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Rapid Antimicrobial Susceptibility Testing by Stimulated Raman Scattering Imaging of Deuterium Incorporation in a Single Bacterium

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TITLE:

Rapid Antimicrobial Susceptibility Testing by Stimulated Raman Scattering Imaging of Deuterium Incorporation in a Single Bacterium

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SUMMARY:

This protocol presents rapid antimicrobial susceptibility testing (AST) assay within 2.5 h by single-cell-stimulated Raman scattering imaging of D_2O metabolism. This method applies to bacteria in the urine or whole blood environment, which is transformative for rapid single-cell phenotypic AST in the clinic.

ABSTRACT:

To slow and prevent the spread of antimicrobial resistant infections, rapid antimicrobial susceptibility testing (AST) is in urgent need to quantitatively determine the antimicrobial effects on pathogens. It typically takes days to complete the AST by conventional methods based on the long-time culture, and they do not work directly for clinical samples. Here, we report a rapid AST method enabled by stimulated Raman scattering (SRS) imaging of deuterium oxide (D_2O) metabolic incorporation. Metabolic incorporation of D_2O into biomass and the metabolic activity inhibition upon exposure to antibiotics at the single bacterium level are monitored by SRS imaging. The single-cell metabolism inactivation concentration (SC-MIC) of bacteria upon exposure to antibiotics can be obtained after a total of 2.5 h of sample preparation and detection. Furthermore, this rapid AST method is directly applicable to bacterial samples in complex biological environments, such as urine or whole blood. SRS metabolic imaging of deuterium incorporation is transformative for rapid single-cell phenotypic AST in the clinic.

INTRODUCTION:

Antimicrobial resistance (AMR) is a growing global threat to the effective treatment of infectious disease[1]. It is predicted that AMR will cause an additional 10 million deaths per year and $100

trillion global GDP loss by 2050 if no action for combating antibiotic-resistant bacteria is taken[1,2]. This stresses the urgent need for rapid and innovative diagnostic methods for antibiotic susceptibility testing (AST) of infectious bacteria to slow down the emergence of antibiotic-resistant bacteria and reduce the related mortality rate[3]. To ensure the best possible clinical outcome, it is crucial to introduce effective therapy within 24 h. However, the current gold standard method, like disk diffusion or broth dilution method, usually requires at least 24 h for the preincubation procedure for clinical samples and an additional 16-24 h to obtain the minimal inhibitory concentration (MIC) results. Overall, these methods are too time-consuming to guide an immediate decision for infectious disease treatment in the clinic, which leads to the emergence and spread of antimicrobial resistance[4].

Genotypic AST methods, such as polymerase chain reaction (PCR)-based techniques[5], have been developed for rapid detection. Such techniques measure the specific resistance genetic sequences in order to provide rapid AST results. They do not rely on time-consuming cell culture; however, only specific known genetic sequences with resistance are tested. Therefore, its application is limited to various bacterial species or different mechanisms of resistance. Also, they cannot provide MIC results for therapy decisions[6,7]. Besides, novel phenotypic methods for rapid AST are under development to overcome these limitations[8], including microfluidic devices[9-13], optical devices[14-16], phenotypic AST quantifying the nucleic acids copy number[17,18], and Raman spectroscopic methods[19-24]. These methods reduce time to guide AST results, however, most of them are only applicable to bacterial isolates, not directly to clinical specimens, and still require long-time preincubation.

In this work, we present a method for rapid determination of the susceptibility of bacteria in the urine and whole blood via monitoring of the cellular metabolic activity by SRS imaging. Water (H_2O) takes part in the vast majority of essential biomolecular synthesis processes in living cells. As an isotopologue of water, through enzyme-catalyzed H/D exchange reaction between the redox-active hydrogen atom in NADPH and the D atom in D_2O, deuterium can be incorporated into biomass inside a cell[25,26]. A deuterated fatty acid synthesis reaction is mediated by the deuterium labeled NADPH. The D_2O incorporation into reactions of amino acids results in the deuterated protein production[26] (**Figure 1**). In this way, the newly synthesized C–D bond-containing biomolecules in single microbial cells can be employed as a general metabolic activity marker to be detected. To read out *de novo* synthesized C-D bonds, Raman spectroscopy, a versatile analytical tool providing specific and quantitative chemical information of biomolecules, is widely used to determine antimicrobial susceptibility and significantly reduce the testing time to a few hours[27-30]. However, due to the inherent low efficiency of the Raman scattering process, the spontaneous Raman spectroscopy is of low detection sensitivity. Therefore, it is challenging to obtain real-time image results using spontaneous Raman spectroscopy. Coherent Raman scattering (CRS), including coherent anti-Stokes Raman scattering (CARS) and stimulated Raman scattering (SRS), has reached high detection sensitivity because of the coherent light field to generate orders of magnitude larger than that of spontaneous Raman spectroscopy, thereby rendering high-speed, specific, and quantitative chemical imaging at the single cell level[31-39].

Here, based on our most recent work[40], we present a protocol for rapid determination of the

metabolic activity and antimicrobial susceptibility by femtosecond SRS C-D imaging of D_2O incorporation of bacteria in the normal medium, urine, and whole blood environment at the single-cell level. Femtosecond SRS imaging facilitates monitoring single cell metabolism inactivation concentration (SC-MIC) against antibiotics at the single bacterium level within 2.5 h. The SC-MIC results are validated by standard MIC test via broth microdilution. Our method is applicable for determining antimicrobial susceptibility of bacteria urinary tract infection (UTI) and bloodstream infection (BSI) pathogens with a much reduced assay time compared to the conventional method, which opens the opportunity for rapid phenotypic AST in the clinic at the single-cell level.

PROTOCOL:

The use of human blood specimens is in accordance with the guidelines of the IRB of Boston University and NIH. Specifically, the specimens are from a bank and are completely deidentified. These specimens are not considered to be human subjects by IRB office at Boston University.

1. Preparation of bacteria and antibiotics stock solution

1.1 Prepare the antibiotics (gentamicin sulfate or amoxicillin) stock solution at a concentration of 1 mg/mL dissolved in sterile 1x phosphate-buffered saline (PBS) or dimethyl sulfoxide (DMSO) solvent in 1.5 mL micro tubes. Dissolve gentamicin sulfate in a sterile PBS solution and amoxicillin in sterile DMSO solvent. Thereafter, store the antibiotics solution at 2-8 °C as suggested.

1.2 To make D_2O containing cation-adjusted Mueller-Hinton Broth (MHB) media, add 220 mg of MHB broth base to 10 mL of D_2O to make 100% D_2O containing medium. Sterilize the solution by filtering with filters of 200 nm pore size.

NOTE: Use this protocol always for making and sterilizing medium solutions in further steps.

1.3 To prepare bacterial samples for SRS imaging, add 2 mL of normal MHB media, which does not contain deuterium, to a sterile round-bottom culture tube, and then prewarm it at 37 °C.

1.4 Use a sterile loop to select one bacterial (*Escherichia coli* BW25113 or *Pseudomonas aeruginosa* ATCC 47085) colony from the fresh culture on a tryptic soy agar plate. Then suspend it in the prewarmed culture media and gently vortex to prepare the bacteria suspension.

1.5 Incubate bacteria at 37 °C in a shaker at 200 revolutions per minute (rpm) until it reaches the logarithmic phase.

2. D_2O incorporation treatment in the presence of antibiotics (Figure 2a)

2.1 Check the bacterial concentration by measuring the optical density with a photometer at a wavelength of 600 nm.

2.2 Dilute the bacterial solution using the normal MHB medium, which does not contain deuterium, to reach a final cell concentration of 8 x 10^5 CFU/mL. Vortex gently to mix the bacterial cells.

2.3 Prepare 300 µL aliquots of the bacterial solution in seven 1.5 mL micro tubes, and 600 µL aliquots of the bacterial solution in one 1.5 mL micro tubes.

2.4 Add 4.8 µL of antibiotic (gentamicin or amoxicillin) stock solution (1 mg/mL) into the micro tube containing 600 µL of the bacterial solution, to make the final antibiotic concentration to 8 µg/mL.

2.5 Take 300 µL of solution out of the 8 µg/mL of antibiotic-containing bacteria solution, and add to another 300 µL of bacterial solution, to make two-fold diluted antibiotic- (4 µg/mL) containing bacteria solution.

2.6 Repeat the two-fold serial dilution of the test antibiotics, gentamicin, or amoxicillin, until the micro tube with the lowest concentration (0.25 µg/mL) is reached, and discard 300 µL from the tube. For both gentamicin and amoxicillin, the serial concentrations range from 0.25 µg/mL - 8 µg/mL.

2.6.1 Leave one tube with no antibiotics for blank control. This will be the positive control to inspect the bacterial metabolic activity without antibiotics treatment but with D_2O treatment.

2.7 Incubate the bacterial aliquot with the certain antibiotic (gentamicin or amoxicillin) containing MHB medium for 1 h.

2.8 During incubation, prepare a serial dilution of antibiotics with 100% D_2O containing medium with the same concentration gradient of antibiotics prepared in step 2.6. For both gentamicin and amoxicillin, the serial concentrations range from 0.25 µg/mL - 8 µg/mL.

2.9 After 1 h antibiotic treatment, add 700 µL of serially diluted antibiotic and 100% D_2O-containing MHB medium to the 300 µL of antibiotic-pretreated bacteria in the same antibiotic concentration (prepared in steps 2.6), respectively.

2.9.1 For example, add 700 µL of 100% D_2O-containing MHB medium (contianing 8 µg/mL of antibiotic) to the 300 µL of 8 µg/mL antibiotic-pretreated bacteria. In the same manner, transfer to the corresponding tubes of the next concentration, and homogenize by pipetting up and down several times.

2.9.2 Add 700 µL of antibiotic-free 100% D_2O-containing MHB medium to 300 µL of antibiotic-free bacteria (prepared in Step 2.6) as a blank control.

2.9.3 Incubate at 37 °C in an incubation shaker at 200 rpm for an additional 30 min.

NOTE: In this step, the final concentration of D_2O in the medium for the test is 70%.

2.10 First centrifuge the 1 mL of antibiotic and D_2O-treated bacterial sample at 6200 x *g* for 5 min at 4 °C, and then wash twice with purified water. Finally, fix samples in 10% (w/v) formalin solution and store them at 4 °C.

3. Preparation of bacteria in urine environment (Figure 2b)

3.1 To prepare *E. coli* BW25113 at the logarithmic phase, follow the steps at 1.4 and 1.5.

3.2 Check the bacterial concentration by measuring the optical density (OD) with a photometer at a wavelength of 600 nm.

3.3 To mimic the clinical UTI samples[14,18,41], spike the *E. coli* solution into 10 mL of deidentified urine to reach a final cell concentration of 10^6 CFU/mL.

3.4 Filter the *E. coli* spiked urine using a 5 µm filter, and then divide the bacterial solution in 300 µL aliquots into ten 1.5 mL micro tubes.

3.5 Perform D_2O incorporation treatment in the presence of antibiotics as described in the previous steps from 2.4 to 2.9.

4. Preparation of bacteria in blood environment (Figure 2c)

4.1 To prepare *Pseudomonas aeruginosa* ATCC 47085 at the logarithmic phase, follow the steps at 1.4 and 1.5.

4.2 To mimic the clinical bloodstream infections samples[42,43], spike *P. aeruginosa* in 1 mL of deidentified human blood to reach a final concentration of 10^6 CFU/mL.

4.3 Add 9 mL of sterile purified water to lyse the blood.

4.4 Filter the *P. aeruginosa* spiked blood using a 5 µm filter. Then harvest bacteria to 1 mL volume by centrifugation at 6200 x *g* for 5 min at 4 °C.

4.5 Divide the *P. aeruginosa* spiked blood solution in 300 µL aliquots into ten 1.5 mL micro tubes.

4.6 Perform D_2O incorporation treatment in the presence of antibiotics as described in the previous steps from 2.4 to 2.9.

5. SRS imaging of D_2O metabolic incorporation in a single bacterium

5.1 Wash 1 mL of fixed bacteria solution with purified water and then centrifuge at 6200 x *g*

for 5 min at 4 °C. Remove the supernatant. Enrich the bacterial solution to about 20 µL.

5.2 Deposit the bacterial solution on a poly-L-lysine coated coverglass. Sandwich and seal the sample for SRS imaging.

5.3 Image bacteria at the C-D vibrational frequency at 2168 cm^{-1} using an SRS microscope.

5.3.1 Input and tune the pump wavelength to 852 nm using the control software on a computer.

5.3.2 Measure the laser power using a power meter. Set the power of pump laser at the sample to ~8 mW and the power of Stokes laser at the sample to ~40 mW by adjusting the ND filter in front of the laser output.

NOTE: In the SRS microscope, a tunable femtosecond laser with an 80-MHz repetition rate provides the pump (680 to 1300 nm) and Stokes (1045 nm) excitation lasers.

5.4 By adjusting the screws of the reflection mirrors, spatially align the pump and Stokes beams and direct the two beams into an upright microscope equipped with 2D galvo mirror system for laser scanning.

5.4.1 Use a 60x water immersion objective to focus the pump and Stokes lasers on the sample.

5.4.2 Use an oil condenser to collect the signals from the sample in the forward direction.

5.4.3 Use a bandpass filter to filter out the Stokes laser before directing it into a photodiode.

5.4.4 Extract the stimulated Raman signal by a lock-in amplifier and detect the signals by the photodiode.

5.5 Set each SRS image to contain 200 x 200 pixels and the pixel dwell time for 30 µs in the software's control panel. The total acquisition time for one image is ~1.2 s. Set the **Step size** to 150 nm, so the image size is about 30 x 30 μm^2. Image at least three field of views for each sample.

6 Image processing and data analysis (Figure 3)

6.1 To obtain the average C-D signal intensity, open and process SRS images with ImageJ software.

6.2 First, convert SRS images into 8-bit type images with inverted color by clicking **Image | Type | 8-bit**, and then **Edit | Invert** buttons in the ImageJ software.

6.3 Then, filter the images with Gaussian blur by clicking **Process | Filters | Gaussian blur** buttons and set the **Sigma (Radius)** to 1.

6.4 Use image threshold adjustment to select the bacterial area. Click **Image | Adjust | Threshold** to ensure the selected bacterial sizes match those in the original SRS images. Eliminate small particles by adjusting the size threshold to determine the particles. Click **Apply**.

6.5 Apply **Analyze | Particles Analysis** buttons to label and determine the area of bacteria.

6.6 By clicking the **Show All** button in the **ROI manager** to the original unprocessed SRS image, label the same area of bacteria, determine the average intensity of each data point by clicking the **Measure** button in the **ROI manager**.

6.7 Circle the background area in the original SRS image and measure the average intensity of the background. The average C-D intensities of each bacterium is obtained by deducting the background signal intensity.

7 Quantitation of antimicrobial susceptibility via SC-MIC

NOTE: The cut-off value at 0.60 to determine the SC-MIC is established according to the statistical analysis of the SRS C-D intensities of the metabolism-active and metabolism-inhibited conditions for bacteria upon various concentrations of drug exposure[40]. The C-D intensities for the antibiotic-susceptible and antibiotic-resistant groups were fitted with normal distribution.

7.1 Plot the receiver operating characteristic (ROC) curve and evaluate the cut-off threshold at 0.60. Based on this cut-off value, the SC-MIC as an indicator of the efficacy of antibiotics can be defined to determine the metabolically inactive and metabolically active group.

7.2 To quantitatively analyze the SRS imaging data, plot the histograms of C-D signal intensities for each bacteria group treated with the serially diluted antibiotic concentration in the a software (see **Table of Materials**). The colored data points stand for a different individual bacterium.

7.3 Normalize the C-D intensities of antibiotic-treated group to the mean intensity of the control group without antibiotic treatment. Determine the SC-MIC results of different bacteria and antibiotic combinations by quantifying the SRS signal intensities at C-D region versus various concentration of antibiotics using the cut-off value at 0.60.

7.4 Validate and compare the SC-MIC readout with the MIC determined using conventional broth microdilution assay.

7.5 According to the Clinical and Laboratory Standards Institute (CLSI), the susceptibility category based on the SRS metabolic imaging results for each tested bacterial strain is interpreted as “susceptible”, “resistant”, or “intermediate”.

REPRESENTATIVE RESULTS:

The effect of incubation time on deuterium incorporation is measured by spontaneous Raman microspectroscopy at the C-D (2070 to 2250 cm^{-1}) and C-H (2,800 to 3,100 cm^{-1}) region (**Figure 4a**). The time-lapse single-cell Raman spectra of *P. aeruginosa* cultured in 70% D_2O containing medium show increasing CD/CH intensity over incubation time from 0 to 180 min. (**Figure 4b**) The increasing C-D abundance in single microbial cells reveals that D_2O is incorporated into deuterated biomolecules inside the cell.

D_2O labeling above 50% affects bacterial metabolism significantly during a 23 h incubation period[27]. We observed bacterial growth inhibition when D_2O labeling concentration is above 70% during a 25 h incubation period (**Figure S1**). We have performed MIC by gold standard broth dilution and obtained SC-MIC results in two *P. aeruginosa* strains (*P. aeruginosa* ATCC 47085 and *P. aeruginosa* ATCC 1133) (**Table S1**). Our current results show that 70% D_2O does not affect the performance of our method in *P. aeruginosa*. The category agreement of our SC-MIC method with the conventional culture-based method is 100% for all the tested *P. aeruginosa* and antibiotic combinations, as shown in **Table S1**. We attribute this good agreement to the minimal toxicity of 70% D_2O on *P. aeruginosa* during the 30 min incubation period in SC-MIC determination.

Following the protocol, *P. aeruginosa* was incubated with serially diluted gentamicin for 1 h and then 70% D_2O for additional 30 min. SRS metabolic imaging at ~2168 cm^{-1} (**Figure 5a**) was conducted. The C-D intensities upon antibiotic treatment are divided by the mean value of the control group, which is without antibiotic treatment. The quantitative statistical analysis (**Figure 5b**) showed that C-D signals of *P. aeruginosa* were significantly lower at 2 μg/mL or higher gentamicin concentration than that without gentamicin treatment (0 μg/mL). Using the cut-off threshold at 0.60, the *P. aeruginosa* was metabolically inhibited at 2 μg/mL and higher concentrations of gentamicin. The dotted line shows the defined cut-off value at 0.60 in **Figure 5b**. In this way, the SC-MIC for *P. aeruginosa* against gentamicin in normal MHB medium was determined to be 2 μg/mL. This SC-MIC value is verified to be within the one-fold difference range with the MIC (4 μg/mL) determined by the broth microdilution method (**Figure 5c**). Taken together, SC-MIC determined by our technology enables quantification of antimicrobial susceptibility.

To explore the potential of rapid AST by SRS imaging of deuterium metabolic incorporation for clinical applications, especially for the most prevalent UTI infection, we tested bacteria-spiked urine sample using *E. coli*, the most common pathogen to cause UTI infection[44]. To mimic the clinical UTI samples at a relavent bacterial concentration, *E. coli* is added to the deidentified urine to a final concentration of 10^6 CFU/mL. After sample purification, the urine sample were incubated with amoxicillin and D_2O. The clean background in the SRS images showed that the sample preparation protocol was applicable for rapid AST measurement (**Figure 5d**). The SC-MIC for the *E. coli*-spiked urine sample against amoxicillin was determined to be 4 μg/mL (**Figure 5e**), which has the same susceptibility readout with the MIC (8 μg/mL) by conventional broth dilution method for pure *E. coli* in normal MHB medium (**Figure 5f**). These results collectively showed that rapid AST by SRS imaging of deuterium metabolic incorporation is of great potential for clinical diagnosis to UTI infectious pathogens.

As compared with UTI infection, rapid AST for BSI pathogens is much more challenging for *in situ* study of bacterial metabolic activity, as a lot of blood cells presenting in blood. To investigate the applicability of rapid AST by SRS imaging of D_2O metabolic incorporation for clinical BSI samples, *P. aeruginosa* spiked in deidentified human blood was detected. As shown in **Figure 5g**, the C-D intensity of SRS image at 2168 cm^{-1} was dominated by bacterial signals. Since the red blood cells do not have metabolic activity to uptake D_2O for further biosynthesis, the C-D signals were originated from the metabolic deuterium incorporation of live bacteria. The cross-phase modulation or photothermal signal of debris or red blood cells species contributed to the weak background signals, without affecting the quantitative analysis of the SC-MIC. The SC-MIC result for *P. aeruginosa* in blood was determined to be 2 μg/mL (**Figure 5h**), which agreed well with the conventional standard MIC result for *P. aeruginosa* in normal growth medium (**Figure 5i**). Taken together, these results showed that SRS metabolic imaging of deuterium metabolic incorporation can be a rapid AST method to determine the SC-MIC for bacteria in BIS infections.

FIGURES AND TABLES:

Figure 1: Scheme for D_2O incorporation into deuterated lipid and protein[25,26]. Deuterium can be incorporated into biomass inside a cell through enzyme-catalyzed H/D exchange reaction between the redox-active hydrogen atom in NADPH and the D atom in D_2O. Deuterated fatty acid synthesis reaction is mediated by the deuterium labeled NADPH. The D_2O incorporation into reactions of amino acids results in the deuterated proteins production. This figure has been modified from ref.[40].

Figure 2: Workflow of rapid AST by SRS metabolic imaging of deuterium incorporation. (**a**) D_2O incorporation treatment in the presence of antibiotics in MHB medium, and the following SRS imaging procedures. (**b**) Preparation of bacteria in urine environment. (**c**) Preparation of bacteria in the blood environment. This figure has been modified from ref.[40].

Figure 3. Automated image processing and data interpretation. (**a**) Raw SRS image. (**b**) Image after intensity threshold adjustment to determine the area of bacterial cells. (**c**) Data points selected after particle analysis step. (**d**) The corresponding data points are selected in the raw image. (**e**) Results of the corresponding data points in the raw image. (**f**) Statistical results of the average intensity of the data points after subtraction of background. Scale bar: 10 μm. This figure has been modified from ref.[40].

Figure 4. Effect of incubation time on deuterium incorporation into bacteria. (**a**) Time-lapse measurement at C-D (2070 to 2250 cm^{-1}) and C-H (2,800 to 3,100 cm^{-1}) region by spontaneous Raman microspectroscopy (averaged from 20 spectra). (**b**) Histogram plot of CD/CH intensity ratio plot over D_2O incubation time for bacteria in (**a**). Each colored point stands for a measurement from a single bacterium. Error bars represent the standard error of the mean (SEM).

Figure 5. SC-MIC determination using SRS imaging of bacterial metabolic incorporation of D_2O

against antibiotics in normal medium, urine and blood environment. (**a**) SRS imaging at C-D vibration (2168 cm^{-1}) and the corresponding transmission images of *P. aeruginosa* in the presence of D_2O with the addition of serially diluted gentamicin in normal MHB medium. (**b**) Quantitative analysis of SRS C-D intensity of *P. aeruginosa* in (**a**). The colored data points in the histogram stand for the different individual bacterium. The dotted line indicates the cut-off value at 0.60. (**c**) The comparison of the SC-MIC readout with the MIC by broth microdilution method, and susceptibility category for *P. aeruginosa* according to the CLSI. (**d**) SRS imaging at C-D vibration (2168 cm^{-1}) and corresponding transmission images of *E. coli* in urine after incubation in D_2O with the serially diluted amoxicillin. (**e**) Quantitative analysis of SRS C-D intensity in (**d**). (**f**) The comparison of the SC-MIC readout and susceptibility category for *E. coli* in normal MHB and in urine. (**g**) SRS imaging at C-D vibration (2168 cm^{-1}) and corresponding transmission images of *P. aeruginosa* in blood after incubation in D_2O with the serially diluted gentamicin. (**h**) Quantitative analysis of SRS C-D intensity in (g). (**i**) The comparison of the SC-MIC readout and susceptibility category for *P. aeruginosa* in normal MHB and in blood. S: sensitive. Number of cells $N \geq 10$ per group. Error bars represent the SEM. Scale bar: 10 µm. This figure has been modified from ref.[40].

Table 1: Troubleshooting table.

Figure S1: Testing D_2O toxicity on *P. aeruginosa* cultured in Lauria-Bertani (LB) medium with different D_2O concentrations. Error bars indicate standard deviation values (number of measurements = 5).

Table S1. **Comparison of SC-MICs and MICs in normal MHB of *P. aeruginosa* upon antibiotics treatment**. S: sensitive; R: resistant; I: Intermediate.

DISCUSSION:

Rapid AST can be obtained by assessing the response of bacterial metabolic activity to antibiotic treatment using single-cell SRS metabolic imaging within 2.5 h from the sample to SC-MIC results. The response of bacterial metabolic activity and antimicrobial susceptibility can be detected by monitoring the metabolic incorporation of D_2O for biomolecule synthesis using SRS imaging of C–D bonds. Since water is ubiquitously used in living cells, SRS metabolic imaging provides a universal method for rapid AST. The rapid AST method is applicable to detect bacteria in complex biological environments, such as urine or whole blood at a single bacterium level. The SC-MIC can be determined after 1.5 h culture of bacteria in urine and blood, which is considered transformative to shift the paradigm of UTI and BSI diagnosis from a time-consuming culture-dependent procedure to a culture-independent in situ approach. Therefore, it means a tremendous reduction in diagnosis time as compared with the conventional broth microdilution method, which paves the way towards clinical translation allowing for on-time identification of appropriate antimicrobial agents for precise treatment.

The protocols for antibiotics treatment described here follow the guidelines of the CLSI, in which the suggested MHB medium can be generally used for the cultivation of a wide variety of microorganisms. A key parameter is that the bacterial cell number used for antimicrobial susceptibility testing is kept at about 5×10^5 CFU/mL as recommended in CLSI. This is of critical

importance for obtaining accurate and reproducible results. In antibiotics treatment experiments, the bacteria concentration is set at 8 x 10^5 CFU/mL. Higher bacteria concentration can lead to an increase in the MIC results. Once the bacterial suspension is adjusted, it must be used within 30 min to avoid changes in the bacterial cell concentration.

For susceptibility testing of an antibiotic such as daptomycin, it is recommended to supplement 50 mg/L of calcium in the media. The cation adjusted MHB medium contains 20-25 mg/L of Ca^{2+}. Therefore, ensure that the medium is further supplemented with additional Ca^{2+} (solubilized in water and filter sterilized) in the concentration of 30 mg/L.

Another critical step in the presented method is the incubation time of bacteria upon antibiotics exposure and D_2O incorporation. Because the generation time of the bacterial life cycle is roughly 30 to 60 min, it is important to influence bacterial metabolic activity upon antibiotic exposure for a certain time. This test has been evaluated for a variety of bacteria-antibiotic combinations to antibiotic exposure for 1 h prior to following 0.5 h D_2O treatment. The first 1 h antibiotic treatment step is essential to influence the bacterial metabolic activity. Next, bacteria are incubated with D_2O-containing and antibiotic-containing medium for an additional 30 min. The final antibiotic concentrations are maintained at the same level, and the final concentration of D_2O is adjusted to 70%. Overall, after 1 h antibiotic preculture and following 0.5 h of D_2O and antibiotic incorporation, the SC-MIC results are then determined by SRS metabolic imaging of the bacterial metabolic activity. This design minimizes the impact of D_2O influence of antimicrobial activity to bacteria and also leads to a comparable readout of SC-MIC results with the MICs by the conventional method.

In the SC-MIC measurements, we prepare in parallel 40 samples, including 5 different antibiotics, each with 8 concentrations at the same time. However, because there are a lot of manual operation procedures, the total assay time for detecting the AST for five different bacteria-antibiotics combinations is longer than 2.5 h. In our method, each SRS image containing ~20 individual bacterial cells was obtained within ~1.0 s in one single shot at 30 μs pixel dwell time. We estimate the total AST assay time to study 10 antibiotics for one bacterial strain would be less than 2.5 h from sample to SC-MIC readout, which has tremendous possibility to perform high throughput measurement. In future work, an automated sample preparation and imaging data acquisition method will be employed to further improve the throughput. The troubleshooting details are provided in **Table 1.**

In conventional culture-based AST methods, to obtain bacterial isolates for further measurements, it is necessary to pre-incubate clinical specimens for hours. Advanced AST methods for clinical UTI sample, such as Raman spectroscopy[29], nanoliter array[6], and digital nucleic acid quantification[18], have been developed to get rid of long-time preincubation. Compared with UTI infection, the BSI or sepsis is much more life-threatening[18,45], where rapid AST is urgently needed for precise diagnostics in the clinic. A microscopic imaging method to measure bacterial colony formation from positive blood cultures has been reported to provide MIC results[46]. However, it takes at least 6 h to grow bacteria to conduct the AST assay. Furthermore, commercial automatic systems[47] and mass spectrometry[48,49] strategies can provide

AST readout from positive blood cultures. However, the MIC results for the clinical decision cannot be provided. The AST results and the MIC readout are significant to avoid excess dosage of antibiotics to patients to cause potential side effects in clinics, to slow and prevent the spread of the antimicrobial resistant infections[50,51]. Compared with the existing spontaneous Raman microscopy-based AST methods, our technology tremendously reduces data acquisition time (ca. 600 times less) due to orders-of-magnitude signal enhancement. In this work, we demonstrate rapid AST by SRS imaging of deuterium metabolism in single bacteria at a clinically relevant bacterial concentration (10^5~10^6 CFU/ml) in either urine or whole blood environment. As shown in previous results, the MIC results are determined after 1 h antibiotic treatment and 30 min mixture of D_2O and antibiotics incubation into bacteria in urine and blood. Our method can provide MICs and susceptibility classification for each strain-antibiotic combination within 2.5 h, and, therefore, opens a new avenue to clinical translation. To summarize, without the need of preculturing and bacterial division, our method has an enormous potential in the field of rapid and high throughput AST in infectious diseases.

Our SC-MIC method by SRS metabolic imaging is applicable to detect MICs and provide susceptibility classification for infectious pathogens when dealing with abundant varieties of strain-antibiotic combinations for clinical use. The SC-MIC is determined after 30-min of D_2O incorporation into bacteria in urine and blood, which means a tremendous reduction in diagnosis time compared with the conventional broth dilution method costing 16 to 24 h for preincubation. To deliver pathogen identification information for clinical decision-making, SRS metabolic imaging technology can be further integrated with diagnostic platforms capable of rapid pathogen identification, such as, matrix-assisted laser desorption ionization-time-of-flight mass spectrometry[49,52,53]. Combining *in situ* pathogen identification and rapid AST diagnosis could be of great potential for translation into clinic that allows for on-time identification of appropriate antimicrobial agents for precise treatment.

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DISCLOSURES:

The authors have no conflicts of interest to disclose.

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Figure 1

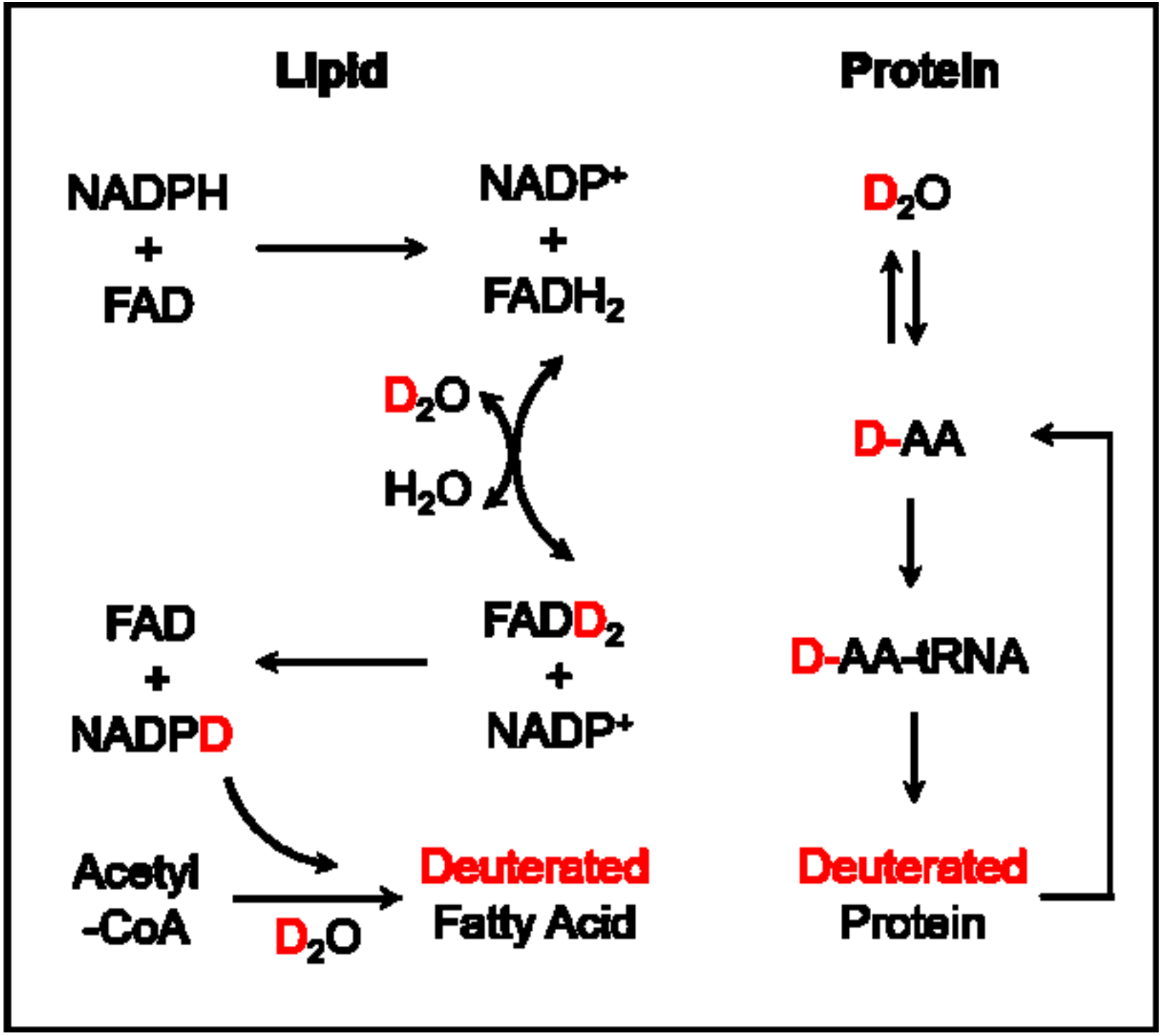


Figure 2

Click here to access/download;Figure;Figure 2.tif

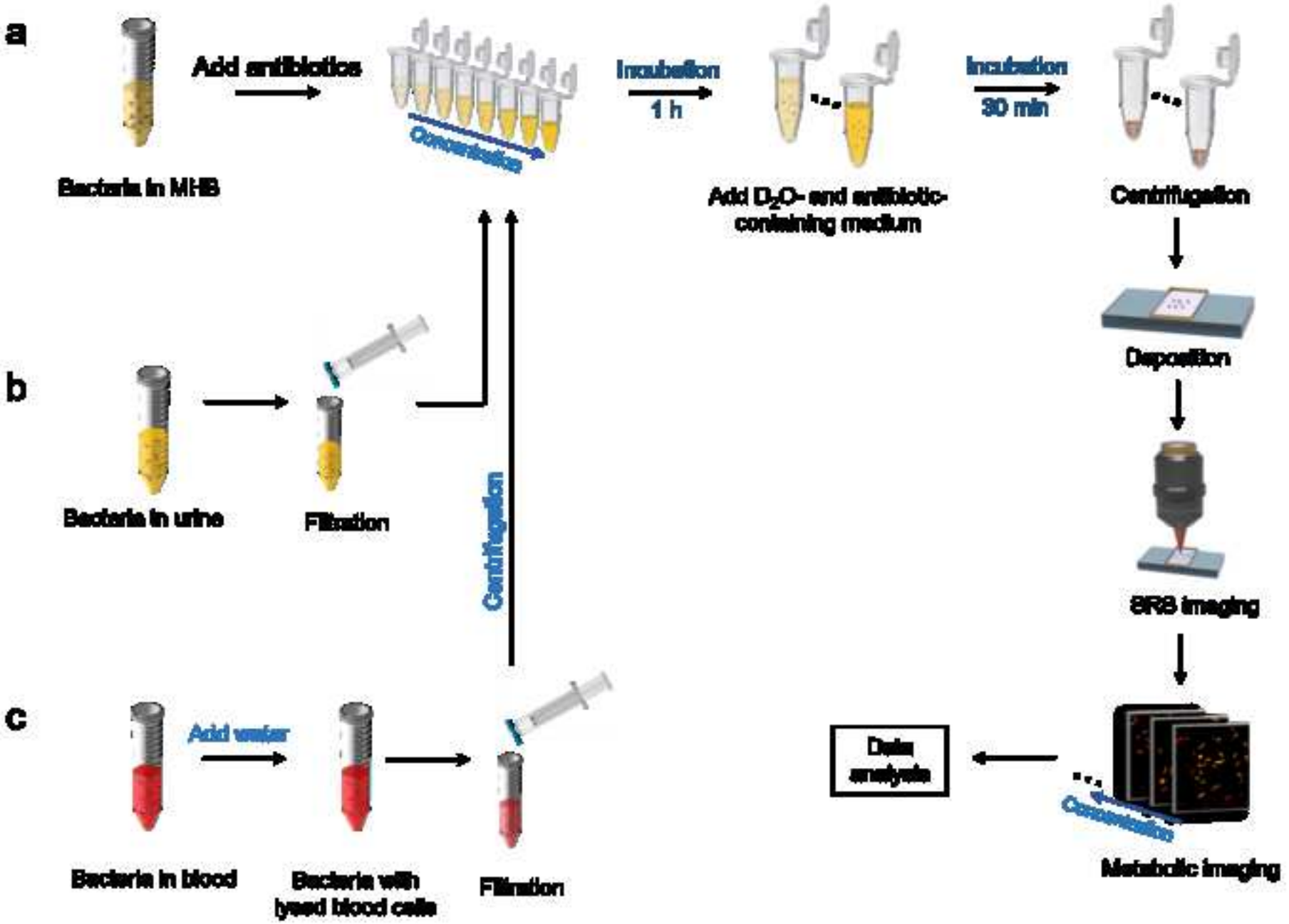


Figure 3

Click here to access/download;Figure;Figure 3.tif

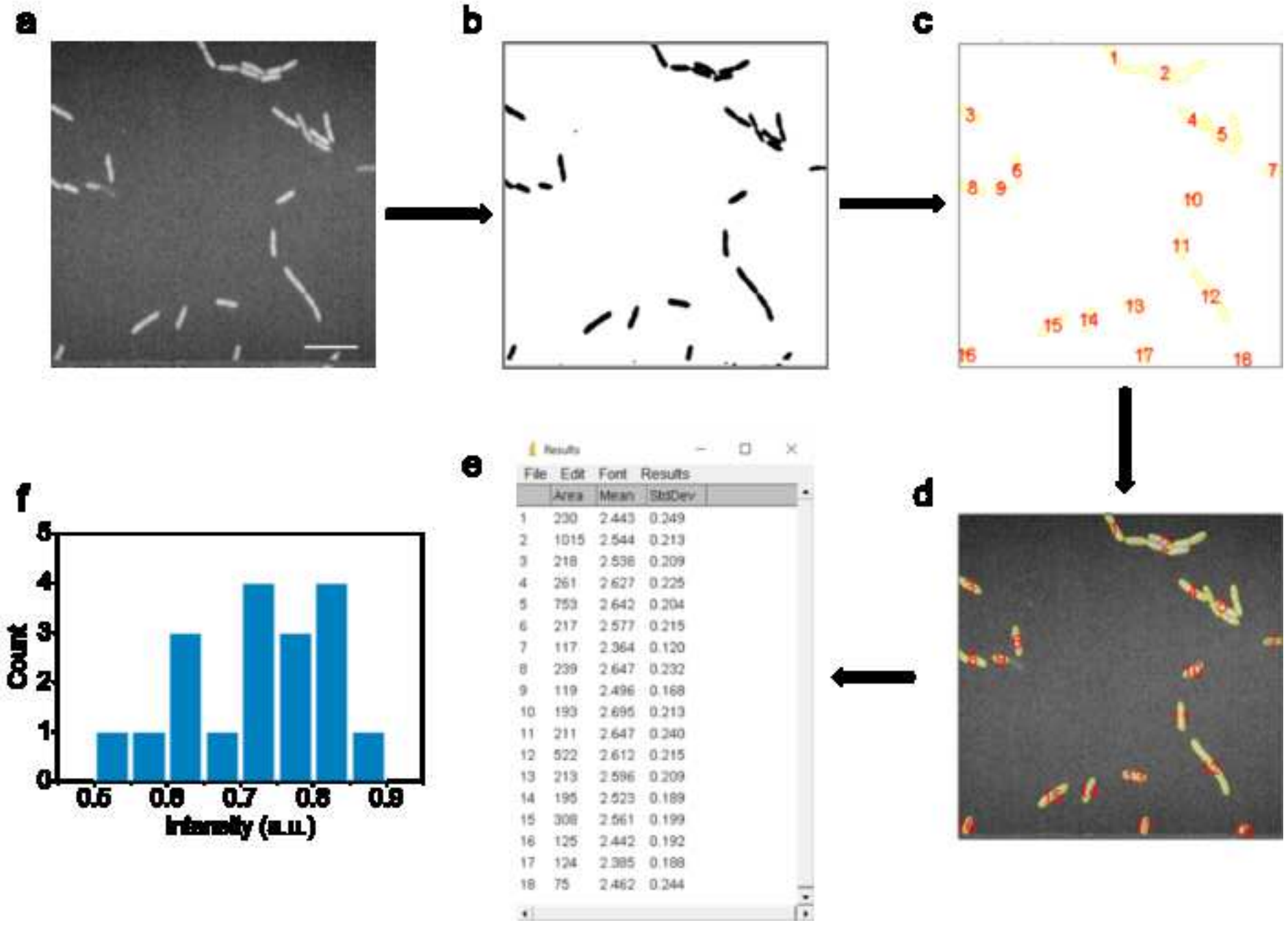


Figure 4

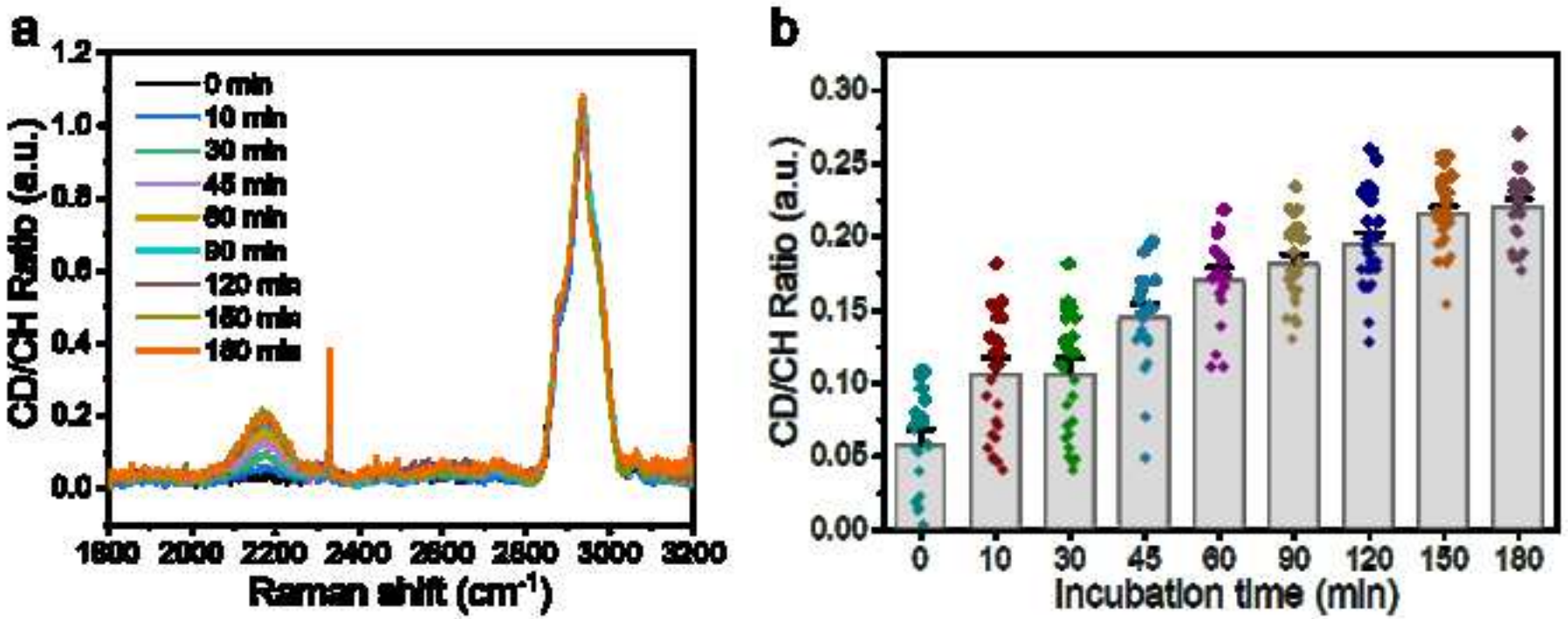
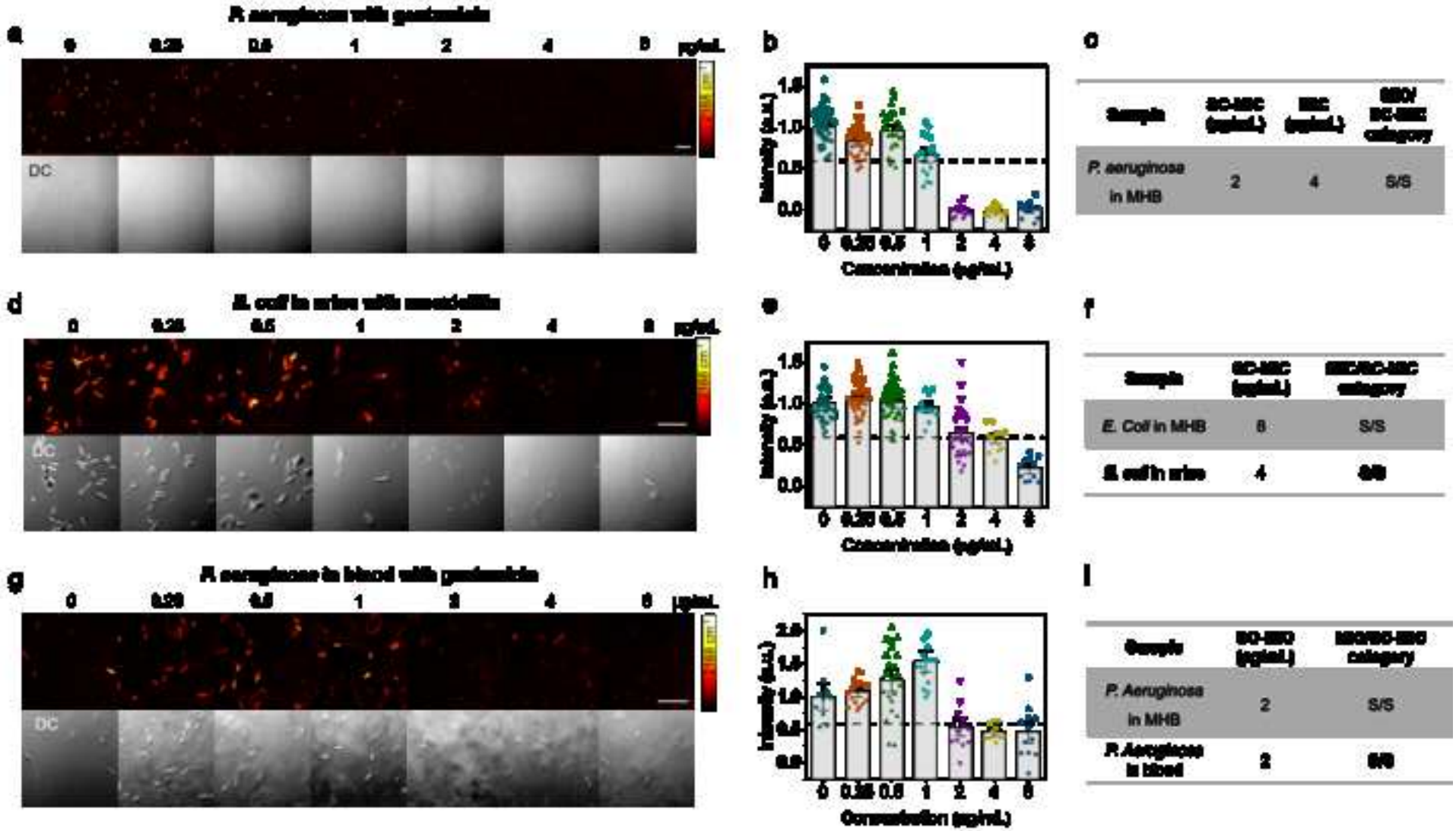


Figure 5

Click here to access/download;Figure;Figure 5.tif



Troubleshooting Table

Click here to access/download;Table;Troubleshooting table.xlsx

Problem	Possible reason	Solution
Little or no bacterial cell number in imaging field of view	Bacterial density in the solution is too low	Centrifuge for longer time to further enrich bacteria
Photodamage of the bacterial cells during SRS imaging	The laser power used is too high	Tune down the laser power to an appropriate value
No SRS signal is detectable	The spatial and temporal overlap of the pump and Stokes beam is not optimized	Align the pump and Stokes beams using a standard sample deuterated dimethyl sulfoxide

Name of Material/ Equipment	Company	Catalog Number
Acousto-optic modulation	Gooch&Housego	R15180-1.06-LTD
Amoxicillin	Sigma Aldrich	A8523-5G
Bandpass filter	Chroma	HQ825/150m
Calcium chloride	Sigma Aldrich	C1016-100G
Cation-adjusted Mueller-Hinton Broth	Fisher Scientific	B12322
Centrifuge	Thermo Scientific	75002542
Cover Glasses	VWR	16004-318
Culture tube with snap cap	Fisher brand	149569B
Daptomycin	Acros	A0386346
Deuterium oxide		151882
Deuterium oxide-d6	Sigma Aldrich	156914
Escherichia coli BW 25113	The Coli Genetic Stock Center	7636
Eppendorf polypropylene microcentrifuge tubes 1.5 mL	Fisher brand	05-408-129
Gentamicin sulfate	Sigma Aldrich	G4918
Hydrophilic Polyvinylidene Fluoride filters	Millipore-Sigma	SLSV025NB
ImageJ software	NIH	Version: 2.0.0-rc-69/1.52t
Incubating orbital shaker set at 37 °C	VWR	97009-890
Inoculation loop	Sigma	BR452201-1000EA
InSight DeepSee femtosecond pulsed laser	Spectra-Physics	Model: insight X3
Lock-in amplifier	Zurich Instrument	HF2LI
Oil condenser	Olympus	U-AAC

Pseudomonas aeruginosa ATCC 47085 (PAO1)	American Type Culture Collection	ATCC 47085
Photodiode	Hamamatsu	S3994-01
Polypropylene conical tube 15 mL	Falcon	14-959-53A
Polypropylene filters	Thermo Scientific	726-2520
Sterile petri dishes	Corning	07-202-031
Syringe 10 mL	Fisher brand	14955459
UV/Vis Spectrophotometer	Beckman Coulter	Model: DU 530
Vortex mixer	VWR	97043-562
Water objective	Olympus	UPLANAPO/IR

Comments/Description
Modulating stokes laser beam
Block the stokes laser beam before the photodiode
Cation adjustment
Antimicrobial susceptibility testing of microorganisms by broth dilution methods
Organic solvent to dissolve antibiotics
Organic solvent as a standard to calibrate SRS imaging system
pore size 5 µm
Image processing and analysis
Tunable laser source and fixed laser source at 1045 nm for SRS imaging
Demodulate the SRS signals
NA 1.4

Detector
pore size 0.2 µm
Measuring optical density at wavelength of 600 nm
60×, NA 1.2

Response Letter to Editorial Team and Reviewers

Editorial comments:
Changes to be made by the Author(s):
1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.
Re: We appreciate the editorial comment. We have checked and revised the manuscript to avoid spelling or grammar issues.

2. Please provide an email address for each author.
Re: We appreciate the editorial comment. We have added the email address for each author.

3. Please rephrase the Short Abstract/Summary to clearly describe the protocol and its applications in complete sentences between 10-50 words: "This protocol presents…"
Re: We appreciate the editorial comment. We have rephrased the Summary as following:
"This protocol presents rapid antimicrobial susceptibility testing (AST) within 2.5 h by single-cell stimulated Raman scattering imaging of D_2O metabolism. This method is applicable to bacteria in urine or whole blood environment, which is transformative for rapid single-cell phenotypic AST in clinic."

4. Please ensure that the Abstract is within 150-300-word limit.
Re: We appreciate the editorial comment. We have confirmed the Abstract is in 151 words.

5. Please revise the following lines to avoid overlap with previously published work: 30-31, 49-51, 54-56, 60-62, 75-76, 85-88, 160-163, 167-170, 199-200, 375-377, 386-388, 405-408,
Re: We appreciate the editorial comment. We have modified these sentences to avoid overlap.

6. Please include an ethics statement before the numbered protocol steps, indicating that the protocol follows the guidelines of your institution's human research ethics committee since human urine and blood is used in the study?
Re: We appreciate the editorial comment. This protocol doesn't include an ethics statement because the human urine and blood are deidentified samples.

7. 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."
Re: We appreciate the editorial comment. We have revised the protocol section to avoid usage of phrases such as "could be," "should be," and "would be".

8. Please add more details to your protocol steps. Please ensure you answer the "how" question, i.e., how is the step performed? This can be done by including mechanical actions, button clicks in the software, knob turns, command lines, etc.
Re: We appreciate the editorial comment. We have modified part 5, 6, 7 to including mechanical actions and button clicks in the software.

9. 1.4 What bacterial strain is used?
Re: We appreciate the editorial comment. We added more information in the text: *Escherichia coli* BW25113 or *Pseudomonas aeruginosa* ATCC 47085.

10. 2.5: Which antibiotic is used in this experiment? Concentration used?

Re: We appreciate the editorial comment. We added more information and clarified in part 1.1 and part 2.4:

1.1 Prepare the antibiotics (gentamicin sulfate or amoxicillin) stock solution at a concentration of 10 mg/mL dissolved in 1x phosphate-buffered saline (PBS) or dimethyl sulfoxide (DMSO) solvent in 1.5 mL micro tubes. Gentamicin sulfate is dissolved in sterile PBS solution. Amoxicillin is dissolved in sterile DMSO solvent.

2.4 2.4 Prepare two-fold serial dilutions of the test antibiotics, gentamicin or amoxicillin, using the aliquots of bacteria solution as a dilution medium. For both of gentamicin or amoxicillin, the serial concentrations cover from 0.25 to 8 µg/mL.

11. 3.3: Citation to show that this is mimicking UTI?

Re: We appreciate the editorial comment. We added the following citations:

14 Baltekin, Ö., Boucharin, A., Tano, E., Andersson, D. I. & Elf, J. Antibiotic susceptibility testing in less than 30 min using direct single-cell imaging. *Proceedings of the National Academy of Sciences.* **114** (34), 9170-9175 (2017).

18 Schoepp, N. G., Schlappi, T. S., Curtis, M. S., Butkovich, S. S., Miller, S., Humphries, R. M. *et al.* Rapid pathogen-specific phenotypic antibiotic susceptibility testing using digital LAMP quantification in clinical samples. *Science Translational Medicine.* **9** (410), eaal3693, doi:10.1126/scitranslmed.aal3693, (2017).

41 Michael, I., Kim, D., Gulenko, O., Kumar, S., Kumar, S., Clara, J. *et al.* A fidget spinner for the point-of-care diagnosis of urinary tract infection. *Nature Biomedical Engineering.* **4** (6), 591-600, doi:10.1038/s41551-020-0557-2, (2020).

12. 4.2: Citations to show that is mimicking blood stream infection?

Re: We appreciate the editorial comment. We added the following citations:

42 Bhattacharyya, R. P., Bandyopadhyay, N., Ma, P., Son, S. S., Liu, J., He, L. L. *et al.* Simultaneous detection of genotype and phenotype enables rapid and accurate antibiotic susceptibility determination. *Nature Medicine.* **25** (12), 1858-1864, doi:10.1038/s41591-019-0650-9, (2019).

43 Stupar, P., Opota, O., Longo, G., Prod'hom, G., Dietler, G., Greub, G. *et al.* Nanomechanical sensor applied to blood culture pellets: a fast approach to determine the antibiotic susceptibility against agents of bloodstream infections. *Clinical Microbiology and Infection.* **23** (6), 400-405, doi:10.1016/j.cmi.2016.12.028, (2017).

13. 4.4. Please check and reword for clarity… centrifugation speed in x g, time and temperature?

Re: We appreciate the editorial comment. We added more information in part 4.4:

"…, and then harvest bacteria to 1 mL volume by centrafuged at 8000 rpm for 5 min at 4 °C."

and in part 2.9:

"… was first centrifuged at 8000 revolutions per minute (rpm) for 5 min at 4 °C, …"

14. 5.3.1: What is C-D vibrational frequency?

Re: We appreciate the editorial comment. We added: "To image bacteria at the C-D vibrational frequency at 2167 cm^{-1}, …"

15. 5,6, 7: How is this done?

Re: We appreciate the editorial comment. We have modified part 5, 6, 7 to including mechanical actions and button clicks in the software.

16. 8: We do not have paragraph of text in the protocol section. The Protocol should contain only action items that direct the reader to do something. This can be moved to representative result/discussion section as appropriate. If this needs to be here, please make numbered action step and include how this is done.

Re: We appreciate the editorial comment. The “Conclusion” part is moved down to the place between Representative result and Discussion.

17. Please include a single line space between each steps of the protocol and then highlight 3 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.
Re: We appreciate the editorial comment. The line space is set to 1.0. And the essential steps of the protocol for the video are highlighted in bold.

18. Please remove the embedded figure(s) from the manuscript. All figures should be uploaded separately to your Editorial Manager account. Each figure must be accompanied by a title and a description after the Representative Results of the manuscript text.
Re: We appreciate the editorial comment. We have removed the figures, and kept the captions for each figure.

19. Please remove the embedded Table from the manuscript. All tables should be uploaded separately to your Editorial Manager account in the form of an .xls or .xlsx file. Each table must be accompanied by a title and a description after the Representative Results of the manuscript text. Table of materials should be uploaded separately as a .xlsx file.
Re: We appreciate the editorial comment. We have removed the “TABLE OF MATERIALS” part and the “Troubleshooting table”. The two tables are uploaded separately in the form of an .xlsx file.

20. Please obtain explicit copyright permission to reuse any figures from a previous publication. Explicit permission can be expressed in the form of a letter from the editor or a link to the editorial policy that allows re-prints. Please upload this information as a .doc or .docx file to your Editorial Manager account. The Figure must be cited appropriately in the Figure Legend, i.e. “This figure has been modified from [citation].”
Re: We appreciate the editorial comment. The publisher John Wiley and Sons clarified that this is an open access article under the terms of the Creative Commons Attribution License, which permits use, distribution and reproduction in any medium, provided the original work is properly cited.

21. As we are a methods journal, please ensure that the Discussion explicitly cover the following in detail in 3-6 paragraphs with citations. Please do not make bullet points.
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
Re: We appreciate the editorial comment. The bullet points have been modified to a), b), … as instructed.

22. Please ensure that the references appear as the following: [Lastname, F.I., LastName, F.I., LastName, F.I. Article Title. Source. Volume (Issue), FirstPage – LastPage, (YEAR).] For more than 6 authors, list only the first author then et al.
Re: We appreciate the editorial comment. The references are modified to the format as instructed.

Reviewers' comments:
Reviewer #1:
Manuscript Summary:
In this manuscript the authors describe the protocol for rapid single cell antimicrobial susceptibility testing, by using D_2O incorporated stimulated Raman scattering imaging. This protocol will be of interest to researchers for rapid AST, and may be considered for acceptance after the following concerns have been addressed.
Re: We appreciate the reviewer's positive comments.

Major issue:
The authors did not give enough and appropriate references to related and previous work.
--- SRS imaging of D_2O metabolism in single cells (as well as animals) was first developed by Shi et al. (Nat Commun. 2018. 9,2995), which provides the underlying foundation of the current work, but this reference was ignored in the manuscript. For example, Line 68, "Cellular metabolic activity can be probed via monitoring … so called heavy water (Figure 1)" - lacks appropriate references. Same to Line 80.
--- Appropriate citations should also be added to the description of figures in both the text and figure captions, in addition to the reference cited in Figure 4 caption.
Re: We appreciate the reviewer's comments. We added references in the text and Figure 1 caption.

In "REPRESENTATIVE RESULTS", many sentences are identical to those in the authors' previous publication.
Re: We appreciate the reviewer's comments. We have modified these sentences to avoid overlap.

All through the Protocol, the authors used inconsistent tenses (present/past) and active/passive voice.
Re: We appreciate the reviewer's comments. We corrected all the sentences into present and active voice.

In addition, many places in the Protocol the description needs to be clearer and more straightforward. For example, Line 120. It is unclear what the concentration of D_2O is, 100%? And what is the final concentration of D_2O in the medium for the test?
Re: We appreciate the reviewer's comments. We modified the sentences in part 2.7 as following:
"2.7 In the meantime of incubation, prepare the serial diluted concentration of antibiotics in the 100% D_2O containing medium with the same concentration gradient of antibiotics."
In part 2.8, we added explanation as following:
"... In this step, the final concentration of D_2O in the medium for the test is 70%."

Did the authors conduct the D_2O toxicity testing in bacteria?
Re: We appreciate the reviewer's comments. Yes, we conducted the D_2O toxicity testing in bacteria. We note that D_2O labelling above 70% affects bacterial metabolism significantly was observed during a 25-hour incubation period (Figure S1). Our current results show that 70% D_2O does not affect the performance of our method in *P. aeruginosa*, because we have performed MIC by gold standard broth dilution and obtained SC-MIC results in two *P. aeruginosa* strains (Table S1). The category agreement of our method (SC-MIC) with the conventional culture-based method is 100% for all the tested *P. aeruginosa* and antibiotic combinations, as shown in the following Table. We attribute this good agreement to the minimal toxicity of 70% D_2O on *P. aeruginosa* during the 30 min incubation period in SC-MIC determination.

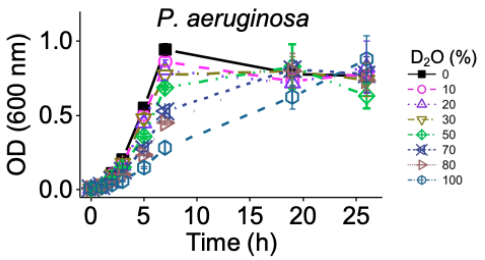


Figure S1. Testing D_2O toxicity to *P. aeruginosa* cultured in LB medium with different D_2O concentrations. Error bars indicate standard deviation values (number of measurements = 5).

Table. Comparison of SC-MICs and MICs in normal MHB of *P. aeruginosa* upon antibiotics. S: sensitive; R: resistant; I: Intermediate.

Bacteria strains	**Antibiotics**	**SC-MIC (μg/mL)**	**MIC in normal MHB (μg/mL)**	**MIC/SCMIC category**
P. aeruginosa ATCC 47085 (PAO1)	Cefotaxime	32	16	I/I
	Colistin	0.5	2	S/S
	Gentamicin	2	4	S/S
	Amikacin	≤4	2	S/S
	Tobramycin	≤1	0.5	S/S
	Ciprofloxacin	≤0.25	0.125	S/S
P. aeruginosa ATCC 1133	Colistin	8	≥16	R/R

Reviewer #2:

Manuscript Summary:

A rapid antibiotic susceptibility testing (AST) method for a single bacterium is proposed in this article by adopting D_2O and Stimulated Raman Spectroscopy (SRS). Living bacteria can incorporate deuterium from D_2O into their organic compounds that form a C-D bond, which reflected their metabolism activity. The intensities of C-D bond Raman signals from a single bacterium with different antibiotic concentration treatments are measured by the SRS image with further data process. By comparing with the standard minimum inhibition concentration (MIC) result, the single-cell metabolism inactivation concentration (SC-MIC) of the bacterium is determined by the relative intensity threshold of 0.6. The major contribution of this manuscript is to provide a detailed SC-MIC protocol, including bacteria sample preparation, treatments, SRS image measurement, data processing, and analysis. Representative results of this method are also provided.

Re: We appreciate the reviewer's positive comments.

Major Concerns:

Overall, the experimental information and the description are quite clear. My only concern is that most figures used in this manuscript are duplicated from the authors' previous article (ref 38), not sure if it is common in JoVE. If the above issue is acceptable, I think the manuscript can be considered as acceptable. Some minor comments are shown below.

Re: We appreciate the reviewer's comments. We have confirmed with the editor Dr. Vineeta Bajaj that we can reuse the figures from previous publications as long as reprint permission is obtained and the original publication is cited in the figure legend.

Minor Concerns:

1. Some experimental descriptions are not complete. For example, how to determine the relative intensity threshold of 0.6 is not described in this manuscript. The authors only add a citation of ref 38 without any explanation, which may be confused to the readers (line 196 on page 6).

Re: We appreciate the reviewer's comments. We added a paragraph on how to determine the threshold in part 7.1:
7.1 The cut-off value at 0.60 to determine the SC-MIC is established according to the statistical analysis of the SRS C-D intensities of the metabolism-active and metabolism-inhibited conditions for bacteria upon various concentrations of drug exposure.[40] The C-D intensities for the antibiotic-susceptible and antibiotic-resistant groups were fitted with normal distribution. Plot the receiver operating characteristic (ROC) curve and the cut-off threshold is evaluated at 0.60. Based on this cut-off value, the SC-MIC as an indicator of the efficacy of antibiotics can be defined to determine the metabolically inactive and metabolically active group.

2. Various spelling errors or repeated words appeared in the manuscript. (Ex. line 167 171 on page 5; "spliked" line 250 on page 7)
Re: We appreciate the reviewer's comments. We corrected the typos in the manuscript.

Reviewer #3:
Manuscript Summary:
The paper titled "Rapid Antimicrobial Susceptibility Testing by Stimulated Raman Scattering Imaging of Deuterium Incorporation in a Single Bacterium" presents excellent results based on the investigation of bacterial metabolic capacity when exposed to antibiotics, with bacterial response measured via the C-D vibrational band on Raman spectrum using Stimulated Raman scattering imaging. The authors demonstrate that bacteria which are susceptible to antibiotics can be identified within 2.5 h which is ideal for timely intervention of appropriate prescription of antibiotic drugs.
Re: We appreciate the reviewer's comments.

Major Concerns:
Figures of Raman spectra showing C-D and C-H bands and their intensity dynamics are needed to make the article clearer. Include more explanation on the dynamics of C-D intensities for antibiotic-susceptible bacteria; is there any time course or concentration level dependence demonstrated by the C-D band? Computing the C-D/C-H ratios between microbes exposed to D_2O/antibiotics and microbes in the control class (i.e., microbes that are not treated with antibiotics) would also help with elucidating the extent of D incorporation into biomass and level of antimicrobial susceptibility.
Re: We appreciate the reviewer's comments. We added a paragraph and a figure discussing effect of incubation time on deuterium incorporation measured by spontaneous Raman microspectroscopy as following.

The effect of incubation time on deuterium incorporation is measured by spontaneous Raman microspectroscopy at the C-D (2070 to 2250 cm^{-1}) and C-H (2,800 to 3,100 cm^{-1}) region (Figure 4a). The time-lapse single-cell Raman spectra of *P. aeruginosa* cultured in 70% D_2O containing medium show increasing CD/CH intensity over incubation time from 0 to 180 min. (Figure 4b) The increasing C-D abundance in single microbial cells reveals that D_2O is incorporated into deuterated biomolecules inside the cell.

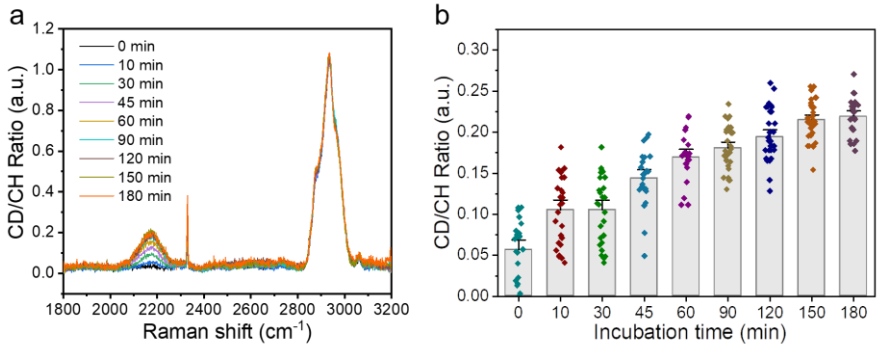


Figure 4. Effect of incubation time on deuterium incorporation into bacteria. (a) Time-lapse measurement at C-D (2070 to 2250 cm^{-1}) and C-H (2,800 to 3,100 cm^{-1}) region by spontaneous Raman microspectroscopy (averaged from 20 spectra). (b) Histogram plot of CD/CH intensity ratio plot over D_2O incubation time for bacteria in (a). Each colored point stands for a measurement from a single bacterium. Error bars represent the standard error of the mean (SEM).

Minor Concerns:
Page 3, line 71: "tob e" should be "to be"
Re: We appreciate the reviewer's comments. We corrected this typo.

Page 4, line 90: Delete "time" repeated word
Re: We appreciate the reviewer's comments. We corrected this typo.

The "Protocols section" is written in both passive and active voices; authors should choose one format and be consistent.
Re: We appreciate the reviewer's comments. We corrected all the sentences into active voice.

Page 4, line 105: It is not clear if the 2mL of normal MHB medium drawn from D_2O-labelled or H_2O-labelled MHB medium? Does "normal MHB" imply H_2O-labelled medium? Please make this clear.
Re: We appreciate the reviewer's comments. We modified this sentence as following:
"… add 2 mL of normal MHB media, which does not contain deuterium, …"

Page 4, line 120: Please mention specific antibiotics and concentration at this stage.
Re: We appreciate the reviewer's comments. We modified this sentence as following:
"Prepare two-fold serial dilutions of the test antibiotics, gentamicin or amoxicillin, using the aliquots of bacteria solution as a dilution medium. For both of gentamicin or amoxicillin, the serial concentrations cover from 0.25 to 8 μg/mL."

Page 4, line 123: What were the actual antibiotic concentrations in serially diluted samples?
Re: We appreciate the reviewer's comments. We clarified the actual antibiotic concentrations in parts 2.4 and 2.7:
"…For both of gentamicin and amoxicillin, the serial concentrations cover from 0.25 to 8 μg/mL."

Page 4, line 125-129: Please include specific shaking and centrifugation parameters.
Re: We appreciate the reviewer's comments. We added specific shaking and centrifugation parameters as following:
2.8 "…and then incubate at 37 °C in a shaker at 200 revolutions per minute (rpm)…"
2.9 "First centrifuge the 1 mL of antibiotic and D_2O-treated bactria sample at 8000 rpm for 5 min at 4 °C, …"

Page 5, line 169: What was the step size of the SRS mapping?
Re: We appreciate the reviewer's comments. We added the step size information as following:
5.5 "Set the step size for 150 nm, ..."

Page 7, line 230: Bacteria were grown in 70% D_2O-labelled MHB. D_2O labelling above 50% affects bacterial metabolism significantly (e.g., Berry et al., Proc. Natl. Acad. Sci. USA, 2015, 112, E194). So, did you observed any 70% D_2O-mediated metabolic response in microbes, in addition to the influence of antibiotics? Any justification as to why such a high concentration of D_2O was used? Would the results be different if say, 30-40% D_2O was used instead?
Re: We appreciate the reviewer's comments. We note that D2O labelling above 50% affects bacterial metabolism significantly was observed during a 23-hour incubation period in this paper (Berry et al., Proc. Natl. Acad. Sci. USA, 2015, 112, E194). We observed bacterial growth inhibition when D_2O

labelling concentration is above 70% during a 25-hour incubation period (Figure S1). Our current results show that 70% D_2O does not affect the performance of our method in *P. aeruginosa*, because we have performed MIC by gold standard broth dilution and obtained SC-MIC results in two *P. aeruginosa* strains (Table S1). The category agreement of our method (SC-MIC) with the conventional culture-based method is 100% for all the tested *P. aeruginosa* and antibiotic combinations, as shown in the following Table. We attribute this good agreement to the minimal toxicity of 70% D_2O on *P. aeruginosa* during the 30 min incubation period in SC-MIC determination.

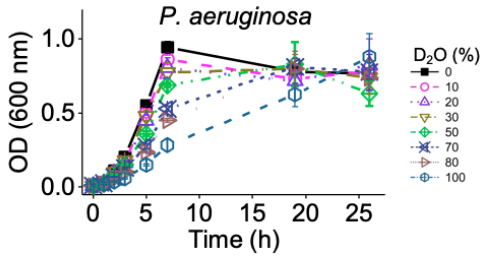


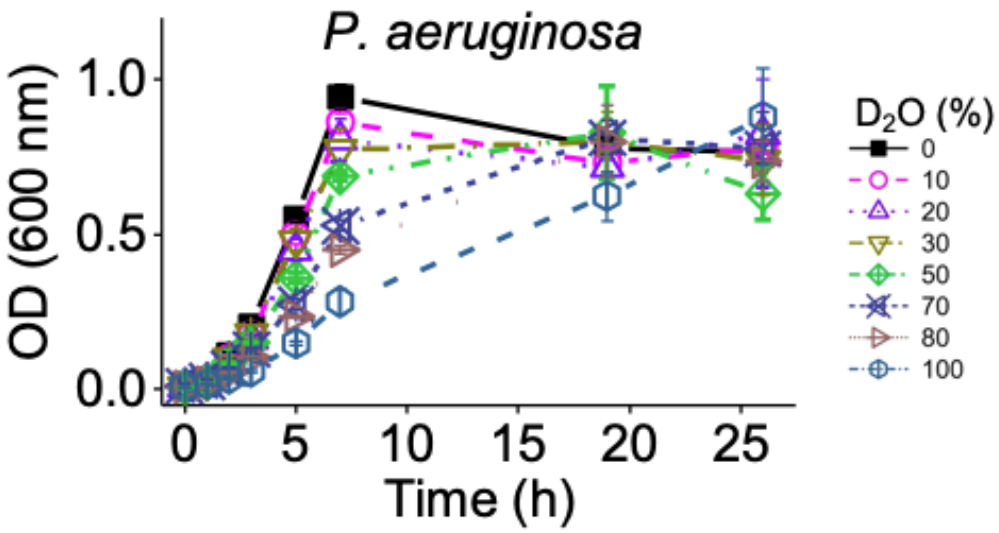
Figure S1. Testing D_2O toxicity to *P. aeruginosa* cultured in LB medium with different D_2O concentrations. Error bars indicate standard deviation values (number of measurements = 5).

Table. Comparison of SC-MICs and MICs in normal MHB of *P. aeruginosa* upon antibiotics. S: sensitive; R: resistant; I: Intermediate.

Bacteria strains	**Antibiotics**	**SC-MIC (μg/ml)**	**MIC in normal MHB (μg/ml)**	**MIC/SCMIC category**
P. aeruginosa ATCC 47085 (PAO1)	Cefotaxime	32	16	I/I
	Colistin	0.5	2	S/S
	Gentamicin	2	4	S/S
	Amikacin	≤4	2	S/S
	Tobramycin	≤1	0.5	S/S
	Ciprofloxacin	≤0.25	0.125	S/S
P. aeruginosa ATCC 1133	Colistin	8	≥16	R/R

Page 14, line 353, Table 1 row number 2: Would "centrifuge for longer time" advice not damage or lyse bacterial cells?

Re: We appreciate the reviewer's comments. According to the protocol listed in the paper [Anal Bioanal Chem (2017) 409:3043], the high-speed centrifugation at 10,000 rpm for 20 min can be applied to the bacterial cells. In our protocol, we usually centrifuge less than 10 min for the fixed bacterial cells.



Bacteria strains	Antibiotics
P. aeruginosa ATCC 47085 (PAO1)	Cefotaxime
	Colistin
	Gentamicin
	Amikacin
	Tobramycin
	Ciprofloxacin
P. aeruginosa ATCC 1133	Colistin

SC-MIC (μg/ml)	MIC in normal MHB (μg/ml)	MIC/SCMIC category
32	16	I/I
0.5	2	S/S
2	4	S/S
≤4	2	S/S
≤1	0.5	S/S
≤0.25	0.125	S/S
8	≥16	R/R