Supporting Information

Estimating Bacterial Concentration in Fibrous Substrates through a Combination of Scanning Electron Microscopy and ImageJ

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Experimental Details:

Bacterial cultures:

The following designated ATCC bacterial cultures were grown overnight in 10 mL of Heart Infusion Broth (Becton Dickson, purchased through Fisher) following the standard procedure at the temperatures described. *Escherichia coli* O157 (ATCC 43894), *Enterococcus faecalis* (ATCC 19433), *Staphylococcus aureus* (ATCC 25923) and *Bacillus anthracis* (avirulent Sterne strain) were grown overnight at 37 ^{o}C. *Listeria innocua* (ATCC 33090) was grown overnight at 30 ^{o}C. The bacterial suspensions, subsequently obtained from the overnight growth of the bacteria, were purified (*i.e*., precipitated and re-suspended) with PBS through centrifugation three times, each at ~1,800g for 7 min. The purification allowed us to achieve the desired cell concentration in the suspension, as well as to obtain bacterial cultures containing bacteria with more consistent shape and size.

Sample preparation for Adherent Bacteria:

Commercially available polymer (*e.g*., olefins: polypropylene, nylon) fibers both as spun bound and woven mats were used as the model for 3D fibrous substrates (0.5 and 20 μm diameter fibrous substrates were gifts obtained from Dr. Grondin of Avintiv Polymers; 50 and 250 μm diameter fibrous substrates were purchased from Spectra Mesh Woven Filters through Fisher Scientific). The substrates were modified or functionalized for either specific or nonspecific binding. Since we did not observe much difference in morphology or orientation between the two binding processes. Most of the estimation process was carried out where bacteria were bound through non-specific binding. The polymer substrates were first thoroughly cleaned with deionized (DI) water (Millipore) and ethanol, then air-dried. For nonspecific binding, the substrates were emerged in 0.01% polylysine solution (Sigma-Aldrich) for ~2 hrs and then dried (in an enclosed chamber to avoid any dust or other airborne contamination). Afterwards, bacterial suspensions (of measured volume and calculated concentration through Flow Cytometry) were exposed to polylysine coated substrates and incubated at 4 ^{0}C for ~12 hrs to ensure complete interactions. The positively charged polylysine coating helped bacteria to bind to the substrate nonspecifically through electrostatic interaction. For specific binding, the polymer substrates were modified to add –OH functionality, which was subsequently modified further to attach antibodies specific to the pathogen[1]. In this case, 2 hrs of exposure were enough to complete the binding process. Once the substrate was exposed to the bacteria, they were then incubated in PBS with 4% glutaraldehyde solution (Sigma-Aldrich) for ~5 min to inactivate the pathogen. Subsequently, the samples were then lightly rinsed with PBS and finally preserved in PBS containing 1% paraformaldehyde (Sigma-Aldrich) at 4 °C for up to one month.

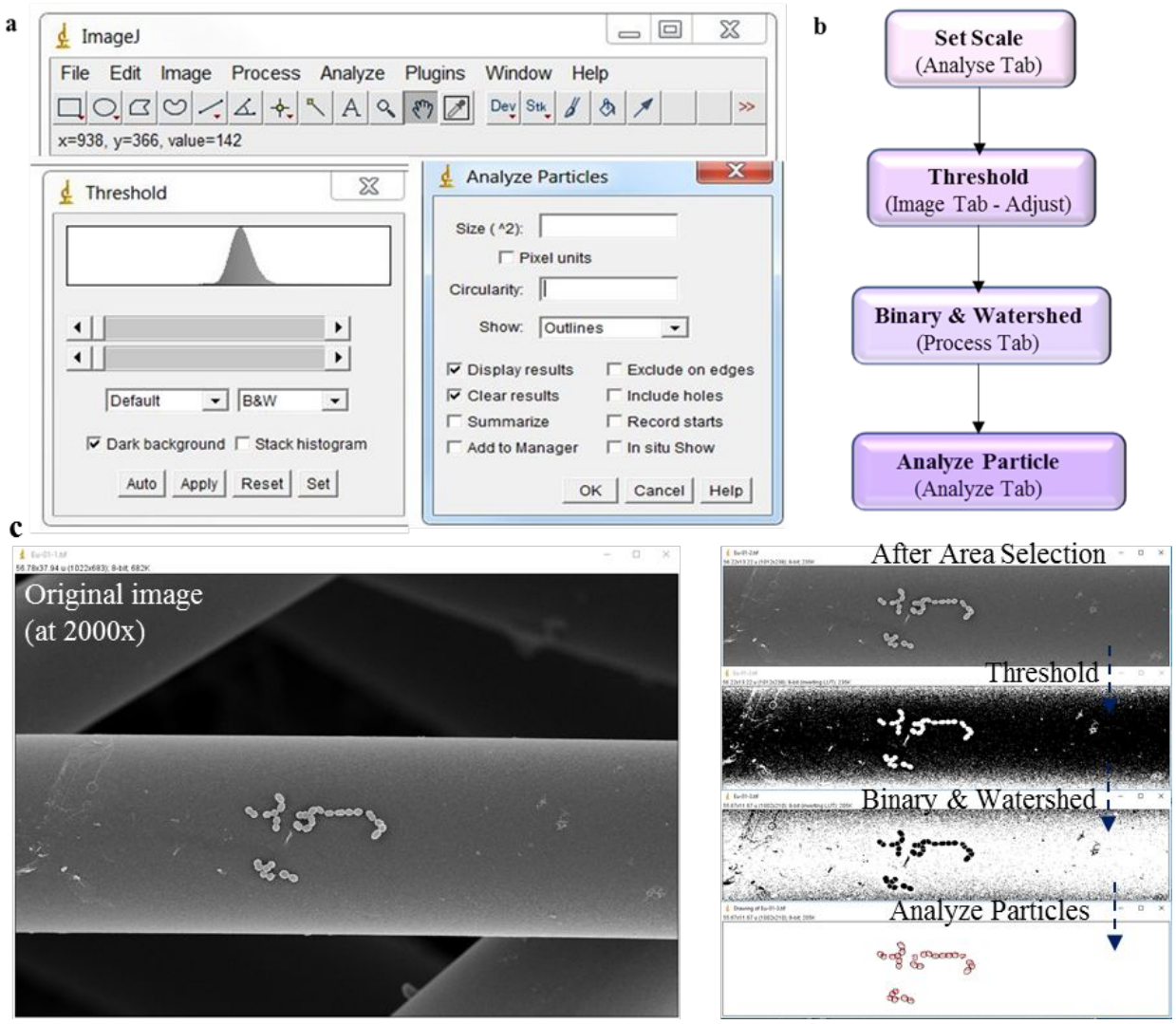
Sample preparation for SEM:

The paraformaldehyde-fixed and preserved samples were first sequentially dehydrated. The dehydration process involved immersing the samples into alcohol solutions of increasing ethanol concentrations (30%, 50%, 70%, 90% and 100%) (200 proof, biological grade from Decon Labs, purchased through Fisher Scientific) with each exposure lasting about 30 min. To ensure complete dehydration, the final incubation with 100% ethanol was repeated 3 times. Next, the samples were critical point dried (Autosamdri®-815, from Tousimis Research Corporation) using liquid carbon dioxide to ensure that the actual morphology of bacteria is preserved during EM. Due to the non-conducting nature of the samples, the critical point dried samples were fixed on sticky carbon tapes (Ted-Pella) and subsequently sputter coated (Cressington, Sputter Coater 208 HR) with Pt-Pd

(Ted-Pella) before being imaged with FE-SEM (Zeiss Merlin) operated at 5 keV. About 10-20 images per sample were collected. In order to better represent the sample, images were captured from various regions of the sample, without straying too close to the edges (to avoid artifacts).

Image Processing:

Grayscale SEM images (as tiff format) were analyzed using ImageJ with the sequential steps, namely, *Set Scale*, *Threshold*, conversion to *Binary* and *Watershed,* and finally *Analyze Particle*, as illustrated schematically in the supplementary information Supplementary Figure S1. This yielded an outline of the bacteria in an output image, along with a list of number of bacteria per image, and their respective sizes. The *Analyze Particles* step needed 2 input parameters: *Circularity* and *Size,* which were optimized to get the best estimate. First, an initial optimization process was carried out using about 5-6 images (from different regions of the same sample) to obtain the initial values. The results were validated against manually counted values and the estimation errors were plotted to obtain the circularity value that yielded maximum accuracy. The size values acquired from the output list were also used to construct the size histogram that yielded the initial size range. In the next iteration, if needed, these circularity and size range values were used to further refine the parameters for a slightly larger pool of images (*i.e*., 6-10). Such sequential iterative steps were helpful to minimize errors. Finally, the optimized parameters were plugged in, in order to process about 20-25 images. The same images were analyzed manually to obtain the actual counts. Once the estimation errors (*i.e*., percentage difference between the automated and manual estimation) were calculated for individual images, they were then averaged over the total number of analyzed images (**n**) to obtain ***i***. an Estimation Error (***E%***), ***ii***. the Magnitude of the Estimation Error (***Abs E%***) and ***iii***. the Cumulative Estimation Error ($\boldsymbol{E_n\%}$) calculated by the formula given below. The ***E%*** and $\boldsymbol{E_n\%}$ were then sorted from highest to lowest values (absolute number and not sign) and plotted against the number of images analyzed to obtain the average estimation error for each condition. During our study, we observed that thresholding played the most critical role, as it influenced the Circularity and Size parameters. It was also observed that the images with a minimum depth of field (variation of focal planes) could be processed with lower threshold values, which resulted in minimum estimation errors. Thus, it is important to note that this method is susceptible to image quality, region of interest, the pathogen itself and substrate of interest, and can be tuned accordingly. Accordingly, the same steps were repeated for various samples and also for different bacteria to obtain their total volume, and most importantly, their size distribution.



Supplementary Figure S1: **a:** general screen tabs of ImageJ; **b:** flow chart of the steps and **c:** the corresponding images to obtain the counts: ***i.*** '*set scale*' of an image according to the magnification or the built-in scale bar; ***ii.*** Adjust the '*Threshold*' to include the maximum number of bacterial cells and to eliminate the background as such as possible (this is an important step to be done carefully to reduce errors); ***iii.*** Convert an image to '*Binary*' and then '*Watershed*' to separate out the features from one another; ***iv.*** Finally, 'Analyze Particles' (with appropriate input of size and circularity) to obtain images with outlines, number of bacteria (N) and their corresponding sizes.

The following is the list of parameters and their definitions that had been used in this analysis

i. Total number of adherent bacterial cells estimated $= \boldsymbol{N}$

ii. *Estimation Error* $(\boldsymbol{E\%}) = \frac{(\boldsymbol{N} \text{ estimated by ImageJ} - \boldsymbol{N} \text{ estimated by Manual counting})}{\boldsymbol{N} \text{ estimated by Manual counting}} \times 100,$

This is estimated by first calculating for individual images then averaging over all images analyzed.

iii. *Magnitude of Estimation Error (**Abs E%**)* =

$$\left(\frac{\left|\text{ImageJ estimated number} - \text{Manually counted number}\right|}{\text{Manually counted number}}\right) \times 100$$

This is estimated for an individual image as an absolute value of the error and represents how suitable an image is for this method.

iv. *Cumulative Estimation Error (**E_n%**)* $= \sum_{n}\left(\frac{\text{Estimated number} - \text{Manually counted number}}{\text{Manually counted number}}\right) \times 100$

This is estimated by taking the cumulative average of Estimation Errors for the number of images (**n**) analyzed.

v. *Minimum no. of images required for estimation* (***ń***) = the minimum number of images (**n**) that are needed to be analyzed for the E_n to converge and remain below ± 5%. This number provides a logical and statistically consistent basis of analysis, especially for the conditions where *Abs E%* is very high.

Conventional Estimation of Bacterial Concentration:

The freshly prepared purified bacterial suspensions were subjected to both flow cytometry and optical density (OD) measurements. For flow cytometry, we adopted the standardized method developed at our facility by other researchers. The detailed steps of optimization and standardization of this method can be found in several reports[2-5]. In short, 667 µL of a bacterial suspension was mixed with 333 µL fluorescent dye (*i.e.*, thiazole orange and propidium iodide) solution and incubated for 5 min. The mixture was then subjected to the flow cytometer (RAPID-B model 9013) that took an average of 2 cycles (readings) and yielded the total bacterial concentration. For the OD, the UV-Vis spectra of the suspension (and its serial dilutions) were recorded. The average height of the spectrum between 600-650 nm, was taken as the measure of OD and converted into CFU/mL by multiplying the standard conversion constants for *E. coli*[4]. For the measurement of bacterial concentrations on a substrate, the substrates with adherent bacteria were first soaked in dilute SDS (Sigma-Aldrich) solution which was then purified through centrifugation (3,000 g for 7 min) to collect their genetic material. This was then subjected to Polymerase Chain Reaction (PCR) in real time (qPCR) against primers specific to *E. coli* O157, following the protocol mentioned in Chapter 4A, section O of FDA's Bacteriological Analytical Manual (BAM)[6].

Sample Preparation for Detection Limit:

To determine the detection limit using this method (*i.e.*, estimation of bacteria using SEM images), purified bacterial suspensions were first serially diluted and subjected to flow cytometry measurement to obtain their concentrations. For example, if the stock *E. coli* suspension was found to have a bacterial concentration of about ~7.8×10^7 CFU/mL through flow cytometry, then the suspension would first be serially diluted to yield concentrations of ~7×10^5 CFU/mL, ~7×10^3 CFU/mL and ~7×10^2 CFU/mL. Next, 100 µL of each suspension was then exposed to fibrous substrates of identical dimensions (20 mm × 20 mm = 400 mm^2). The substrates were then sealed

and incubated at 4 ^{o}C, followed by fixation and preservation. Subsequently, half of the sample was used for sample preparation and EM imaging, and the other half for qPCR measurement. Each measurement was repeated to ensure consistent measurements. For the SEM method, the total number of adherent bacteria were obtained through the estimation method. They were then used to first estimate the surface concentration (by dividing them with total number of images and the area per image) and subsequently to volume concentration (by multiplying the total area of the substrate and the volume of suspension used).

Sample Preparation for simulating contamination scenarios:

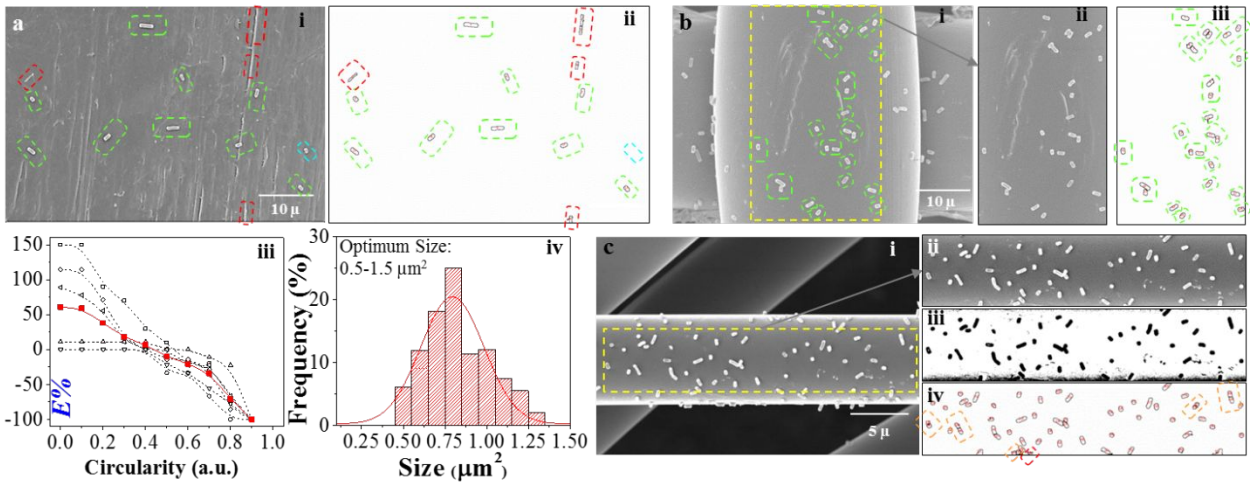
In order to mimic a medical contamination, commonly used bandages were soaked in dilute polylysine solution and air-dried. They were then soaked in a *S. aureus* suspension of a concentration of ~10^3 CFU/mL. Cotton swabs and tissue papers (Kimwipe™), used as substrates for environmental and bioterrorism samples, were also modified with polylysine, then soaked in *E. faecalis* and *B. anthracis* suspensions respectively, each having a concentration of ~10^3 CFU/mL. For food samples, about 100 µL of 10^5 CFU/mL *E. faecalis* suspensions were dropped on top of a glass coverslip covered with pieces of lean ground beef. All the samples were incubated overnight in covered petri dishes in a refrigerator (4 ^{o}C). They were then processed and imaged as explained earlier.

<u>References:</u>

1. Bhattacharyya, D. , Senecal, K. , Marek, P. , Senecal, A. and Gleason, K. K. (2011), High Surface Area Flexible Chemiresistive Biosensor by Oxidative Chemical Vapor Deposition. Adv. Funct. Mater., 21: 4328-4337.
2. Buzatu, D. A.; Cooper, W. M.; Summage-West, C.; Sutherland, J. B.; Williams, A. J.; Bass, D. A.; Smith, L. L.; Woodruff, R. S.; Christman, J. M.; Reid, S.; Tucker, R. K.; Haney, C. J.; Ahmed, A.; Rafii, F.; Wilkes, J. G., Photobleaching with phloxine B sensitizer to reduce food matrix interference for detection of Escherichia coli serotype O157:H7 in fresh spinach by flow cytometry. *Food Microbiology* **2013,** *36* (2), 416-25.
3. Buzatu, D. A.; Moskal, T. J.; Williams, A. J.; Cooper, W. M.; Mattes, W. B.; Wilkes, J. G., An integrated flow cytometry-based system for real-time, high sensitivity bacterial detection and identification. *PLoS One* **2014,** *9* (4), e94254.
4. Williams, A. J.; Cooper, W. M.; Summage-West, C. V.; Sims, L. M.; Woodruff, R.; Christman, J.; Moskal, T. J.; Ramsaroop, S.; Sutherland, J. B.; Alusta, P.; Wilkes, J. G.; Buzatu, D. A., Level 2 validation of a flow cytometric method for detection of Escherichia coli O157:H7 in raw spinach. *Int J Food Microbiol* **2015,** *215*, 1-6.
5. Volkmer, B.; Heinemann, M., Condition-Dependent Cell Volume and Concentration of Escherichia coli to Facilitate Data Conversion for Systems Biology Modeling. *PLoS ONE* **2011,** *6* (7), e23126.
6. https://www.fda.gov/food/foodscienceresearch/laboratorymethods/ucm070080.htm

Supplementary Results:

Steps for Parameter Optimization and Improving the Estimation:

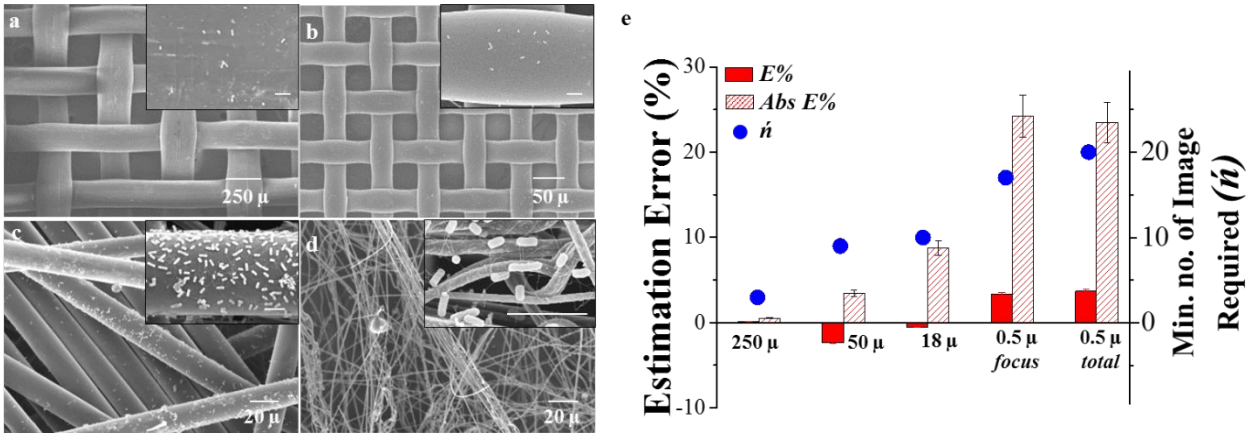


Supplementary Figure S2: Detailed method of estimation. **a**: for fiber with ~250 µm diameter. ***i:*** Representative SEM micrograph; ***ii:*** corresponding output image showing the bacterial outline and their counts: green line – correct identification (signal), red line – wrong identification (noise) and blue line – undetected (mishits). ***iii:*** the initial histogram constructed for about 5 images for rudimentary approximation of *Circularity* (the black dotted lines are for individual images and the red solid line represents their average); ***iv:*** Histogram plotted using the approximate circularity values which were used to analyze a larger set of images. Such histograms yielded good approximation of the *Size* parameter, which were refined with another iteration (over a larger number of images) to optimize *Circularity* and *Size* parameters and the *Estimations Errors* (***E%***, $\boldsymbol{E_n\%}$ and ***Abs E%***). Addition step of zone selection was required for fibers with **b**: ~40 µm; and **c**: ~20 µm diameter, as the diameters of fibers are smaller than the image frame. Consequently, central parts of the images (indicated by yellow lines in both cases) were selected for the analysis to obtain more accurate estimations.

Effects of Fiber Diameter of the Substrate:

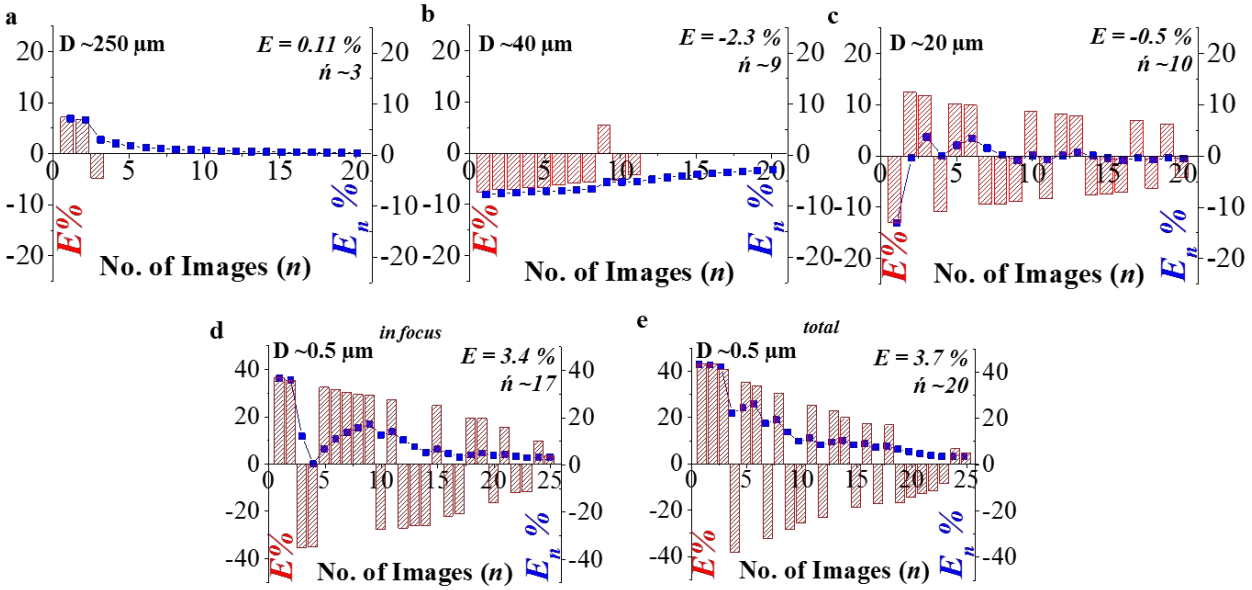
Supplementary Table S1: The list of parameters, the errors and minimum number of images to be used using this method for substrates with Fiber Diameters.

Sub. Dia. (µm)	Circularity (a.u.)	Size (μm^2)	Avg. E (%)	Avg. Abs. E (%)	Min. no. of images required ($\acute{n}$)
250	0.3-0.9	0.5-1.5	0.11	0.54	~5-10
40	0.3-0.9	0.5-1.5	-2.35	3.45	~10
20	0.3-0.9	0.4-1.5	-0.53	8.76	~10
0.5-1 In focus Total	 0.4-0.9 0.3-0.9	 0.3-1.5 0.2-1.5	 3.39 3.69	 24.22 23.46	 ~15-20 ~20-25



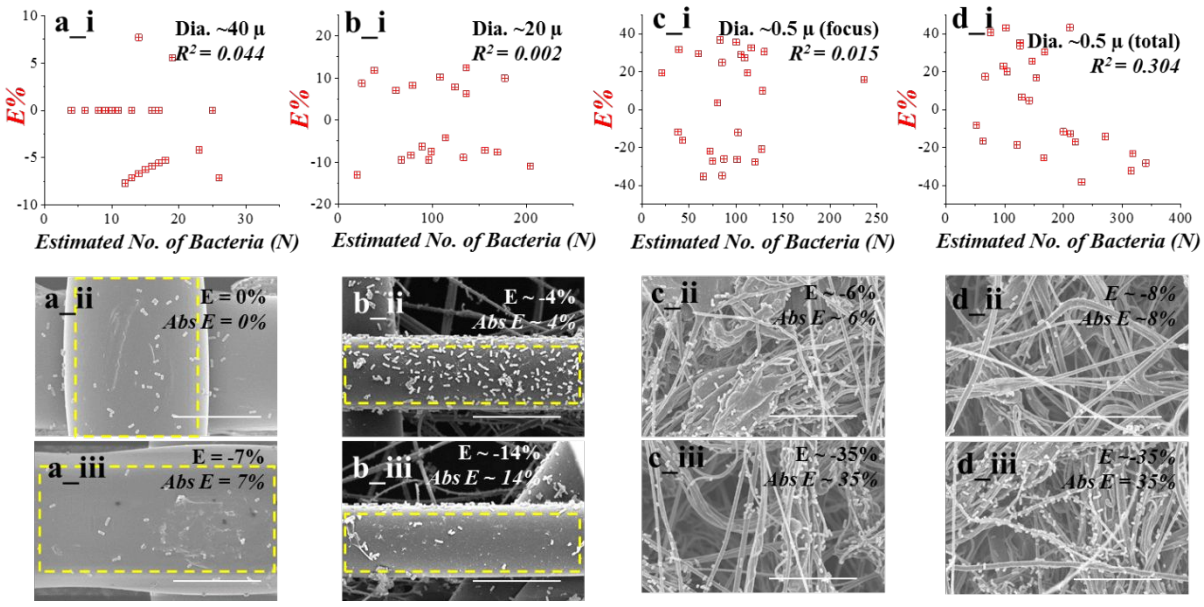
Supplementary Figure S3: Effects of Fiber Diameter on bacterial estimation and the corresponding errors. **a** to **d:** represents SEM images of fibers of different diameters. Their insets highlight the dimensional difference/similarity of the fiber diameter to that of the bacterial dimensions. **e:** shows the average errors (***E%***) as well as their magnitude (***Abs E%***) for all 4 substrates using this method. It is important to note that the magnitude of the errors increases significantly when the dimension of the fibers appear close to that of bacteria, and hence, a higher number of images are to be analyzed in order to maintain the average error below ± 5%.

Exploring if the order in which images were analyzed played any role:



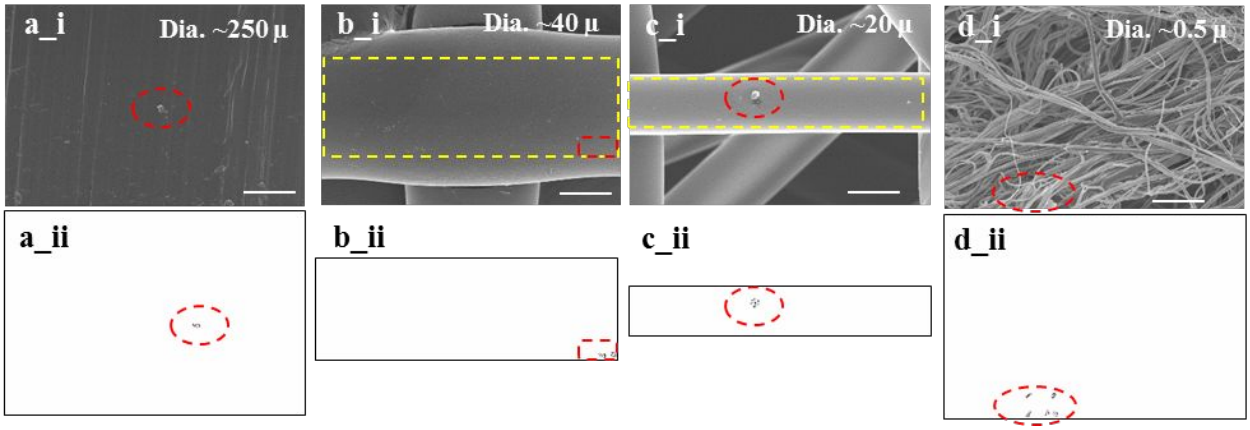
Supplementary Figure S4: Errors of estimation (***E%*** and ***E_n%***) for substrates with fiber diameters, **a** ~250 µm; **b:** ~40 µm, **c:** ~20 µm, and **d**& **e**: ~0.5 µm. Instead of plotting the estimations errors in the order in which the images were captured, the ***E%*** were first sorted from high to low before plotting. We did not observe significant change in the estimation parameters, especially ***ń***, showing the method is not greatly perturbed by the order in which images were analyzed.

Estimation error (E%) on the number of adherent bacteria (N):



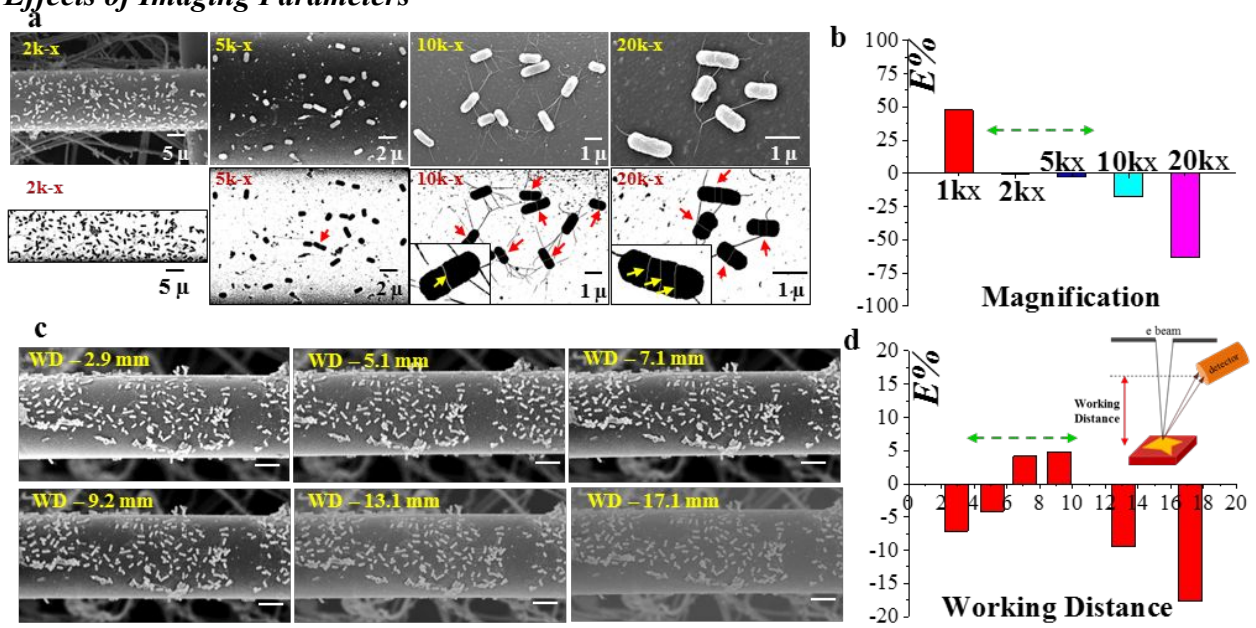
Supplementary Figure S5: Estimation error (***E%***) as a function of the number of bacteria estimated (***N***) for substrates with fiber diameters, **a** ~250 µm; **b:** ~40 µm, **c:** ~20 µm, and **d**: ~0.5 µm. The top-row (a_i to d_i) shows the distribution of E% as a function of N, which shows no significant co-relation as the correlation coefficient (R^2) were quite low. Middle row (a_ii to d_ii) shows representative images were the ***E%*** was small or close to zero and the bottom row (a_iii to d_iii) shows representative images were the ***E%*** was high. These observations suggest that the E% could be low even for large number of bacterial adhesion but would yield high values if images with defects and anomalies were used for the analysis. They collectively highlight that selecting defect free areas while acquiring images helps in achieving lower ***E%***.

Negative control to better understand the base Substrates Effect:



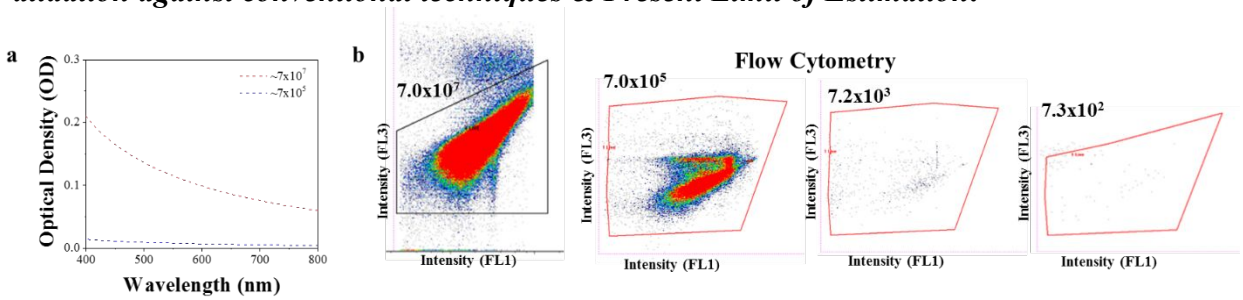
Supplementary Figure S6: Estimation errors when images of bare substrates were used. a to d: represents SEM images of fibers of different diameters. Top row (a_i to d_i) shows as captured SEM images and bottom row (a_ii to d_ii) the corresponding processed output image. The ***E%*** was found close to 0 for most images, except for a few. In these case, we observed the errors resulted from singular defects [defects (a_i and c_i), edges (b_i) or high differences in focal planes (d_i)]; which are too random to be generalized as substrate effect or reference error.

Effects of Imaging Parameters



Supplementary Figure S7: Effects of Magnification and Working Distance on bacterial estimation. **a**: representative SEM images of adherent bacteria on a fiber surface, at different magnifications and their corresponding processed image. **b:** errors of estimation for different magnifications. At higher magnifications, bacterial shapes tend to break into multiple fragments, as shown in the insets, which imparts significant errors during estimation. **c**: SEM images, (with the scale bars being 5 µm) and **d:** errors of estimation when images were taken at different working distances (shown schematically in the inset). Intermediate magnifications and working distances, as highlighted by the horizontal green arrows, yielded minimum errors of estimation. It is important to note that these parameters depend on the size of the pathogen, substrate or other sample/imaging parameters.

Validation against conventional techniques & Present Limit of Estimation:

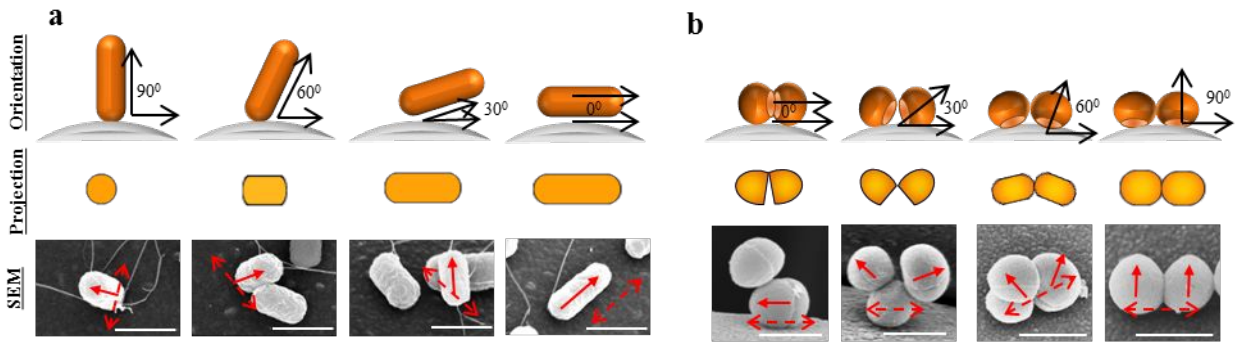


Supplementary Figure S8: a: Optical Density and **b:** Flow Cytometry measurements showing different bacterial concentrations exposed to the substrates. For concentrations less than 7×10^5, only Flow Cytometry measurements were done for the sake of better accuracy. Once the bacterial concentration of the suspension was measured, the suspensions were then exposed to the substrates. Post-exposure, each substrate was divided into two portions, one for SEM-based and another for qPCR-based quantification. We observed an overall good correlation amongst flow cytometry (measured before exposure), SEM estimation and qPCR techniques (measured after exposure) within this range (~10^5 to ~10^2 CFU/mL).

Extension to other bacteria species and the effect of Orientation:

Supplementary Table S2: List of parameters (Size and Circularity), the errors and minimum images to be analyzed for 4 strains of bacteria (adherent to substrates with a 20 µm dia. fiber)

Species	**Circularity (a.u.)**	**Size (μm^2)**	***E %***	***Abs. E %***	***ń***
E. coli	0.3-0.9	0.4-1.5	-0.53	8.76	~10
L. innocua	0.3-0.9	0.2-1.0	0.21	6.82	~10
E. faecalis	0.5-1.0	0.1-1.0	-0.92	2.74	~10
S. aureus	0.4-1.0	0.1-1.0	-2.32	10.18	~15



Supplementary Figure S9: The adhesion-dependent orientation of bacteria and their effect of their projected sizes. **a:** for rod-shaped *E. coli* O157 and **b:** spherical *S. aureus*, showing the effect of their morphologies and orientations on this estimation method. This method works best when bacteria are well dispersed and adhere uniformly onto the matrix.

Simulated contamination scenarios

Supplementary Table S3: The list of ***E%*** for various bacterial contamination samples

Field of Application	**Bacterial Species**	**Substrate**	**N**	***E%***	***n***
Medical waste	*S. aureus*	bandage	61	5.4	15
Environmental/ Forensic	*E. faecalis*	cotton swab	98	-3.2	15
Bioterrorism	*B. anthracis*	tissue paper	113	6.3	15
Food Safety	*E. faecalis*	ground beef	137	-3.6	15