home Depot	1S11143A
ScanAcquire Software	TeraView, Ltd	N/A
Stainless steel low-profile blade (#4689)	VWR	25608-964
Stainless steel metal tray	Quick Medical	10F
Tissue Marking Dyes	Ted Pella, Inc	Yellow Dye #27213-1 Red Dye #27213-2 Blue Dye #27213-4
TPS Spectra 3000	TeraView, Ltd	N/A
TPS Spectra Software	TeraView, Ltd	N/A

**Comments/Description**

Contains 70% USP grade isopropanol and 30%  
USP grade deionized water

Concentrated detergent to remove organic  
contaminants from glass, metal, stainless steel,  
porcelain, ceramic, plastic, rubber, and fiberglass

Justrite FM-Approved Biohazard Waste

Container Replacement Bags

Scrap cardboard to keep tissue imaging face  
intact when immersed in formalin

Centrifuge Tubes with Flat Caps, Conical-Bottom,  
Polypropylene, Sterile, Standard Line

Q-tips Original Cotton Swabs used to dye the  
tissue

KOPTECH Pure (undenatured) anhydrous (200  
proof/100%) ethyl alcohol

Kimberly-clark professional safety glasses

DUKAL Corporation surgical masks

Whatman grade 1 cellulose filters

10% neutral buffered formalin

A protocol is signed with the NDRI for the type of  
tumors required

Pre-diluted Sodium Hypochlorite Bleach solution

Kimberly-clark professional Kim wipes

This catalog number is for medium size coat  
Any laboratory tweezers can be used as long as it  
does not damage the tissue

1" diameter, and 0.1452" thick quartz windows

This catalog number is for medium size gloves

NITROGEN UHP GR 5.0 SIZE 300

11 x 8.78" Sheets, 1 Ply

Flexible thermoplastic. Rolled, waterproof sheet  
interwound with paper to prevent self-adhesion.

VWR Petri Dish, Slippable, Mono Plate

(undivided bottom)

~ 10 x 10 cm square piece cut from a 11" x 14" x

0.05" Non-glare styrene sheet

System Software for THz reflection imaging  
measurements

Tissue-Tek Accu-Edge Disposable Microtome  
Blades

Polar Ware Stainless Steel Medical Instrument  
Trays

Used to orient excised tissue samples  
sent to the histopathology laboratory

THz imaging and spectroscopy system

System Software for THz transmission  
spectroscopy measurements

## **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  $\mu\text{m}$  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 is 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 4:

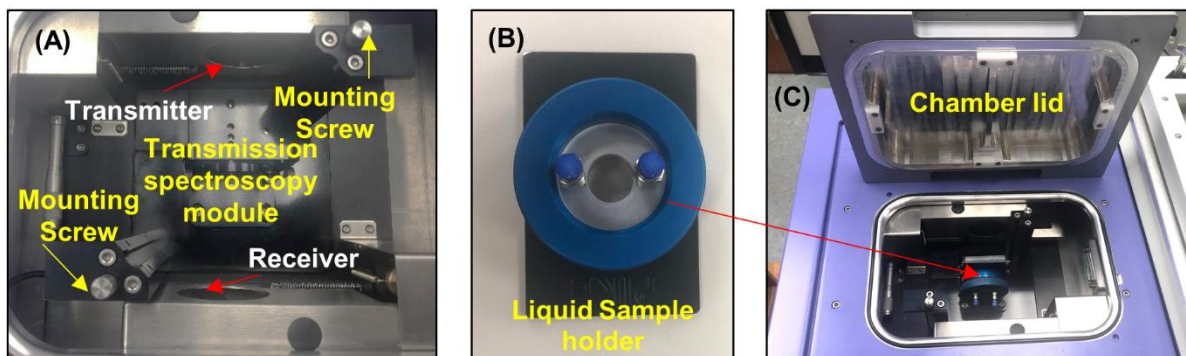
“Set the transmission spectroscopy module inside the THz core chamber by aligning the module handles over the mounting posts in the core system and sliding the stage down into the system. Tighten the two mounting screws in the upper right and lower left corners of the module as shown in Figure 3A.”

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.

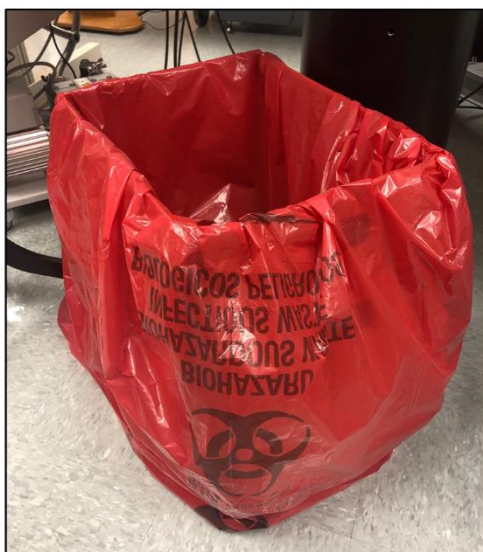
**Comment 9:** There is a 10-page limit for the Protocol, but there is a 2.75-page limit for filmable content. Please highlight 2.75 pages or less of the Protocol (including headings and spacing) that identifies the essential steps of the protocol for the video, i.e., the steps that should be visualized 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  $\mu\text{m}$ .

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  $\mu\text{m}$  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<sup>8-11</sup>.”

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<sup>29</sup>.”

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  $\mu\text{m}$ . In all our experiments we have maintained the step size of 200  $\mu\text{m}$  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  $\mu\text{m}$  to 500  $\mu\text{m}$  in 50  $\mu\text{m}$  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  $\mu\text{m}$ .

The following sentence has been added to page 15:

“...though the maximum spatial resolution of the system is around 80  $\mu\text{m}$  due to the spectral content of the THz signal.”



\_ Nagma <nvohra@email.uark.edu>

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**Katie Sinclair** <katies@spie.org>

Tue, Nov 5, 2019 at 10:59 AM

To: \_ Nagma <nvohra@email.uark.edu>

Cc: "Magda O. El-Shenawee" <magda@uark.edu>

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- 1) Tyler Bowman, Nagma Vohra, Keith Bailey, Magda El-Shenawee, "Terahertz tomographic imaging of freshly excised human breast tissues," J. Med. Imag. 6(2), 023501 (2019).

Please grant us the permission to use these figures in our JoVE manuscript, we will appreciate your help.

Best Regards,

Nagma Vohra

Ph.D. Student in Electrical Engineering

University of Arkansas

Lab: 479 575 7757

<https://terahertz.uark.edu/>



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Author	Nagma Vohra	Expected publication date	2020-02-15
Publication	Journal of Visualized Experiments	Expected size (number of pages)	20
Publisher	MyJove Corp	Standard identifier	N/A

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