Supplementary

Hua Tian[1], David A. Six[2], Thomas Krucker[2], Jennifer A. Leeds[2], Nicholas Winograd[1]

1 Department of Chemistry, Pennsylvania State University, University Park, PA 16802, USA

2 Novartis Institutes for BioMedical Research, Inc., 5300 Chiron Way, Emeryville, CA 94608-2916, USA

Table of Contents

Section 1 Protocol of ToF-SIMS characterization of ampicillin and tetracycline S-2

Section 2 Detection limit of antibiotics standards using ToF SIMS S-4

Section 3 Stability study of antibiotics during C_{60}^+ depth profiling..... S-9

Section 4 Depth resolution with different C_{60}^+ ion dose S-10

Section 5 Biomarker detection from *E. coli* S-11

Section 6 Software development for the statistical study S-14

Section 7 Time course study of AMP and TET response in *E. coli* wild-type strain using cryo-SEM S-15

Section 8 Comparison the average antibiotics signal levels in *E. coli* aggregates and bare Si surface around *E. coli* S-18

Section 9 Comparison the average antibiotics signal levels in single *E. coli* cells and bare Si surface around *E. coli* S-19

Section 1 Protocol of ToF-SIMS characterization of ampicillin and tetracycline

E. coli lysate preparation

The *E. coli* membrane-free lysate was generated from *E. coli* C41(DE3) (Lucigen, Middleton, WI) containing the empty vector pET28a(+) (EMD Millipore, Billerica, MA). The strain was inoculated into 1 L of Terrific Broth medium supplemented with 20 mM sodium phosphate buffer pH 7.4, and incubated at 37 °C in a shake flask at 250 rpm. The cells were harvested by centrifugation when the culture reached OD_{600} of ~3.0. The cell pellet was re-suspended with ~20 mL of 50 mM tris(hydroxymethyl)aminomethane (Tris), pH 7.4 to wash, centrifuged, and the final pellet was re-suspended in 20 mL of 50 mM Tris, pH 7.4. The cells were lysed with a French pressure cell and centrifuged at 100,000 × g for 1 h to remove membranes and cell debris. The resulting supernatant (membrane-free lysate) was harvested, and 1-mL aliquots were placed in 1.5-mL cryotubes and frozen. The protein concentration was determined by the bicinchoninic acid (BCA) assay method to be ~25 mg/mL using bovine serum albumin as the standard.

Sample preparation of antibiotics in *E. coli* lysate

AMP and TET (BioReagent grade, Sigma, Milwaukee, WI) were initially prepared and analyzed as aqueous trehalose stock solutions. For these tests, trehalose was used to maintain the volume of the thin film during and after dehydration and SIMS analysis under vacuum. These stocks were evaluated to select characteristic SIMS ion peaks and then a dose-response study was performed with C_{60}^+-SIMS to determine the limits of detection.

To evaluate whether any common bacterially-derived molecules could affect the presence or intensity of the characteristic ions, stock solutions of AMP and TET (10 mg/mL) were prepared by dissolving the solid antibiotic into 0.2 M trehalose and the *E. coli* lysate (1:1 v/v), and then diluting to 1000 100, 10, 7.5, 5, 2.5, 1, 0.1 and 0.01 μg/mL with 0.1 M trehalose and *E. coli* lysate (1:1 v/v). 10-μL aliquots of the antibiotic solutions were spin-coated onto a pre-cleaned silicon substrate to form a thin film, which was subject to dehydration under vacuum in preparation for SIMS analysis.[1] The *E. coli* lysate provided a more representative matrix for signature ion detection. It should be noted that the membrane-free lysate lacks the penicillin-binding proteins, the primary targets of AMP.

SIMS measurement

The J105 3D Chemical Imager (Ionoptika, UK) was used for all SIMS measurements. This instrument utilizes a shaped-field buncher to create timed secondary ion pulses for time-of-flight (TOF) mass spectrometry. This configuration allows direct current (DC) operation of the primary ion beam,

offering the fast acquisition and high mass resolution without compromising the high spatial resolution of the image. Details of this instrumentation have been described.[2,3] To determine the effective SIMS sensitivity for the two target antibiotics, six parallel analyses were performed on each sample at a primary ion fluence of 2.5×10^{12} ions/cm^2 using the C_{60}^+ primary ion beam. The C_{60}^+ ions were generated at 40 keV using the IOG C60-40 source provided by Ionoptika.[4] The source operated at a beam current of ~0.5 pA with a spot size of 300 nm. Spectra were acquired by rastering the beam over an area of 100 μm × 100 μm. Because of the high count rate associated with the most intense peaks, data are recorded in analog mode using a Fast Flight 2 (Ametek Technologies, Berwyn, PA) digitizing device. The signal intensity reported using the digitizer is proportional to the number of secondary ions hitting the detector. Under the conditions described here, no evidence for sample charging is observed. The intensities of characteristic peaks for each antibiotic from mass window of Δm 0.2 were plotted as a function of the concentration. From the plot, the linear detection range and the detection limit were determined. The signal-to-noise ratio has been characterized around 1000 to 6000 at mass range of *m/z* 100 to 600,[5] validating the detection of the antibiotic ions at different concentrations.

Reference

(1) Cheng, J.; Winograd, N. *Anal. Chem.* **2005**, *77*, 3651-3659.
(2) Fletcher, J. S.; Rabbani, S.; Henderson, A.; Blenkinsopp, P.; Thompson, S. P.; Lockyer, N. P.; Vickerman, J. C. *Anal. Chem.* **2008**, *80*, 9058-9064.
(3) Hill, R.; Blenkinsopp, P.; Thompson, S.; Vickerman, J.; Fletcher, J. S. *Surf. Interface Anal.* **2011**, *43*, 506-509.
(4) Weibel, D.; Wong, S.; Lockyer, N.; Blenkinsopp, P.; Hill, R.; Vickerman, J. C. *Anal. Chem.* **2003**, *75*, 1754-1764.
(5) Sadia, R. *Advances inTime-of-Flight Secondary Ion Mass Spectrometryfor the Analysis of Single Cells on Sub-Cellular Scale* Doctoral level ETD - final, The University of Manchester, Manchester, UK, 2010.

Section 2 Detection limit of antibiotics standards using ToF SIMS

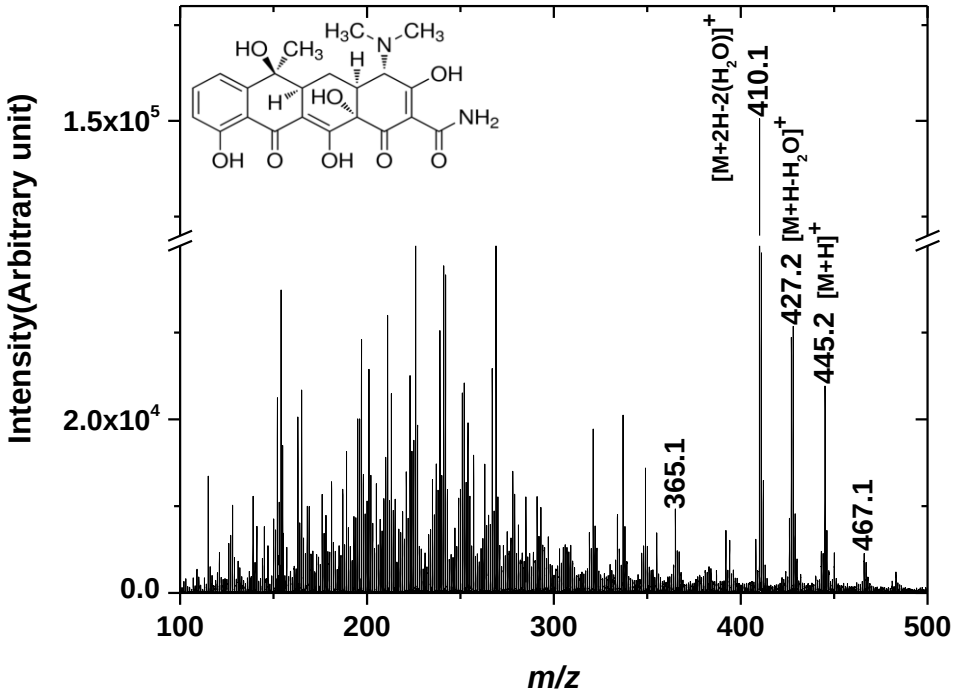


Fig. S 1 Positive SIMS spectrum of TET (10 mg/mL) in *E. coli* lysate and trehalose. The characteristic ions of TET can be detected as $[M+H]^+$ at *m/z* 445.2 and fragment ions at *m/z* 410.1 and 427.2. Sodiated TET $[M+Na]^+$ at *m/z* 467.1 was present with low intensity. A trehalose ion was present in the mixture as $[M+Na]^+$ at *m/z* 365.1.

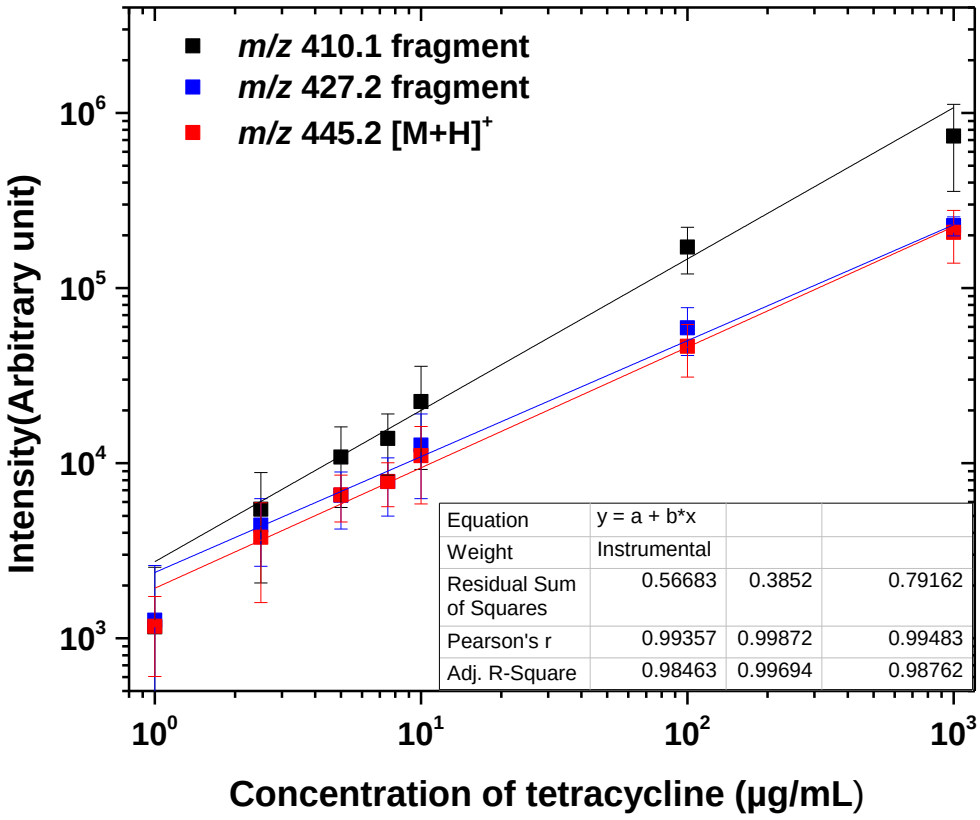


Fig. S 2 Intensity of the signal from TET (molecular ion or two fragment ions) at concentrations from 1 to 1000 μg/mL in *E. coli* lysate and trehalose. The detection limit is 1 μg/mL. Data were plotted as average ± standard deviation from six independent replicates.

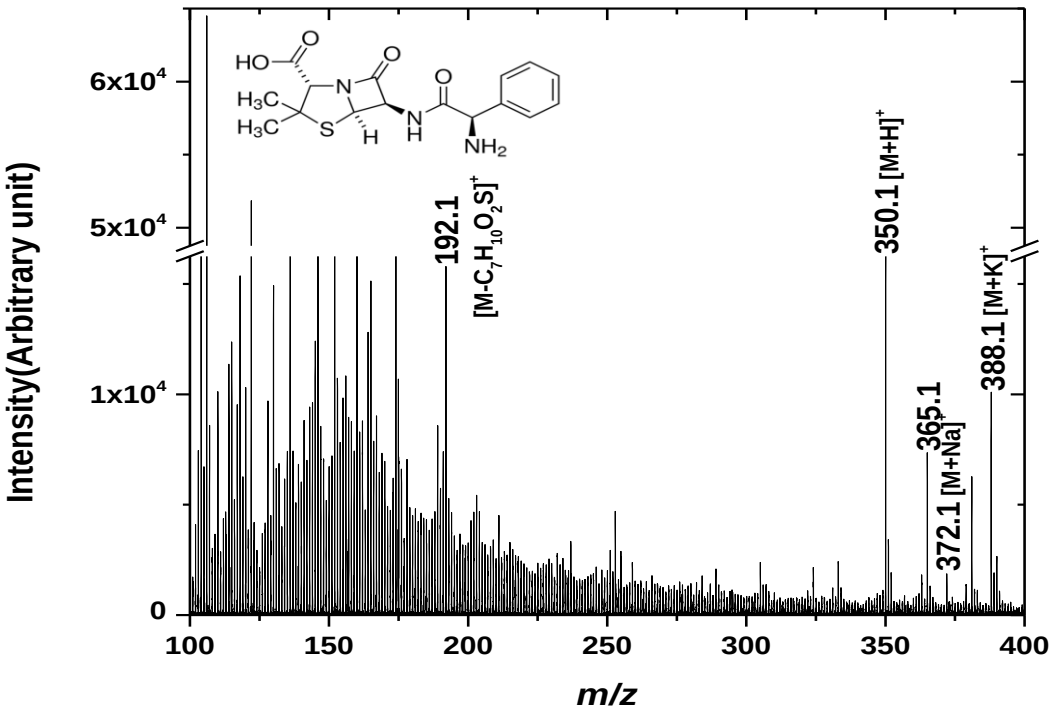


Fig. S 3 Positive SIMS spectrum of AMP (10 mg/mL) in *E. coli* lysate and trehalose. The characteristic ions of AMP can be detected for $[M+H]^+$ at *m/z* 350.1, $[M+Na]^+$ at *m/z* 372.1, $[M+K]^+$ at *m/z* 388.1 and a fragment ion at *m/z* 192.1. A trehalose ion was present in the mixture as $[M+Na]^+$ at *m/z* 365.1.

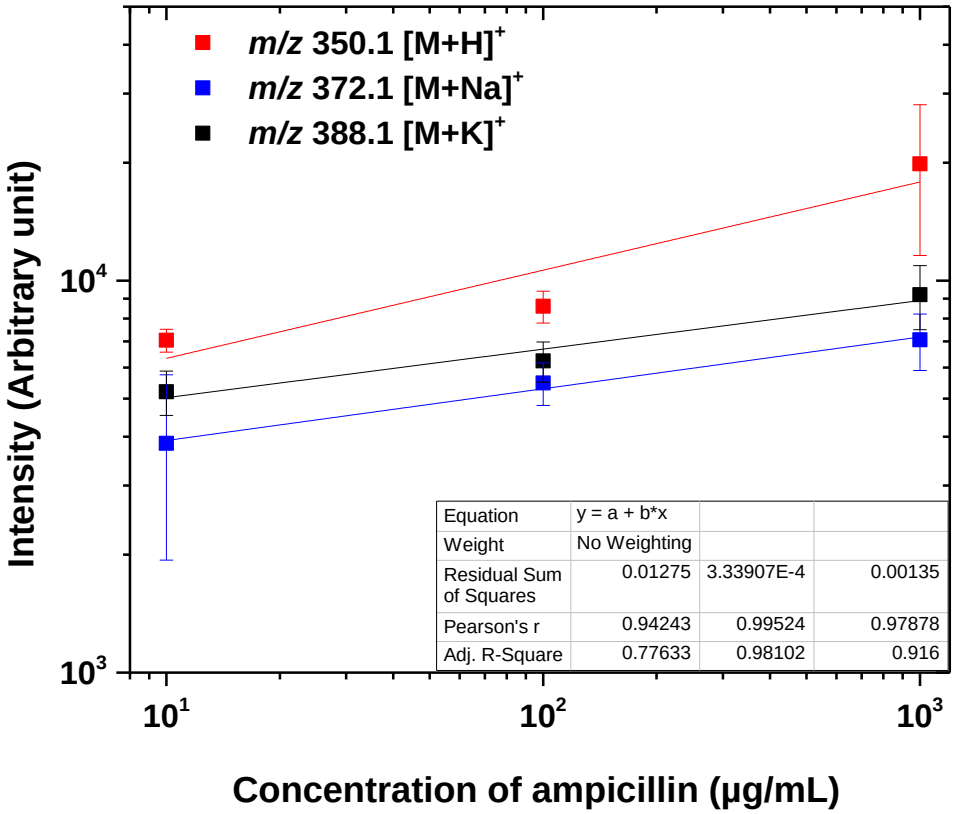


Fig. S 4 Intensity of the signal from AMP (molecular ion or adduct ions) at concentrations from 10 to 1000 µg/mL in *E. coli* lysate and trehalose. The detection limit is 10 µg/mL. Data were plotted as average ± standard deviation from six independent replicates.

Table S 1 Detection limit of selected antibiotics for SIMS in *E. coli* lysate

Antibiotic	MW (g/mol)	Diagnostic SIMS peaks (*m/z*)	ToF-SIMS Limit of Detection (in *E. coli* lysate)	Linear detection range (μg/mL)
Tetracycline	444.2	445.2, 427.2, 410.1	1 μg/mL for all the diagnostic peaks	1 to 1000
Ampicillin	349.1	372.1, 350.1, 192.1	100 ng/mL for *m/z* 350.1	10 to 1000
Ciprofloxacin	331.1	332.1, 314.1, 231.0	1 μg/mL for *m/z* 231.0	10 to 1000
Kanamycin	484.2	523.2, 507.2, 485.2, 324.2, 205.1	100 ng/mL for *m/z* 507.2 and 523.2.	0.1 to 1000
Doxycycline	444.2	445.3, 428.2, 410.2	5 μg/mL for all the diagnostic peaks	1 to 1000
Erythromycin	733.5	558.5, 576.5, 592.5, 720.6, 734.6, 756.6, 772.6	10 μg/mL for *m/z* 576.5, 592.5 and 772.6	10 to 1000
Colistin	1154.7	1156.4, 1178.0	NA	NA

Note: Please refer to Supplementary section 1 for the sample preparation for ToF-SIMS detection limit of all the antibiotics. The protocols for ciprofloxacin, kanamycin, doxycycline, erythromycin and colistin are as same as tetracycline and ampicillin.

Section 3 Stability study of antibiotics during C_{60}^+ depth profiling

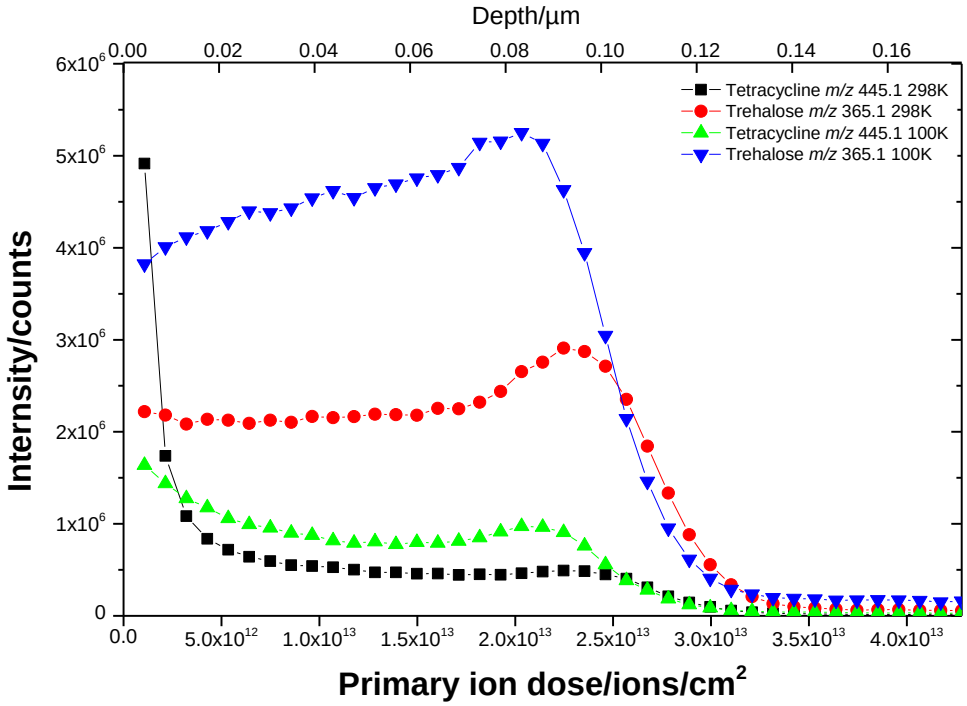


Fig. S 5. C_{60}^+-depth profiling of TET doped into a trehalose thin film at 100 K and 298 K. Each point is acquired using a beam of 30 pA, 10 μm spot size, 50% duty cycle, 32×32 pixels, 300×300 μm^2, and a dwell time of 20 ms, resulting in a primary ion dose 1×10^{12} ions/cm^2 per layer. The molecular ion of TET is monitored at *m/z* 445.1 and the sodiated molecular ion of trehalose is monitored at *m/z* 365.1.

Section 4 Depth resolution with different C_{60}^+ ion dose

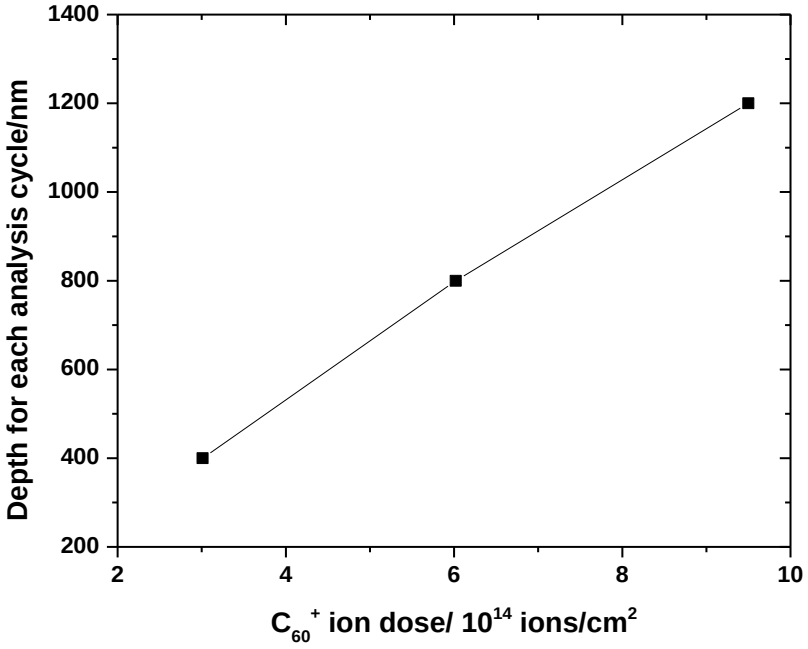
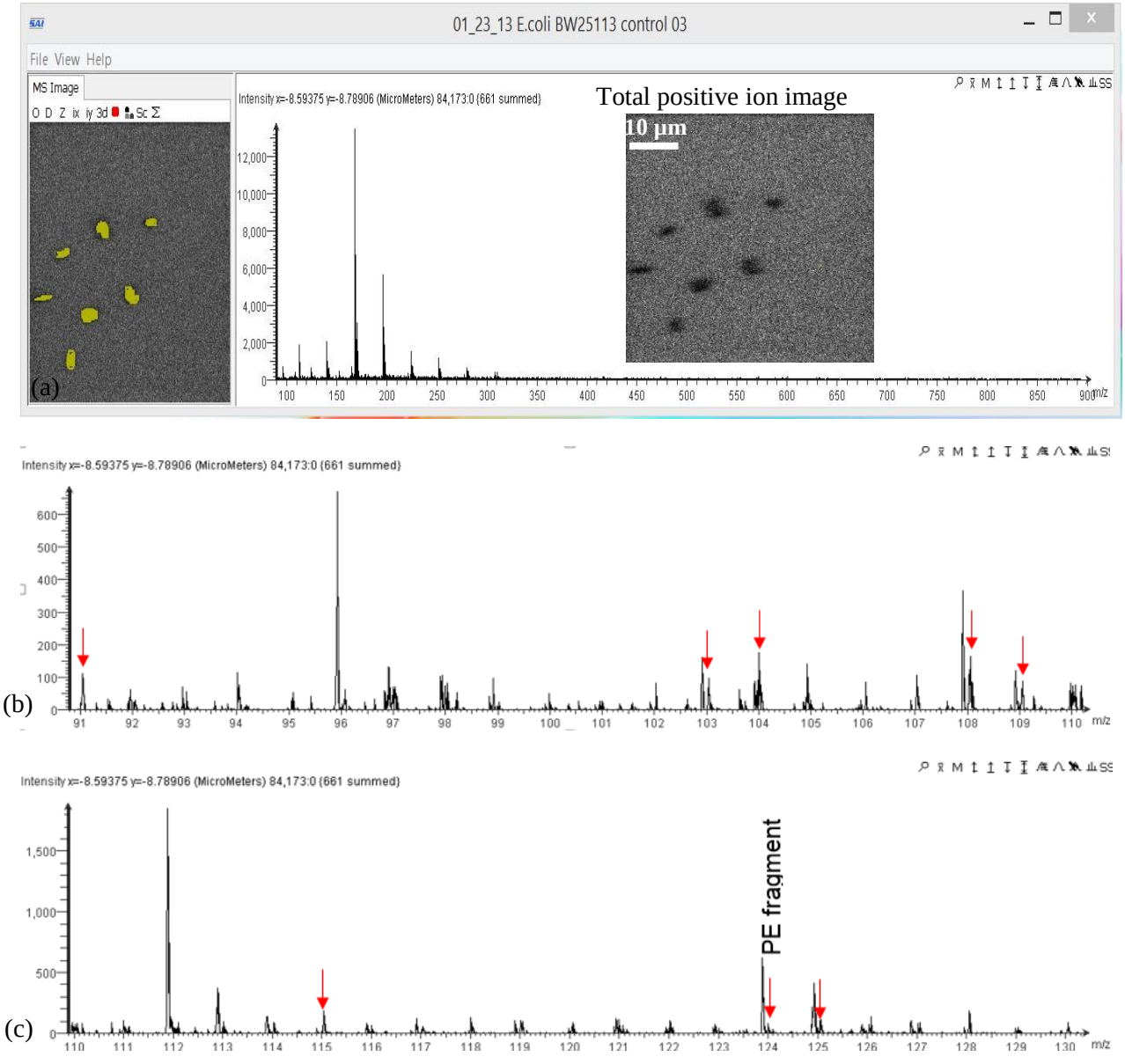


Fig. S 6 Depth for each analysis cycle at different C_{60}^+ ion dose. The different C_{60}^+ ion doses allow for eroding different depths of *E. coli* cultured on the Si substrate, such that the probing depth for each analysis cycle can be tuned. At a C_{60}^+ ion dose of approximately 10^{14} ions/cm^2, the depth of the C_{60}^+ probe is around 50 nm, which would allow depth profiling in *E. coli*.

Section 5 Biomarker detection from *E. coli*

Peaks were detected from an *E. coli* aggregate to identify biomarkers. Putative assignments of the identified peaks include potential diacylglycerol ions at *m/z* 534.5 and *m/z* 598.6 and potential phosphatidylethanolamine (PE) ions at *m/z* 647.6, 675.6 and 718.7. However, these ions were in the higher mass region and usually were observed at low intensity. Further investigation was carried out to detect biological markers in the low mass region from a monolayer of *E. coli* directly cultured on the silicon wafer. As shown in Fig. S 7 (a), the black dots in the total positive ion image are bacteria. Summing the pixels from the bacterial area shows the biological signals as red arrows in Fig. S 7(b-f). The lipid head group fragments from abundant PE and phosphatidylglycerol (PG) in the *E. coli* membranes are also detected at *m/z* 124.1, 142.1 and 195.1, even at low intensity. These low intensity biological signals can be summed to map the *E. coli* as shown in Fig. S 8. The summed biological signal image and Si ion image have an orthogonal distribution.



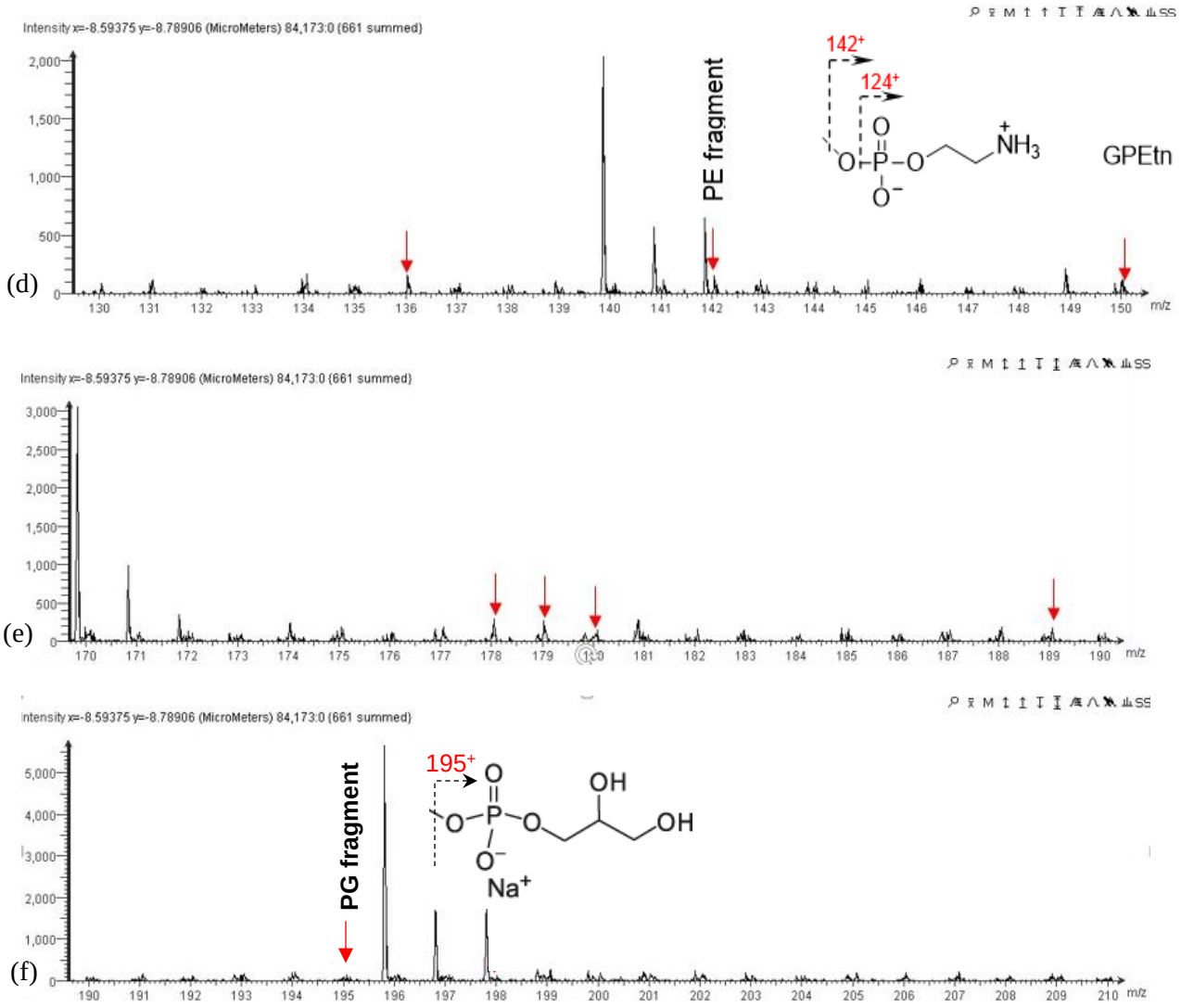


Fig. S 7 Biomarker peaks for *E. coli* in the low mass region of the SIMS spectra.

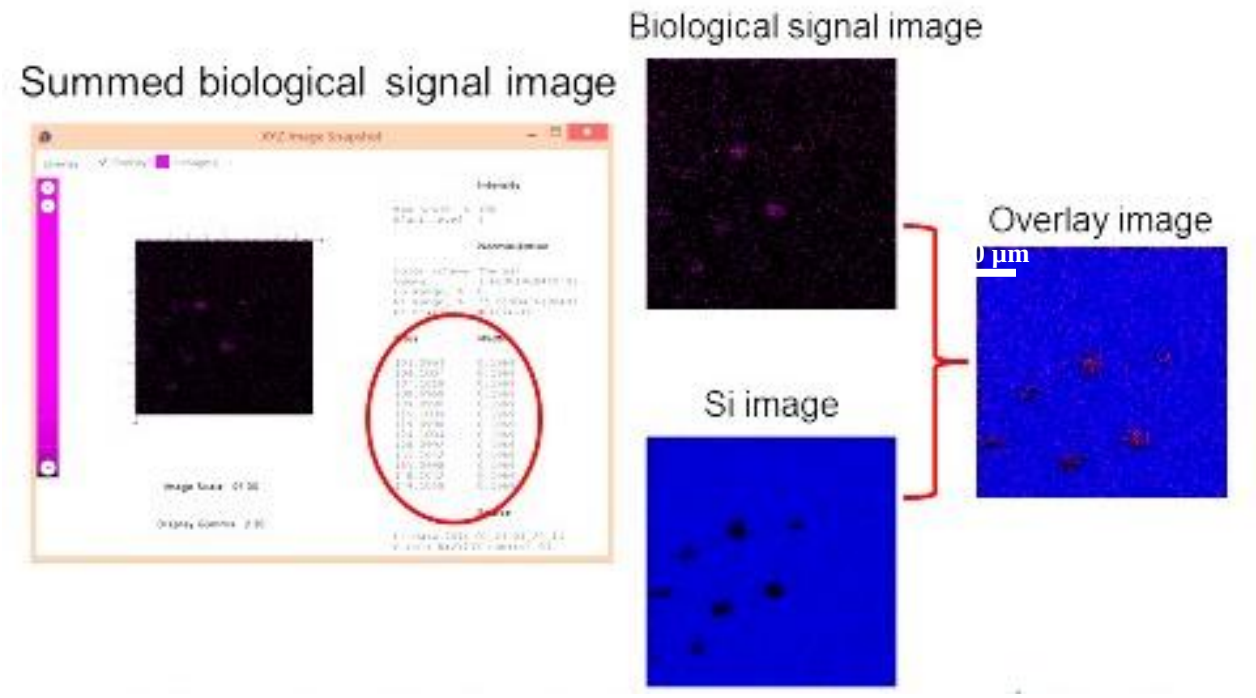


Fig. S 8 Mapping single *E. coli* using low mass biological signals. *E. coli* was directly cultured on the Si substrate overnight.

To enhance the biological signal from *E. coli*, a more confluent bacterial sample was prepared by increasing the culture time from 12 h to 48 h. The culture medium was refreshed every 8 h. The SIMS images in Fig. S 9 show a more confluent *E. coli* sample mapped using biological signals, which are orthogonal to the Si signal.

The results demonstrate that either the biological signal or the absence of Si signal can be used to locate the bacteria. Because the Si signal is more intense than biological signal, it is more convenient to use the absence of a single Si peak at *m/z* 167.9 to map *E. coli*. Where the Si is absent, the bacteria are located. The Si signal image can map the sharp outline of the bacteria.

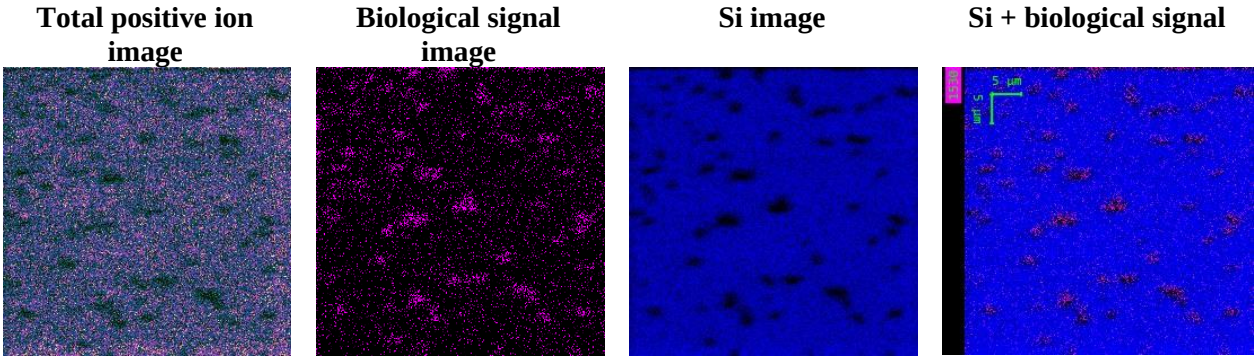


Fig. S 9 *E. coli* was directly cultured on the Si substrate for 48 h. The images allow mapping single *E. coli* cells using low mass biological signals (red, *m/z* 124.1, 142.1 and 198.1) and the Si peak (blue, *m/z* 167.9) against a black background.

Section 6 Software development for the statistical study

ImagingSIMS Version 3.6 (developed by Jay Tarolli, Penn State University) was used to calculate the weighted standard deviation within single bacteria and small cluster cells to replace manual calculation of standard deviation.

The procedure included the following: converting the J105 data to software readable format, generating single ion images of Si and dosed antibiotics, recognizing the valid cellular region, reading the signal counts of cellular region and calculating the standard deviation.

The software interfaces are user friendly as in Fig. S 10. There are a few features worth mentioning. The non-valid cell regions can be removed by deleting the cursors of the cell region. The separated cell cluster areas circled in red in Fig. S 6 can be merged together if those clusters are visually connected and can be considered as one cluster.

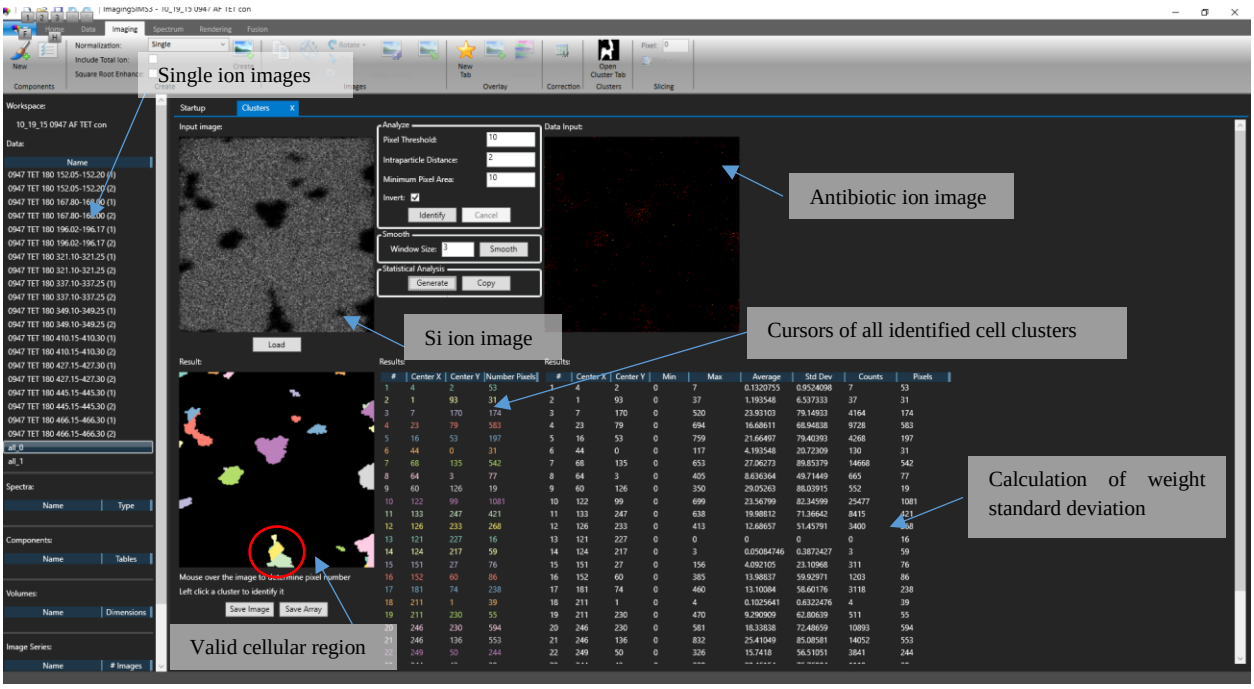
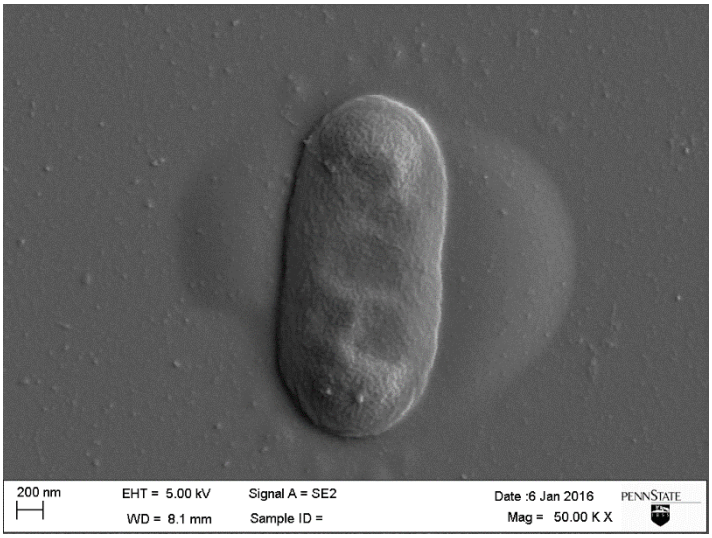


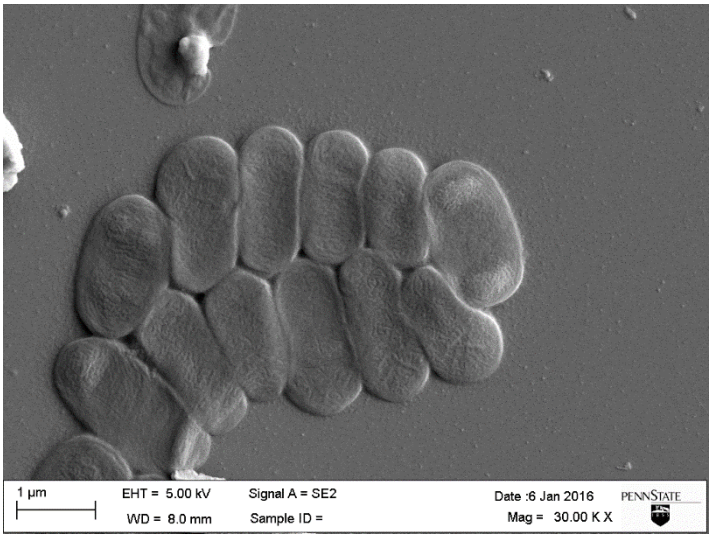
Fig. S 10 ImagingSIMS interface for statistical calculation.

Section 7 Time course study of AMP and TET response in *E. coli* wild-type strain using cryo-SEM

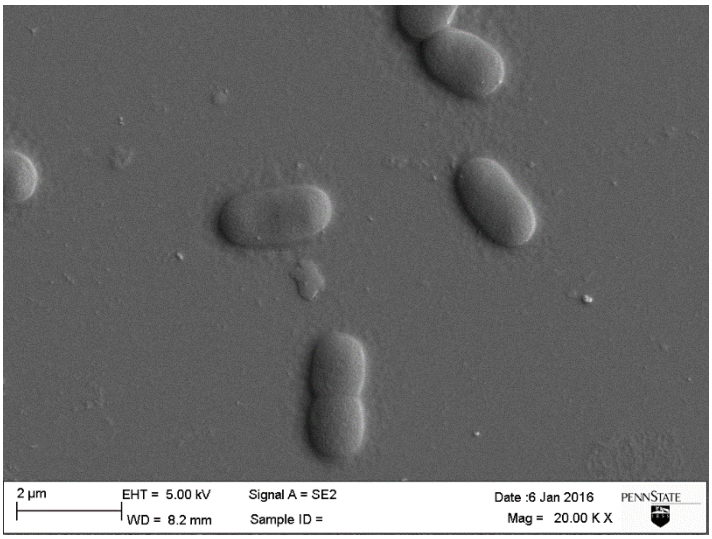
AMP Control



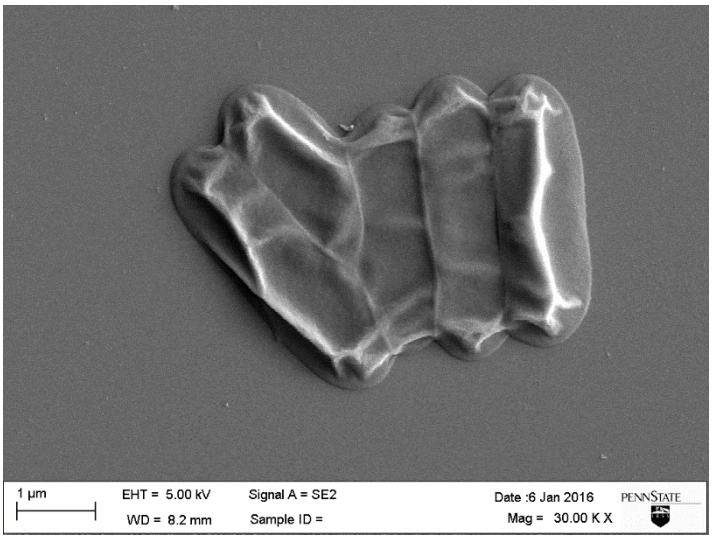
AMP 20μg/mL 1min



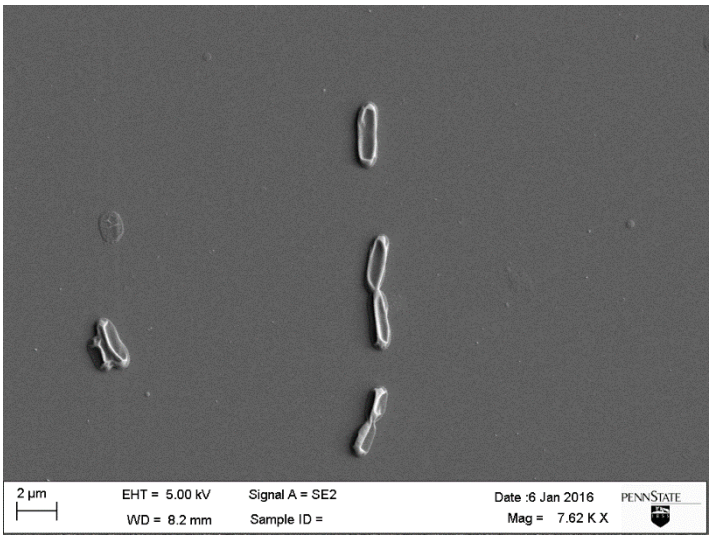
AMP 20μg/mL 5min



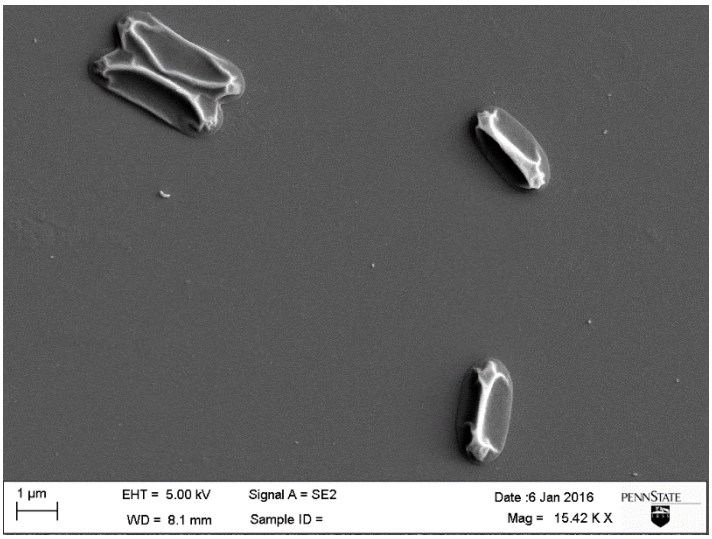
AMP 20μg/mL 20min



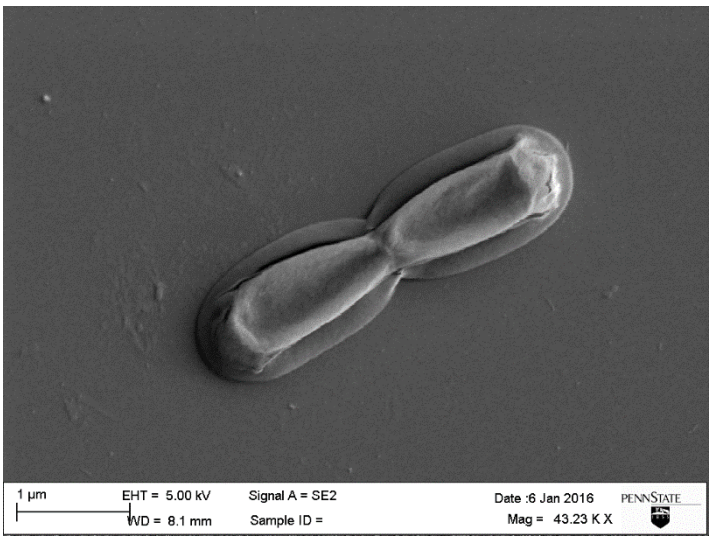
AMP 20μg/mL 40min



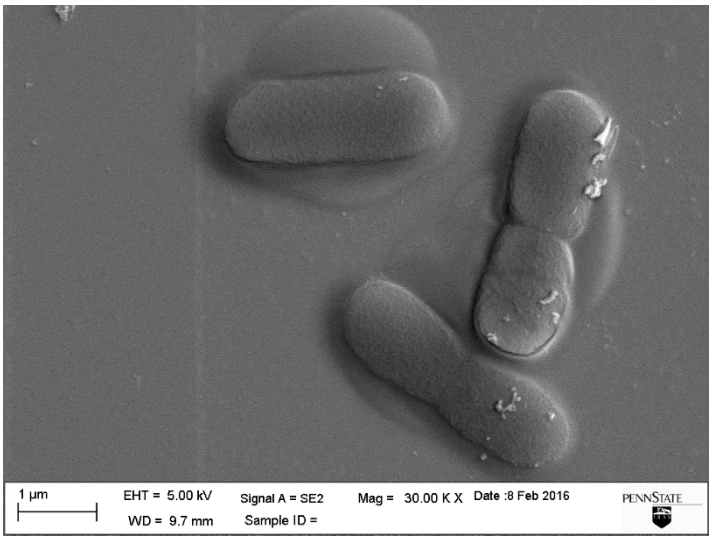
AMP 20μg/mL 60min



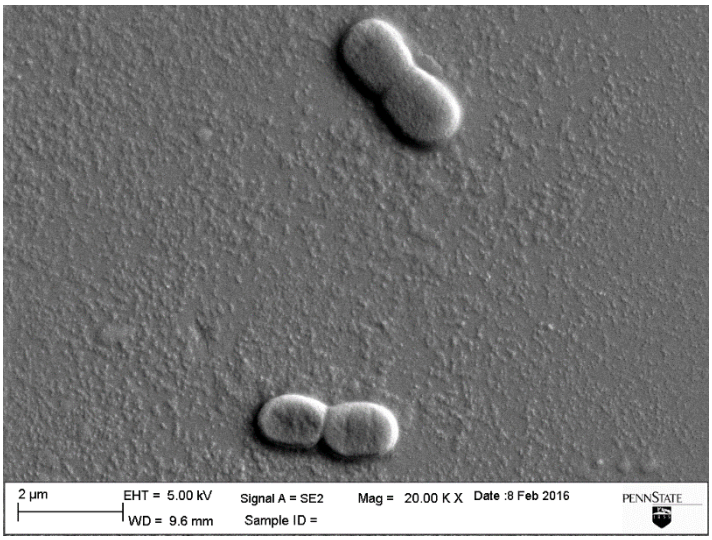
AMP 180μg/mL 1min



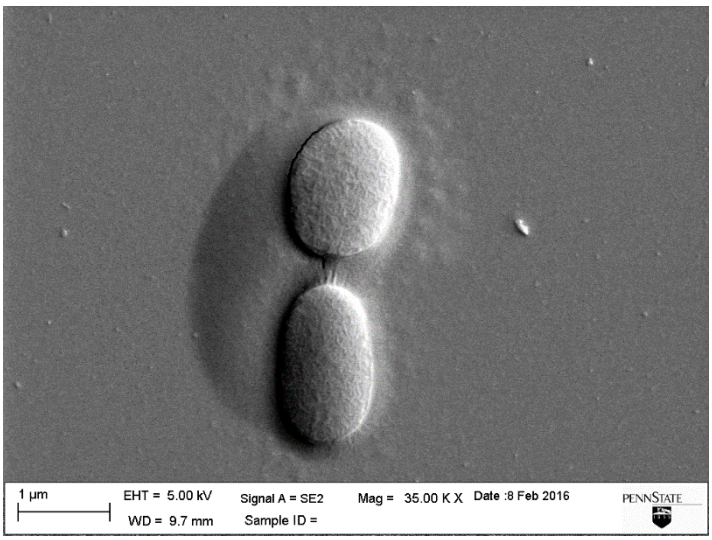
TET Control



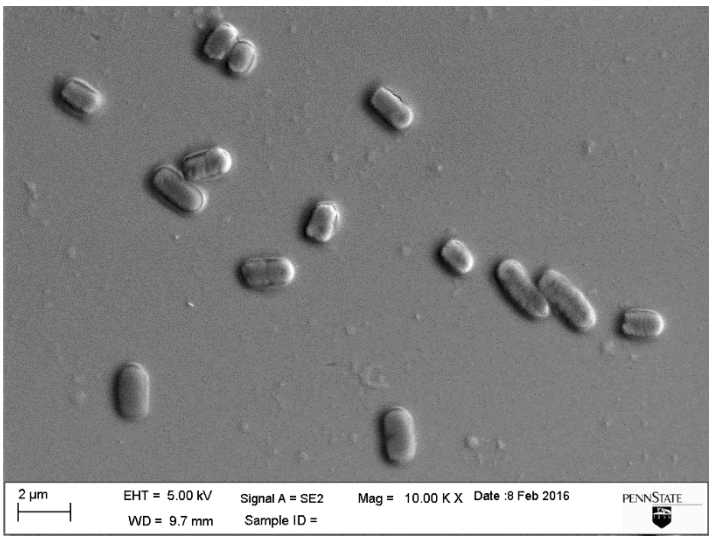
TET 20μg/mL 1min



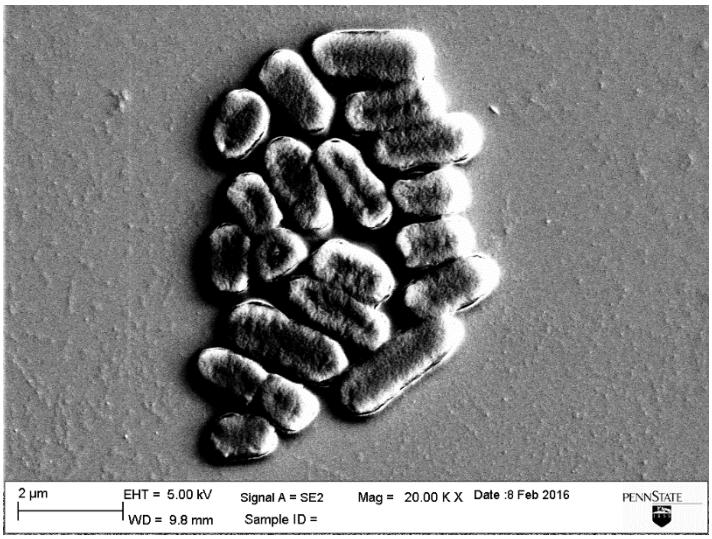
TET 20μg/mL 5min



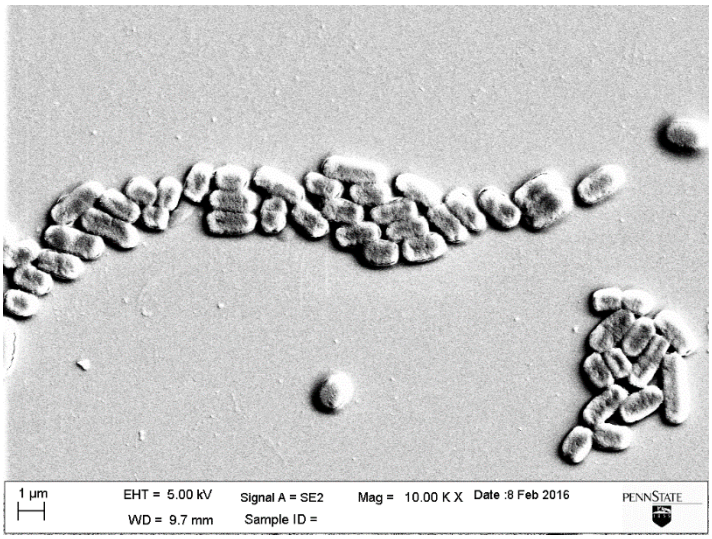
TET 20μg/mL 20min



TET 20μg/mL 40min



TET 20μg/mL 60min



TET 180μg/mL 1min

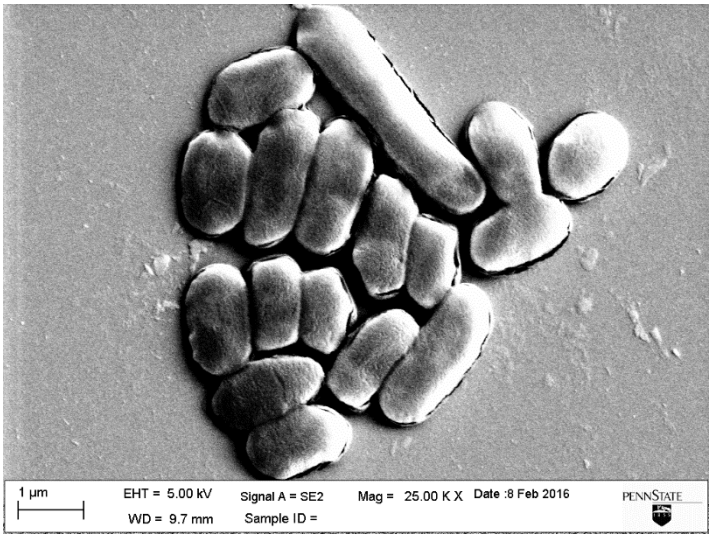


Fig. S 11 Time course study of AMP and TET response in *E. coli* wild-type strain using cryo-SEM.

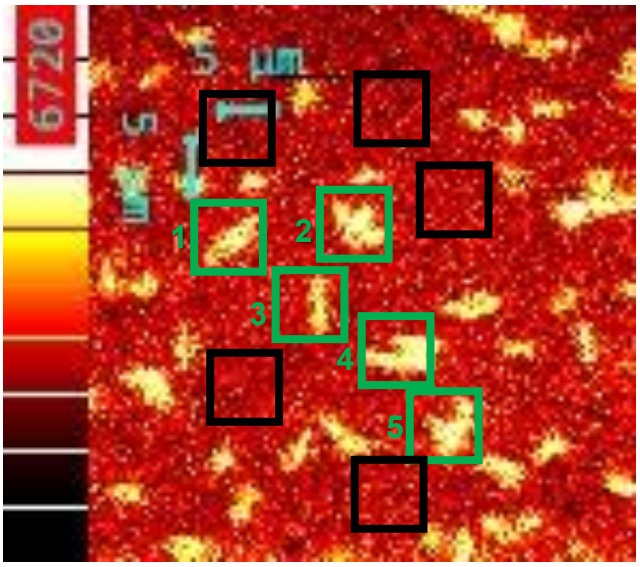
Section 8 Comparison the average antibiotics signal levels in *E. coli* aggregates and bare Si surface around *E. coli*

Table S 2 Comparison the average TET and AMP (dosed at 20 μg/mL respectively) signal levels in *E. coli* aggregates and bare Si surface around *E. coli*.

Region	Average TET signal (counts/pixel)	Average AMP signal (counts/pixel)
***E. coli* cluster area**	**6.8**	**7.0**
Si surface outside *E. coli* cluster	**0.5**	**0.4**

Section 9 Comparison the average antibiotics signal levels in single *E. coli* cells and bare Si surface around *E. coli*

To rule out the interference of noise in the detection of an antibiotic, signal counting is utilized to compare the background signal with the antibiotic signal. As shown in Fig. S 12, five areas with *E. coli* cells (green boxes) and five areas without *E. coli* cells (black boxes) were selected to calculate the signal counts from AMP, represented by the molecular ion at *m/z* 350.1 and the fragment ion at *m/z* 192.1. As shown in the right panel, the molecular ion at *m/z* 350.1 was detected from Areas 1-5, the average 3 ± 1 counts/pixel from the green boxes (*E. coli* regions) are detected compared to 0 counts/pixel in the black boxes (Si only regions). The AMP fragment ion at *m/z* 192.1 showed a more intense signal level in the green boxes, average 14 ± 6 counts/pixel compared to 1±0.4 counts/pixel in the black boxes. This data analysis is an underestimate of the levels of AMP because each green box contains pixels devoid of bacterial signal. Nevertheless, this statistical analysis suggests that AMP was detectable at the single cell level.



Area	AMP ion peak			
	m/z 350.1		*m/z* 192.1	
	Signal counts	Counts /pixel	Signal counts	Counts /pixel
1	**188**	4	**1102**	21
2	**227**	3	**1071**	15
3	**69**	2	**164**	5
4	**304**	3	**1517**	17
5	**137**	2	**707**	12
Ave. ± Std. Dev.		3±1		14±6
Black boxes	**0**	0	**115**	1±0.4

Fig. S 12 Data analysis of AMP-treated (20 µg/mL) *E. coli*. Left panel: Total positive ion image of treated *E. coli*. The yellow patches are *E. coli* cells, red area is Si surface. The five green boxes with *E. coli* cells and five black boxes devoid of *E. coli* cells were selected to calculate the signal counts from AMP, represented by the molecular ion at *m/z* 350.1 and a fragment ion at *m/z* 192.1. As show in the right panel, the AMP molecular ion at *m/z* 350.1 was detected from the green boxes, but not from the black boxes. The AMP fragment ion at *m/z* 192.1 showed more intense signal levels in the green boxes than the molecular ion.