

Label-free bacterial colony detection and viability assessment by continuous-wave terahertz transmission imaging

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ABSTRACT:

Timely and accurate bacterial detection is critical for various health and safety applications, which promotes the continuous development of versatile optical sensors for bacterial investigations. Here, we report a new strategy for bacterial colony sensing using terahertz (THz) imaging with minimal assay procedures. The proposed method utilizes the acute sensitivity of THz wave to the changes in the water content and cellular structures. Single bacterial colonies of four bacterial species were directly

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distinguished using THz imaging by utilizing their differences in THz absorption. In addition, the distribution of mixed bacterial samples has been demonstrated by THz imaging, which demonstrated that the target bacterium could be easily recognized. Furthermore, we investigated the differentiation of bacterial viability, which indicated that bacteria under different living states could be distinguished by THz imaging because of their different hydration levels and cellular structures. Our results suggest that THz imaging has the potential to be used for mixed bacterial sample detection and bacterial viability assessment in a label-free and nondestructive manner.

Key words: Terahertz imaging; bacterial detection; THz absorption; biosensor

1. Introduction

The rapid detection of bacterial species and their living states is of great importance in many fields, including the food industry and clinical medicine. For instance, the detection of dead bacteria in patient specimens is not significant for making a clinical diagnosis¹, because the potential hazards are limited to the living portion of mixed bacterial populations². Matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF MS) and nucleic acid amplification tests (NAATs) have facilitated dramatic advances in bacterial detection. However, in most cases, these methods cannot be used to distinguish between living and dead species; instead, they only confirm the presence of target proteins or nucleic acids, respectively. On the other hand, most routine methods for distinguishing between living and dead bacteria use DNA-binding dyes to evaluate the membrane integrity. Samples are unavoidably destroyed by the added reagents, hindering further confirmatory tests or subsequent experimental assays³. Therefore, a rapid and simple method is urgently needed for identifying bacteria and evaluating their viability in a label-free manner.

The terahertz (THz) region of the electromagnetic spectrum is typically defined as 0.1–10 THz. The energy levels of weak interactions, including hydrogen bonds and van der Waals forces, coincidentally lie within the THz range; thus, water is notoriously highly absorbent of THz wave^{4, 5}. By utilizing this contrast mechanism, various healthy, tumor, dehydrated, and burned tissues could be clearly differentiated by THz spectroscopy and imaging according to their different hydration levels^{6, 7}. Researchers have demonstrated that THz spectroscopy has a greater sensitivity for detecting subtle structural changes of cells than conventional optical phase contrast microscopy⁸. As a result, THz technology has been extensively applied in various biomedical applications, such as probing hydration dynamics⁹, detecting DNA hybridization¹⁰, investigating cytoplasm leakage in real time¹¹, imaging cancer tissues¹², and detecting bacteria¹³. Specifically, some THz signatures (absorption

peaks) have been found for spores of *bacillus* species¹⁴⁻¹⁶, complementing Raman and infrared spectral signatures for bacterial spore identification. Different types of bacterial colonies could also be differentiated by THz spectroscopy because of their different water contents, although no obvious signatures were found at room temperature as a result of the complexity of vegetative cellular components^{17, 18}. In addition, living and dead bacteria also exhibited different THz absorption because of the different hydration levels and structural characteristics¹⁹. Therefore, THz spectroscopy has proven to be a label-free alternative in bacterial detection and viability evaluation.

However, the studies described above focused only on investigating pure bacterial colonies using THz spectroscopy. In practical applications, the rapid detection of the composition and distribution of mixed bacterial samples is also important. For instance, several bacterial colonies may overlap on a culture medium if the parent cells are too close together. This is particularly true when detecting mixed or polymicrobial specimens. The prompt detection of the mixed samples and recognition of the portion of interest would be helpful for more accurately targeting bacterium identification in a short amount of time. Additionally, relatively large numbers of samples were required in the previous spectroscopic study¹⁷, hindering its practical applications in the food industry or clinical laboratories. Consequently, there is an increasing interest in using THz imaging for detecting the distributions of different bacterial colonies, particularly mixed bacterial colonies. Notwithstanding such potential, two issues need to be addressed when applying THz imaging to bacterial detection. First, rapid detection speeds are favorable for timely bacterial identification and reduce the effects of the experimental environment and sample changes. In conventional THz time-domain spectroscopy (THz-TDS), scanning speeds are relatively slow because of the time delay scanning. Additionally, THz-TDS has difficulty in detecting high-moisture products as a result of the relatively low output power²⁰. Second, the sizes of bacterial colonies on a blood agar medium are typically

only 1–4 mm in size (depending on the species); thus, higher THz frequencies are preferable for obtaining a higher image resolution.

In this study, we used a continuous-wave THz imaging system in transmission mode to investigate the characteristics of single bacterial colonies and the distributions of mixed bacterial colonies, to demonstrate its practical usage for bacterial colony sensing. Single bacterial colonies of *Escherichia coli*, *Staphylococcus aureus*, *Acinetobacter baumannii*, and *Pseudomonas aeruginosa* were effectively differentiated by THz imaging from their different THz absorption. Using this contrast mechanism, the distributions of the mixed samples were clearly demonstrated, and the target bacterium was easily recognized. In addition, living and dead bacteria were successfully distinguished based on their different hydration levels and structural characteristics. Moreover, living bacteria in the sample cell were monitored continuously using THz imaging within 3 h, demonstrating the potential of the method for the label-free assessment of bacterial viability.

2. Materials and methods

2.1 Bacterial sample preparation

Standard bacterial strains (*E. coli* ATCC 25922, *S. aureus* ATCC 25923, *A. baumannii* ATCC 19606, and *P. aeruginosa* ATCC 27853) were obtained from the National Institute for the Control of Pharmaceutical and Biological Products, China. After activation from $-80\text{ }^{\circ}\text{C}$, the bacteria were inoculated overnight on Columbia blood agar medium (CNA, Pang tong, Chongqing, China) at $37\text{ }^{\circ}\text{C}$ in an atmosphere containing 5% CO_2 . Then, bacterial colonies were scraped off the surface of the solid medium for direct measurement. Dead bacteria were prepared by treating the bacterial colonies in Eppendorf tubes in a metal bath at $100\text{ }^{\circ}\text{C}$ for 60 min. The Eppendorf tubes were sealed with Parafilm to minimize evaporative water losses during thermal treatment. Then, the samples were evenly mixed and cooled to room temperature before removing the Parafilm for measurement.

2.2 Continuous-wave THz imaging system

A continuous-wave THz imaging system in transmission mode was used to detect bacterial samples, as depicted in Figure 1A. The source was an optically pumped continuous-wave THz gas laser (FIRL100, Edinburgh Instruments Ltd). The THz frequency was fixed at 2.52 THz with an output power of 40 mW. Golay cells (GC-1P, Tydex Ltd) were used as the detectors and operated at room temperature. Considering the responsivity of the detectors, the THz wave output was modulated as a sinusoidal signal using a chopper operating at 50 Hz. In order to reduce the image noises due to slight power fluctuations, THz wave was separated into transmitted and reflected beams as the signal (I_1) and reference (I_0) by a wire grid polarizer. The reference beam was detected by a Golay cell (#1). Meanwhile, the signal beam was focused on the sample with a confocal system consisting of two Tsurupica lenses ($f = 30$ mm) and detected by a Golay cell (#2). The sample was placed on a computer-controlled horizontal x-y scanning stage, and two-dimensional image (P) can be obtained with the pixel value of I_1/I_0 . The resolution of this imaging system was $260\text{ }\mu\text{m} \times 380\text{ }\mu\text{m}$ (measured by the knife-edge method), and the scanning step was fixed at $100\text{ }\mu\text{m}$ in this study.

For every measurement, we employed a sample cell composed of a sample plate and cover quartz plate. The sample plate, which was made of a silicon wafer (thickness = $300\text{ }\mu\text{m}$, resistivity $> 10\text{ k}\Omega\cdot\text{cm}$), was fabricated using conventional photolithography technology (Karl Suss MA6, Suss MicroTec, Germany). Then, a central cavity surrounded by four indicator lines was created in the sample plate. The cavity was 2 mm in diameter and $20\text{ }\mu\text{m}$ in depth, and the volume of the cavity was approximately $0.06\text{ }\mu\text{L}$, allowing single bacterial colonies to be detected.

2.3 Measurement protocol

After culture, a portion of an isolated colony was spotted into the central cavity for direct measurement, as shown in Figure 1B. The sample plate was covered with a

quartz plate, and excessive bacteria cells were poured out of the cavity to ensure that it was completely filled. Then, the sample cell was placed on the x-y stage for THz imaging. After measurement, the sample cavity was treated to reduce bacterial adhesion with 1 mL of a trypsin solution for 3 min at room temperature, followed by a rinsing with distilled water. To minimize the influence of experimental conditions, every species of bacteria was observed in five samples, and three independent experiments were conducted on different days. Hence, 15 samples of each species of bacteria were measured to minimize sample heterogeneity. Bacteria were inoculated and handled safely in a level II biosafety cabinet.

2.4 Data analysis

Considering that both the quartz and silicon plates have high transmittances in the THz band of interest, the reflected THz intensity caused by the marginal Fresnel loss due to their different refractive indices can be neglected during scanning. The sample cell with and without bacteria were measured as the images of P_{sample} and $P_{substrate}$, respectively. Owing to the uniformity of sample cell and the small depth of sample cavity, the averaged value V_{bac} for the pixels in sample cavity of $P_{substrate}$ was taken as the image reference for simplicity. Thus, the distribution of sample transmittance can be calculated by dividing the pixel values in P_{sample} by V_{bac} . Similarly, the transmittance of sample cell is obtained by using the air as the reference. The averaged transmittance, T_{ave} , is defined as the average of multiple pixels within the regions of interest (ROIs). Statistical analyses were conducted by calculating the values of T_{ave} and the standard deviations of the 15 individual data values in each group. A Kruskal–Wallis test was used to compare the imaging results for different bacterial species, followed by a Dunn’s multiple comparison test. An additional independent t-test was used to test for a significant difference between living and dead bacteria. All analyses were conducted using the statistical software SPSS software 20.0; a P value of less than 0.05 was considered to be statistically significant.

3. Results

3.1 THz imaging of different bacterial colonies

To demonstrate the uniformity of the sample cell and the reliability of the measurement protocol, we measured the sample cell without and with bacteria. Figures 2A and 2C show visible images of the sample cell before and after adding *S. aureus* bacteria, respectively. It is noted that the air area beside the sample cell was chosen and simultaneously imaged, which is completely transparent in the THz range. Correspondingly, Figures 2B and 2D show the transmittances of the sample cell before and after adding *S. aureus*, where the air area was used as the reference. The transmittance of air areas was 100%, corresponding to the black areas in Figures 2B and 2D. The transmittances of the cavity area without bacteria and its surrounding area were similar, indicating the uniformity of the sample cell. Moreover, when the quartz cover plate was bonded onto the sample plate through the middle bacterial layer, excess bacteria cells spilled out of the central cavity. Accordingly, the pink areas in Figure 2D indicate the sample in the cavity, and the red areas represent overflowing bacterial cells. We can see that the bacteria were well-distributed in the cavity using our preparation method. Three other bacteria species were measured in the same way.

For a quantitative comparison, the transmittances of bacterial colonies were used in the following analysis, where the reference was calculated from $P_{\text{substrate}}$ by averaging the data obtained in a selected area of the sample cavity (denoted by the black dashed rectangle in the inset of Figure 2B). Figure 3 shows the stereogram imaging results from drawing the sample transmittances on the Z axis. The top surface of the cylinder represents the transmittance distribution of the detected bacteria in the cavity, while the bottom surface represents the overflowing bacterial cells. The top surfaces of the central cylinders were quite flat, indicating that the cavities were fully and evenly filled with samples. The transmittance of the overflowing layer was much higher than that of the cells in the cavity, demonstrating that the depth of overflowing

bacterial cells was considerably shallower compared to that of the cells in the cavity. The four species of bacteria had different surface colors. *A. baumannii* exhibited the highest THz absorption among the four species, while *S. aureus* exhibited the lowest absorption. According to their different surfaces, *P. aeruginosa* was more absorbing than *E. coli*. The results can be explained by the differing water contents of the four species, which agrees with the previous spectroscopic investigation¹⁷. Figure 3B presents a comparison of T_{ave} for the four bacterial species: $10.8\% \pm 1.3\%$ for *A. baumannii*, $16.2\% \pm 1.3\%$ for *P. aeruginosa*, $18.7\% \pm 1.2\%$ for *E. coli*, and $24.6\% \pm 2.1\%$ for *S. aureus*. By conducting nonparametric Kruskal–Wallis tests followed by Dunn’s multiple comparison tests, statistically significant differences were observed between all pairings of different bacteria ($P < 0.05$).

3.2 THz imaging of mixed bacterial samples

After illustrating the differences in the imaging results between bacterial species, we explored the THz imaging analysis of mixed bacterial samples. Considering that *P. aeruginosa* can be preliminarily identified by its characteristic odor²¹ and *S. aureus* can be preliminarily identified by its typical hemolytic zones on blood agar plates²², mixed bacterial samples composed of *E. coli* and *A. baumannii* were investigated. To mimic the overlapping bacterial colonies on an agar medium, different amounts of the two bacteria were spotted onto opposite sides of the inner edge of the central cavity. When the sample plate was covered with the quartz plate, bacteria were squeezed inside to form a mixed sample. Figure 4A shows a THz image of the sample mixed at a proportion of 5:5. The result demonstrated that the two bacterial colonies, which are difficult to discern with the naked eye, could be effectively recognized using THz imaging. The obvious boundary between the purple and light blue colors clearly delineates the sample distribution inside the central cavity. *A. baumannii* samples, which have stronger liquidity because of their higher water content, tended to occupy more space than *E. coli* samples during the squeezing. More precisely, the surface colors were identical to those of pure samples (Figure 3A) for the same color bar scale.

The inherent THz absorption properties of bacteria in mixed samples can still be effectively displayed by THz imaging, allowing for easy and direct recognition of a target bacterium in mixed samples. In addition, an obvious boundary between the purple and light blue colors was also observed in Figure 4B with a proportion of 7:3. This result demonstrated that the two bacterial colonies mixed at different proportions could be effectively distinguished. However, the boundary was not very clear in the Figure 4C when the mixing proportion increased to 9:1. The small amount of *E. coli* in the bacterial colonies was not able to be effectively recognized using THz imaging, which could be attributed to the resolution limit of the imaging system.

3.3 THz imaging for bacterial viability assessment

In addition to using THz imaging to identify different bacterial species, we used THz imaging to assess bacterial viability. Figure 5 shows a comparison of THz imaging results for living and dead *S. aureus*; obvious THz absorption differences can be observed for the two states. According to an independent t-test, the two groups exhibited statistically significant differences ($24.6\% \pm 2.1\%$ vs. $30.0\% \pm 2.3\%$ for living and dead *S. aureus*, respectively; $P < 0.05$). This can be explained by differences in both cellular structures and water content. The structural variability of bacterial cells, such as protein denaturation, during thermal treatment led to changes in low-frequency biomolecular motions, including molecular vibration and rotation²³. As a result, bacterial viability can be effectively displayed by THz imaging because these low-frequency vibration modes coincidentally lie in the THz frequency range. Based on this principle, recent work demonstrated the feasibility of using THz imaging to quantitatively measure the permeability of epithelial cells¹¹. In addition, the change in water content should be a more important factor for their different THz responses. Hydration water (i.e., the intracellular water molecules in the immediate vicinity of biomolecular surfaces) exhibits a different THz response than the surrounding bulk water^{9, 24}. Because water molecules are closely associated with metabolic activities²⁵, the changes in water content (especially the

hydration-water-to-bulk-water ratios) should be responsible for the significant THz absorption differences between living and dead *S. aureus*. A similar investigation demonstrated that THz spectral differences between *Bacillus thuringiensis* samples in the log phase and late stationary phase could be attributed to the tiny changes in hydration levels ¹⁵, which is in agreement with our results.

To investigate the changes in bacterial viability after bacteria were removed from the culture medium, we monitored the same sample with THz imaging for 3 h at 10-min intervals. Figure 6 shows the changes in the average THz transmittances of living *S. aureus* after being spotted into the sample cell. The THz transmittance increased gradually for the first 100 min and then remained at an approximately constant level (~32%). As shown in the insets, no significant changes were observed inside the cavities (pink areas) in the first 40 min. After 40 min, the samples in the cavity became uneven, and the overflowing samples (red areas) become smaller. The increased THz transmittance for these 40 min may be attributable to the reduced metabolic activity of the bacteria after leaving the culture medium (in particular, the changes in intracellular hydration states) ²⁶. Meanwhile, the accumulated metabolic wastes in bacterial cells in the spacer might also be responsible for this phenomenon. The exact mechanisms have not yet been discovered because of the complexity of intracellular components. After 40 min, we observed obvious sample shrinkage due to cell dehydration and water evaporation. Hence, the transmittance of the uneven samples in the cavity increased to an even higher level, and became comparable to that of dead *S. aureus*. Therefore, the measurement results were affected by both 1) the viability changes that occurred after bacteria left the culture medium, and 2) the inevitable shrinkage of the sample over time. Accordingly, a standardized operation procedure including the detection time should be a necessary prerequisite for acquiring accurate bacterial species identification results. Instead of replacing routine use in clinical laboratories, THz imaging would be more useful for continuous bacterial monitoring, such as detecting

intracellular metabolites in high-throughput industrial strain screening processes²⁷, by utilizing its high sensitivity to the hydration levels and structural changes.

4. Discussion

Our results have demonstrated the applicability of THz imaging to detect four bacterial species and mixed bacterial samples. The results indicated that gram-negative bacteria (*E. coli*, *A. baumannii*, and *P. aeruginosa*) tended to absorb more THz wave than gram-positive bacteria (*S. aureus*). This phenomenon was also observed in a previous spectroscopic study of additional species²⁸. It remains unclear whether this trend is biased by the limited number of tested species; further investigation of other species would be worthwhile. As with other optical sensors²⁹, the presence of the target bacteria can be quickly and directly identified from color changes in THz imaging, without the need for further analytical assays. Hence, THz imaging could be very useful in cases where medically important bacteria must be promptly identified before appropriate actions can be taken (for example, when *Bacillus anthracis* must be unambiguously identified). Similar to the spectral profiles for MALDI-TOF MS, identifying an unknown bacterium requires establishing a THz database containing the target bacteria of interest under same culture conditions. In addition, living and dead bacteria possessed different THz responses, hinting at novel strategies for assessing bacterial viability in a label-free manner. Accordingly, THz imaging may lay a foundation for developing a new antimicrobial susceptibility test by monitoring tested bacteria subject to particular antibiotics.

In conventional bacterial detection methods, laborious assay procedures are required for metabolic and phenotypic analyses³⁰. Considering that the reagent cost per identification is approximately \$2.5–5.0³¹, THz imaging offers another tantalizing possibility for label-free and reagentless bacterial detection. Similar to other biophysical properties of bacterial colonies, such as the forward-scattering patterns produced by light-scattering technologies^{3,32}, the THz responses of bacterial colonies

can be directly utilized for bacterial detection within 10 min after culture. Compared with other mainstream bacterial detection methods¹³, THz imaging has several advantages, as summarized in Table 1. In particular, MALDI-TOF MS can only detect isolated pure colonies after culture due to its limitation in separating multiple spectra collected simultaneously¹. Therefore, THz imaging would be helpful for pre-analyzing mixed colonies on a solid medium, which is favorable for detecting mixed or polymicrobial specimens. Unlike dye-based molecular methods, THz imaging enables the label-free investigation of bacterial viability. In this noncontact and nondestructive method, the sample remains intact during the analysis of its biophysical properties, which would be useful when further confirmatory tests or subsequent experimental assays are required.

There are still some challenges facing the widespread application of THz imaging for bacterial detection. First, THz imaging has a poor sensitivity because of the size mismatch between THz radiation and bacterial cells. The wavelength of THz wave at 1 THz is approximately 300 μm , while a single bacterial cell has a typical diameter of 1–3 μm . Accordingly, relatively large numbers of samples are generally required for detection. In addition, the spatial resolution is limited by the diffraction of THz wave. Although post-culture bacterial colonies are large enough to be detected, near-field THz techniques with a greatly improved spatial resolution are preferable for the detection of small amounts of bacteria. Second, the operating performance of THz systems should be further improved for practical applications. For instance, a better sensitivity for detecting small amounts of bacteria requires the use of more sensitive detectors such as Schottky diode mixers³³. Compact THz sources with a high output power may serve as promising substitutes for developing miniaturization systems. Additionally, rapid sampling methods such as compressed sensing³⁴ are worthy for attempting to realize bacterial imaging with accelerated imaging speeds. All of these improvements are deemed essential before THz imaging can be applied in the food industry or medical laboratories.

5. Conclusions

In summary, we used a continuous-wave THz imaging system for bacterial detection with minimal sample preparation. To the best of our knowledge, this is the first study using THz imaging to differentiate between bacterial species and to determine bacterial viability. Utilizing the biophysical properties of bacterial colonies, a fast, simple, cost-effective, and one-step method for analyzing bacterial colonies was proposed. It would be worthwhile to further investigate the selectivity between more or less virulent strains of same bacteria; this might require better instrument performance and standard operating procedures. Because of the high sensitivity of THz wave to the water content and bacterial cell structures, certain future applications can be envisioned, such as the continuous monitoring of fermentation processes. Although our current methodology still needs significant improvements, particularly in terms of the detection sensitivity, our results provide a proof-of-concept demonstration that THz imaging could be developed into an alternative approach for bacterial analysis that is label-free, reagentless, and nondestructive.

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Table**Table 1.** Characteristics of bacterial detection by THz imaging compared with common methods

Characteristic	THz imaging	Traditional culture methods	MALDI-TOF MS	NAATs
Detection principle	THz absorption differences between bacteria	Metabolic and phenotypic analysis	Spectra based on protein mass-to-charge ratios	Specific nucleic acid amplification
Time for bacterial detection	Minutes after culture	Days to weeks	Minutes after culture	One to several hours
Simplicity	Yes	No	Yes	No
Reagentless detection	Yes	No	Yes (only matrix is needed)	No
Living/dead bacteria discrimination	Yes	No	No	No(except reverse transcriptase PCR)
Mixed bacterial sample analysis	Yes	Yes	No	Yes
Limit of detection	0.06 μ L (the sensitivity in this study)	-	10^5 – 10^6 cfu	Several to tens of genome equivalents

Legends

Figure 1 Bacterial colony detection by THz imaging. (A) Schematic of the continuous-wave THz transmission imaging system. (B) Schematic showing sample loading and THz measurement.

Figure 2 THz imaging of the sample cell before and after adding bacterial colony. (A) Visible image of the empty sample cell. (B) Transmittance of the empty sample cell. (C) Visible image of the sample cell after adding *S. aureus*. (D) Transmittance of the sample cell after adding *S. aureus*.

Figure 3 THz imaging of different bacterial colonies. (A) Comparison of THz images for different bacteria in stereo view. Note that four independent tests for different bacteria were drawn in the same coordinate system for easy comparison. (B) Statistical analysis of the average transmittances of four bacterial groups after multiple measurements ($n = 15$). Data are shown as mean $\pm$ standard deviation. The differences between each pair of groups were evaluated using the Kruskal–Wallis method followed by a Dunn’s multiple comparison test ($P < 0.05$).

Figure 4 THz imaging of *A. baumannii* and *E. coli* samples mixed at different proportions. (A) 5:5 mixing. (B) 7:3 mixing. (C) 9:1 mixing.

Figure 5 THz imaging of living and dead bacteria. (A) Comparison of THz images of living and dead *S. aureus*. Note that two independent tests were drawn in the same coordinate system for easy comparison. (B) Statistical analysis of the average transmittances of two groups after multiple measurements ($n = 15$). Data are shown as mean $\pm$ standard deviation. An independent t-test was used to statistically compare the two groups ($P < 0.05$).

Figure 6 Viability monitoring of *S. aureus* by THz imaging for 3 h at 10-min intervals. The insets show representative results at different time points.

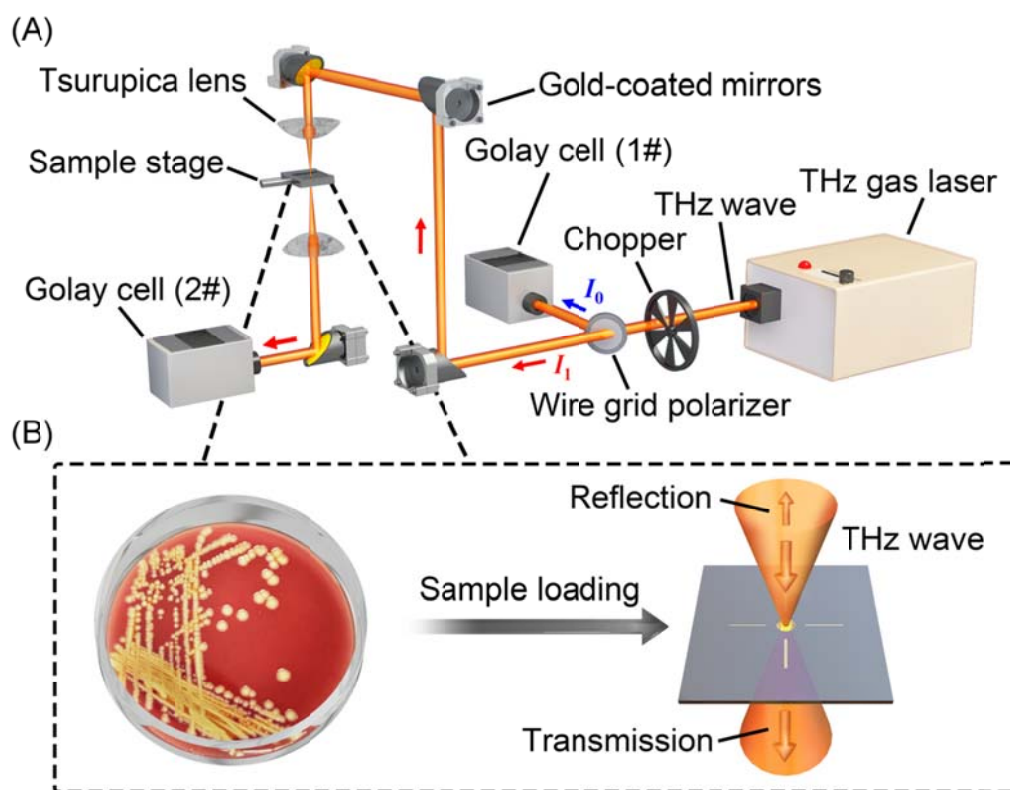


Figure 1

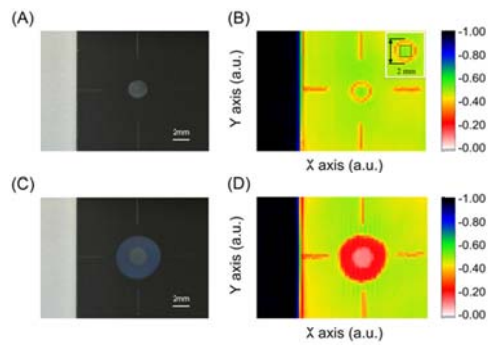


Figure 2

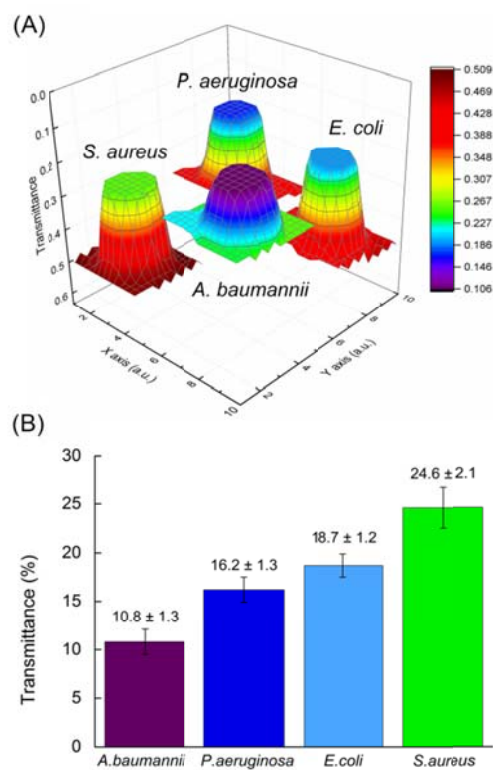


Figure 3

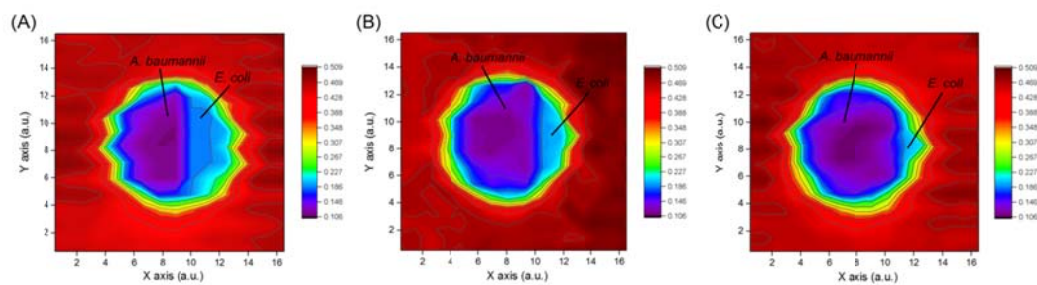


Figure 4

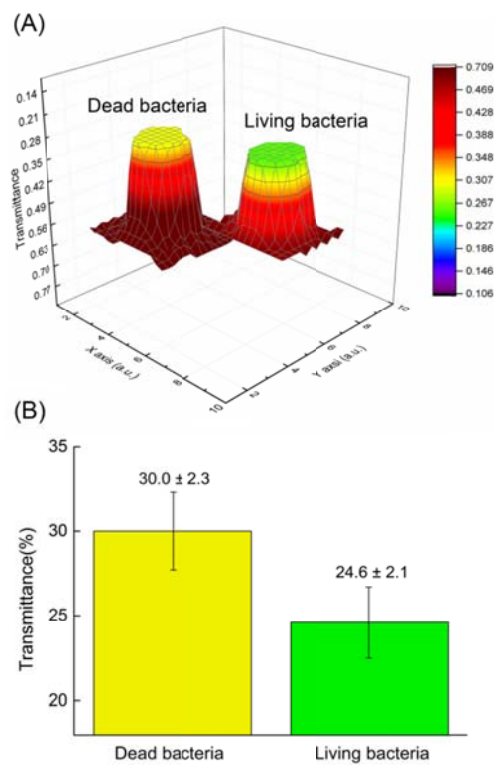


Figure 5

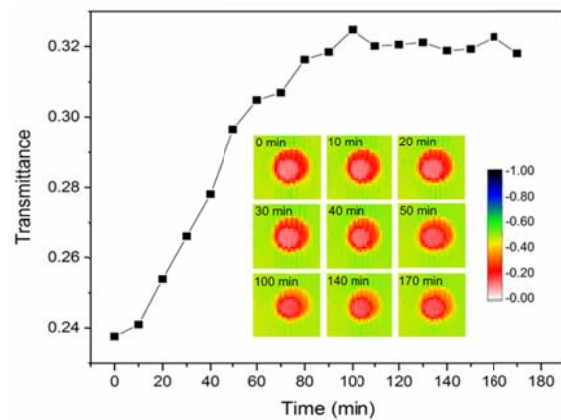
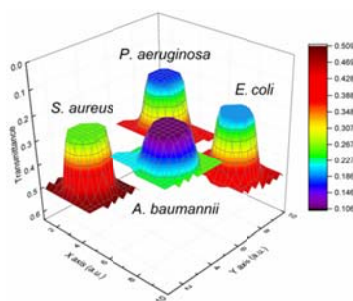


Figure 6

Graphical Abstract



In this study, we used terahertz (THz) imaging to investigate the characteristics and distributions of different bacterial colonies and mixed bacterial samples. Single bacterial colonies of four bacterial species were directly distinguished as a result of their different THz absorption. In addition, same bacteria in different living states were successfully distinguished based on their different hydration levels and structural characteristics, demonstrating the potential of THz imaging for the label-free assessment of bacterial viability.