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TITLE:

Automated, High-Throughput Detection of Bacterial Adherence to Host Cells

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SUMMARY:

Detection of host-bacterial pathogen interactions based on phenotypic adherence using high-throughput fluorescence labeling imaging along with automated statistical analysis methods enables rapid evaluation of potential bacterial interactions with host cells.

ABSTRACT:

Identification of emerging bacterial pathogens is critical for human health and security. Bacterial adherence to host cells is an essential step in bacterial infections and constitutes a hallmark of potential threat. Therefore, examining the adherence of bacteria to host cells can be used as a component of bacterial threat assessment. A standard method for enumerating bacterial adherence to host cells is to co-incubate bacteria with host cells, harvest the adherent bacteria, plate the harvested cells on solid media, and then count the resultant colony forming units (CFU). Alternatively, bacterial adherence to host cells can be evaluated using immunofluorescence microscopy-based approaches. However, conventional strategies for implementing these approaches are time-consuming and inefficient. Here, a recently developed automated fluorescence microscopy-based imaging method is described. When combined with high-throughput image processing and statistical analysis, the method enables rapid quantification of bacteria that adhere to host cells. Two bacterial species, Gram-negative *Pseudomonas aeruginosa* and Gram-positive *Listeria monocytogenes* and corresponding negative controls, were tested to demonstrate the protocol. The results show that this approach rapidly and accurately enumerates adherent bacteria and significantly reduces experimental workloads and timelines.

INTRODUCTION:

Bacterial adhesion is a process whereby bacteria attach to other cells or surfaces. Successful establishment of infection by bacterial pathogens requires adhesion to host cells, colonization of tissues, and in some cases, invasion of host cells[1–3]. Emerging infectious diseases constitute major public health threats, as evidenced by the recent COVID-19 pandemic[4–6]. Importantly, new or emerging pathogens may not be readily discerned using genomic-based approaches, especially in cases where the pathogen has been engineered to evade detection or does not contain genomic signatures that identify it as pathogenic. Therefore, the identification of potential pathogens using methods that directly assess hallmarks of pathogenicity, like bacterial adherence to host cells, can play a critical role in pathogen identification.

Bacterial adherence to host cells has been used to evaluate mechanisms of bacterial pathogenesis for decades[1,7]. Microscopic imaging[8,9] and the enumeration of bacterial colony-forming unit (CFU)[10–13] by post-infection plating are two well-developed laboratory methods for testing microbial adherence and/or infection of host cells[14]. Considering the micrometer scale size of bacterial cells, the enumeration of the adherent bacterial cells generally requires the use of advanced high-magnification microscopy techniques, as well as high-resolution imaging approaches, including electron microscopy, expansion microscopy (ExM)[15,16], and three-dimensional imaging[17]. Alternatively, the enumeration of bacteria bound to or internalized within host cells can be performed by plating the dilution series of harvested bacteria on solid agar and counting the resultant CFUs[10,12,13]. This method is laborious and includes many manual steps, which introduces difficulties in establishing a standardized or automated procedure required for high-throughput analyses[18,19]. Therefore, the development of new methods for evaluating host cell attachment would address current limitations in the field.

One such method is described here that uses automated high throughput microscopy, combined with high throughput image processing and statistical analysis. To demonstrate the approach, experiments with several bacterial pathogens were performed, including *Pseudomonas aeruginosa*, an opportunistic Gram-negative bacterial pathogen of humans, animals, and plants[14,20], which is frequently found to colonize the respiratory tract of patients with impaired host defense functions. This approach optimized the microscopic imaging process described in previous studies[14,20]. The imaging detection was simplified by fluorescence-labeled host cells and bacteria to rapidly track the proximity of them, which dramatically reduced the microscopy workload to get high-resolution images for distinguishing bacteria. In addition, the automated statistical analysis of images in counting host cells and bacteria replaced the hand-on experiment of bacterial CFU plating to estimate the ratio of adherent bacterial counts per host cell. To confirm the compatibility of this method, multiple bacterial strains and host cell types have also been tested, like *Listeria monocytogenes*, *Staphylococcus aureus*, *Bacillus cereus,* and *Klebsiella pneumoniae,* as well as human umbilical vein endothelial cells (HUVECs), and the results support the diversity and effectiveness of the method.

PROTOCOL:

1. A549 cell culture and staining

1.1. Maintain the A549 cell line in F-12K medium supplemented with 10% fetal bovine serum (FBS) and incubate at 37 °C, 5% CO_2.

1.2. Change the medium every 3–4 days and passage at 85%–95% confluency.

1.3. Briefly, rinse the cells with 1 x phosphate-buffered saline (PBS, pH 7.4, unless otherwise indicated) and treat with 1 ml of 0.25% Trypsin-0.53 mM ethylenediaminetetraacetic acid (EDTA) solution (submerging the cell layer) for about 2 min at 37 °C.

1.4. Add additional 6 mL of complete growth medium (F-12K medium + 10% FBS) to stop the protease activity. Then, plate the cells in a sterile polystyrene T-75 tissue culture flask at a subcultivation ratio of 1:3–1:8. The final volume of culture is 12 mL.

1.5. Seed approximately 1 x 10^4 A549 cells (cell concentration: ~1 x 10^5 cells/mL) onto each well of a 96-well plate a day prior to the adherence assays.

2. Bacterial growth and staining

2.1. Perform all bacterial work in a Biosafety Cabinet, Biosafety Level 2 laboratory.

2.2. Inoculate all bacterial cultures, including *P. aeruginosa* (PAO1), *E. coli*, *L. monocytogenes,* and *B. subtilis,* etc., from the frozen glycerol stock and grow them in Tryptic Soy Broth (TSB, 3 mL) in a shaking incubator at 37 °C overnight maintained at 250 rpm.

2.3. The next day, use a 1:100 dilution of the overnight culture to inoculate a subculture and grown them in TSB (1 mL) for 3 h to the exponential phase, measure the OD at 600 nm (OD_{600}) to confirm. Ensure that the OD_{600} is in the range of 0.4–0.6.

2.4. Prior to performing the bacteria-host adherence assay, plate serial dilutions of bacterial suspension onto TSB-agar plates, incubate them overnight at 37 °C, and then establish the bacterial CFUs from the number of colonies. For *P. aeruginosa*, an OD_{600} of 1.0 corresponds to 2 x 10^8 viable bacterial cells/mL, and for *L. monocytogenes*, an OD_{600} of 1.0 corresponds to 9 x 10^8 viable bacterial cells/mL.

2.5. Harvest the bacterial cultures at exponential phase by centrifugation at 13,000 x *g* for 2 min at room temperature (RT), then wash them once using 1x PBS (1 mL). Resuspend the bacterial pellets in 1 mL of 1x PBS and determine the concentrations by measuring OD_{600} of bacterial suspensions. For example, *P. aeruginosa* with OD_{600} of 0.5 represents a concentration of 1 x 10^8 bacterial cells/mL.

2.6. Stain the bacterial suspension using either a green or a red fluorescent dye at RT for 30 min with gentle rotation in the dark. For this, add 2 μL of the 500- fold concentrated stock staining dye into 1 mL of bacterial suspension to dilute the dye 1-fold. To wash off the staining dye,

centrifuge the stained bacteria at 13,000 x *g* for 2 min and resuspend the pellet in 1 mL of 1x PBS for three times.

NOTE: If fluorescence- (GFP-, RFP-, mCherry-, etc.) tagged bacteria are used in the experiment, then skip this bacterial staining step. GFP- tagged *P. aeruginosa* and red fluorescent dye-stained *L. monocytogenes* were used in this protocol.

2.7. Collect the stained bacterial cells or GFP- tagged bacteria by centrifugation at 13,000 x *g* for 2 min. Resuspend in fresh F-12K medium (1 mL) and measure the OD_{600} of each culture. Subsequentially dilute the cultures to the desired concentrations based on the multiplicity of infection (MOI) and host cell concentration. The final volume used in this experiment is 500 µL.

NOTE: For example, if the host cell concentration counted using Trypan Blue staining is 1 x 10^5 cells/mL, the desired concentration of bacterial cells at an MOI of 100 will be 1 x 10^7 cells/mL. Concentration of *P. aeruginosa* at 0.5 OD_{600} is 1 x 10^8/mL. To obtain the desired concentration, dilute the *P. aeruginosa* culture 10-fold, add 50 µL of resuspended culture to 450 µL of fresh F-12K medium.

3. Bacterial adherence assay

3.1. First, wash the seeded A549 cell monolayers three times with warm 1x PBS. For each wash, add 100 µL of 1x PBS to each well, gently pipette up and down three times, dispose of 1x PBS or wait for 10 s after addition and then vacuum to remove 1x PBS. To determine the kinetics of bacterial association, overlay the cells with 100 µL of desired concentrations of bacterial suspension with different MOIs (0, 1, 10, and 100). Spin down the bacteria at 200 x *g* for 10 min and incubate the infected A549 cells at 37 °C, 5% CO_2 for an additional 1 h.

NOTE: In this experiment, each condition had a technical triplicate.

3.2. Remove the unbound bacteria by washing the monolayers five times with warm 1x PBS as described above. Add 100 µL of 4% formaldehyde (in 1x PBS) into each well of the 96-well plates to fix the cells. Let the plates sit on ice for 15 min and then wash off the fixation solution using 1x PBS three times.

3.3. To stain the nuclei, add 50 ng/mL of 4′,6-diamidino-2-phenylindole (DAPI) and incubate it for 10 min at RT. After incubation, wash the wells three times using 1x PBS. Cover the infected A549 cells with 100 µL of 1x PBS to avoid drying. Process for the next step or store the plate at 4 °C for up to 2 days in the dark.

4. Automated fluorescence imaging, processing, and analysis

4.1. To maintain the data integrity, randomly and manually pick five locations of each well to capture the images at 20x magnification. Capture the fluorescent images of A549 cells and

bacteria under DAPI and GFP channels, respectively. Use PE-Cy5 channel for the bacteria stained by the red fluorescent dye.

4.2. To have a better resolution, process all the images for background flattening and deconvolution. Set the parameters as 68 µm diameter of the rolling ball for smoothing and auto-measure the point spread function (PSF) of image deconvolution based on the objective.

4.3. To count all qualified cells and bacteria, measure the fluorescence intensity of the host cells and bacteria and set the weakest fluorescence intensity of host cells and bacteria as the thresholds for cell count. Count all bacteria proximate to a host cell within 15 µm distance as the adherent bacteria as the diameter of A549 cell is 10.59–14.93 µm[21].

NOTE: A default setting in the imaging system selectively counts the host cells with diameters ranging from 5–100 µm and bacteria ranging from 0.2–5 µm in size (width and length).

4.4. Based on the above-mentioned manual image processing results, apply the parameters of image smoothing, deconvolution, objects sizes, distance, and fluorescence intensities to the rest of the automated images. After the automated analysis, consider critical readouts, such as total host cell counts, cell sizes and shapes, total bacterial counts, and the average bacterial count per host cell, which was the most important indicator, to determine bacterial adherence.

4.5. Data export and statistical analysis

4.5.1. Export the analyzed results from all images to a spreadsheet (e.g., 'xlsx' format). The automated system generates two sets of results: 1) the average adherent bacterial count from each image; 2) the informative data of each single host cell, such as adherent bacterial count on a single cell, host size, and the average fluorescence intensity of host cell and bacteria, from the corresponding image.

4.5.2. Calculate the mean number and standard deviation of adherent bacterial counts from all images to represent the bacterial adherence level compared to negative controls. In this method, *E. coli* and *B. subtilis* served as negative controls in testing Gram-negative and Gram-positive bacteria adherence, respectively. Perform two-way ANOVAs to test for significant variation between data points across treatment for three independent experiments.

REPRESENTATIVE RESULTS:

To develop the fluorescence imaging-based bacterial adherence assay, *P. aeruginosa* strain PAO1 and its negative-adherence counterpart *E. coli* were used to test the protocol effectiveness, as the adherence of these bacteria to A549 cells had been reported[14,20,22]. First, GFP- labeled *P. aeruginosa* (PAO1) and GFP-labeled *E. coli* were co-incubated with a human immortalized epithelial cell line A549 at various MOIs, respectively. The results showed that PAO1 adhered to A549 cells in a dose-dependent fashion (**Figure 1A**,**B**); in the meantime, near null adherence of *E. coli* was also verified (**Figure 1A**,**B**). There were 50 images captured and analyzed at each MOI.

Two-way ANOVAs were performed to test the significant variations from three independent experiments.

[Place **Figure 1** here]

Similar results were also observed when Gram-positive bacteria, *L. monocytogens*[23,24] and its negative control *B. subtilis,* were used (**Figure 2A**,**B**). The adherence to A549 cells was significant in *L. monocytogenes* than *B. subtilis* (**Figure 2A**,**B**).

[Place **Figure 2** here]

A representative image of host-adherent *P. aeruginosa* segmentation and counts is shown in **Figure 3** (Yellow masks: selected bacteria, red outline: single bacterial count). The settings for both host and bacteria targets are described in the Protocol section.

[Place **Figure 3** here]

The results of the automated analyses, including host cell sizes and areas, bacterial and host cell counts, as well as the average bacterial counts per host cell, representing the status of host cell health, bacteria adherence level, and bacterial cytotoxicity, are listed in **Table 1** and **Table 2**. **Table 1** represents the adherent bacterial counts at an average cellular level from different images, while **Table 2** represents adherent bacterial counts analyzed on one image at a single A549 cellular level. These representative images were captured from A549 cells infected by PAO1 at an MOI of 100. The analyzed results of the initial image in **Figure 3** were listed as Image 1 in **Table 1** and **Table 2**.

[Place **Table 1** here]

In each image, host sum count and bacteria sum spots represent the total counts of host nuclei and total bacterial counts recognized by the system based on the parameters described above, respectively. In addition, the automated calculation of bacterial spots ratio represents the average bacterial count per host cell, derived from the bacteria sum spots/host sum count. Moreover, additional readings can also be included; for instance, the bacteria-adherent host counts illustrate the universal or specific phenomenon of host-bacteria adherence. When the bacteria-adherent host counts are much less than host sum counts, in contrast, the average bacterial count per host is relatively high, which represents that such adherence phenotype has a significant heterogeneity.

[Place **Table 2** here]

Singular cell analysis provides more details about host cell and bacterial targets in each image, which is more useful when gathering other bacterial features and applying them to the automated computational analysis to develop a more powerful tool in evaluating the potential bacterial pathogenicity, like a Machine Learning model which is being studied. In this protocol,

host size and area represent the diameters and areas of each stained host nuclei, and host fluorescence intensity represents the average intensity of the stained nuclei. Bacterial spot count and area represent the adherent bacterial targets to each host and their total areas. Bacterial fluorescence intensity represents the average intensity of all adherent bacteria.

It is not surprising that some virulent bacteria did not adhere to A549 while some bacteria with high levels of bacterial adherence do not necessarily correlate with pathogenicity. A different host cell type, HUVECs, was also tested to maximize the application of this method. The results showed the effective detection and the different adherence phenotypes of bacteria (**Figure 4**). Different host cells could increase the estimation of potential bacterial pathogens; for instance, pathogenic *Serratia rubid*aea and *Streptococcus agalactiae* were adherent to HUVEC but not to A549 cells (**Figure 4**). Moreover, the cytotoxic Enterohemorrhagic *E. coli* (EHEC) was non-adherent to either A549 or HUVEC (**Figure 4**). Therefore, it is critical to ensure that the method is suitable for multiple host cell types to respond to the specificity of host-bacteria interactions. Both A549 and HUVEC cells were co-incubated with bacteria at an MOI of 100 at 37 °C for 1 h.

[Place **Figure 4** here]

FIGURE AND TABLE LEGENDS:

Figure 1: Gram-negative bacterial *P. aeruginosa* adhered to A549 cells within 1 h of co-incubation. (**A**) Microscopic images for an overview of bacterial adherence where the images were taken using 20x magnification. PAO1 and the negative-adherence control *E. coli* adhered to A549 cells at the indicated multiplicity of infections (MOIs). Bacteria were GFP-fluorescence tagged. A549 cell nuclei were stained by DAPI. The scale bar is 50 µm. (**B**) Quantification of adherent bacterial counts per A549 cell. For each tested bacterial strain (PAO1 and *E. coli*) in each MOI, a total of 50 images were applied to the analysis at each condition. Data are mean ± standard deviation (SD) from one representative of three independent experiments. The two-way ANOVA statistical analysis was performed. * $p < 0.05$, *** $p < 0.001$.

Figure 2: Gram-positive bacterial *L. monocytogenes* adhered to A549 cells within 1 h of co-incubation. (**A**) Microscopic images for an overview of *L. monocytogenes*, as well as the negative control *B. subtilis* adherence to A549 cells at different MOIs. Bacteria were stained using a red-fluorescent dye. A549 cell nuclei were stained with DAPI. The scale bar is 50 µm. (**B**) Quantification of adherent bacterial count per A549 cell. For each tested bacterial strain (*L. monocytogenes* and *B. subtilis*) in each MOI, a total of 50 images were applied to the analysis at each condition. Data are mean ± SD from a representative of three independent experiments. The two-way ANOVA statistical analysis was performed. ** $p < 0.01$, *** $p < 0.001$.

Figure 3: Example images of bacterial segmentation and counting in the automated analysis process. (A) Microscopic images of *P. aeruginosa* adhered to A549 at an MOI of 100 without bacterial segmentation and counting. **(B)** The same image after the statistical analysis of bacterial segmentation and counting. Yellow masks: selected bacteria, red outline: single bacterial count.

Figure 4: **Specificity of bacterial adherence to host A549 and HUVEC cells**. The bacterial strains, including *S. rubidaea, S. agalactiae,* cytotoxic Enterohemorrhagic *E. coli* (EHEC)*,* PAO1*,* PAO-NP*,* *E. coli, S. aureus,* and *S. aureus* Δ*saeR*, were tested on both A549 and HUVEC cells at an MOI of 100 for 1 h of co-incubation at 37 °C, 5% CO_2. Bacteria were stained with a red-fluorescent dye. Adherent bacterial counts were quantified from 45 images/bacterial strain in three independent experiments. Data are mean ± SD from one representative of three independent experiments. * $p < 0.05$, *** $p < 0.001$.

Table 1: Statistical analysis results of 9 representative images at the cellular level.

Table 2: Single cellular statistical analysis of a representative image.

DISCUSSION:

The protocol describes an automated approach for enumerating bacterial attachment to host cells. The described approach has several attractive advantages over conventional methods. First, this approach enables the precise quantification of the number of microbial pathogen cells that are attached to individual host cells. Importantly, this quantification can be performed without the need for laborious bacterial harvesting, serial dilutions, plating on solid media, and determination of CFUs[10–12]. As such, the described technique reduces the overall workload required to quantify bacterial adherence. It should be appreciated that the advantages of the proposed approach are amplified when seeking to detect adherence phenotypes in large-scale screening experiments, including the identification of bacterial or host genes in mutant or CRISPR libraries, respectively, that regulate this process. Second, the direct evaluation of interactions between host and bacterial cells using fluorescence microscopy provides information for elucidating mechanisms of bacterial pathogenicity, including alterations in a host or bacterial cell survival, morphology, or dynamics. Finally, the automated statistical analysis performed on images collected in the analysis not only provides an overall quantification of the average bacterial association with host cells but also enables the evaluation of dynamic, single-cell, host-pathogen interactions.

Despite these advantages, the described methods have some important limitations. First, fluorescence staining of bacteria is required to enumerate their adherence to host cells. Thus, the fluorescent staining dye has to selectively stain bacteria than their host cell counterparts. In addition, the staining dye must not alter the attachment phenotype of the bacteria that carries it. Therefore, the optimization of bacterial fluorescence staining (e.g., concentration, staining duration) must be determined in advance of performing the described adherence studies. In this protocol, fluorescent staining of bacterial strains including *P. aeruginosa, L. monocytogenes, E. coli,* and *B. subtilis* for 30 min does not affect their adherence to host cells. Second, due to the specificity of different host-bacterium interactions, a single host cell type may not be sufficient to evaluate bacterial attachment to host cells. Therefore, multiple host cell types might be required to gain a complete understanding of the degree to which a given microbe can adhere to host cell surfaces. Third, bacterial segmentation was not fully efficient when bacteria propel aggregation during the co-incubation, e.g., *S. aureus*, or when bacteria formed clumps at a higher MOI (>100), e.g., *P. aeruginosa*. In this case, a higher magnification should be applied to capture

the images, or more images should be used in the analysis to reduce the effect of bacterial aggregation.

Human lung epithelial cells (A549) and HUVECs were employed in the studies to demonstrate the plasticity of the approach with respect to host cell type, and both displayed high levels of susceptibility to bacterial adherence. The use of two host cell types also demonstrated that the described method could be widely applied to characterize adherent host-bacterium interactions rapidly.

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DISCLOSURES:

All authors have no conflicts of interest to disclose.

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Figure 1

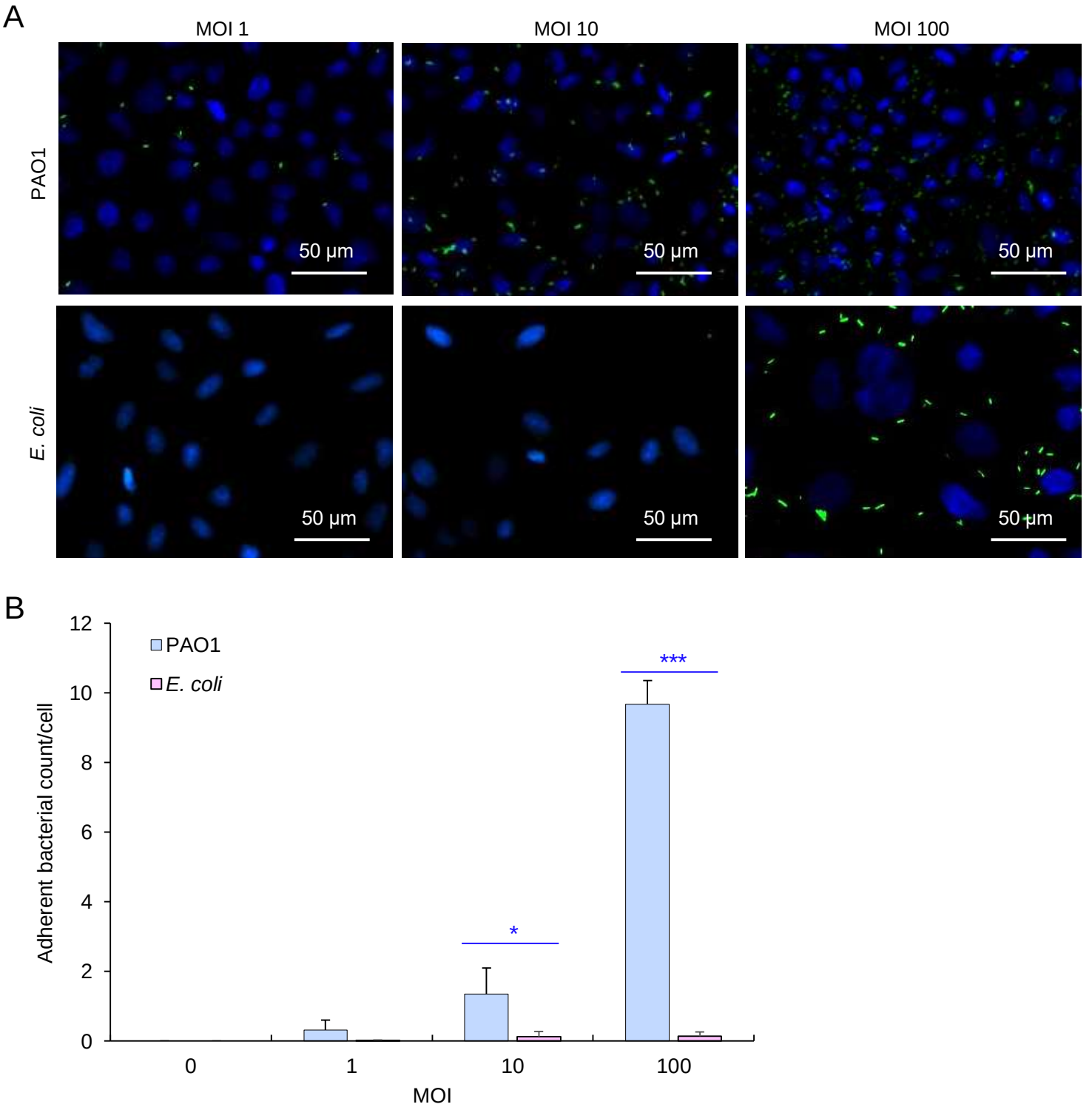

Figure 2

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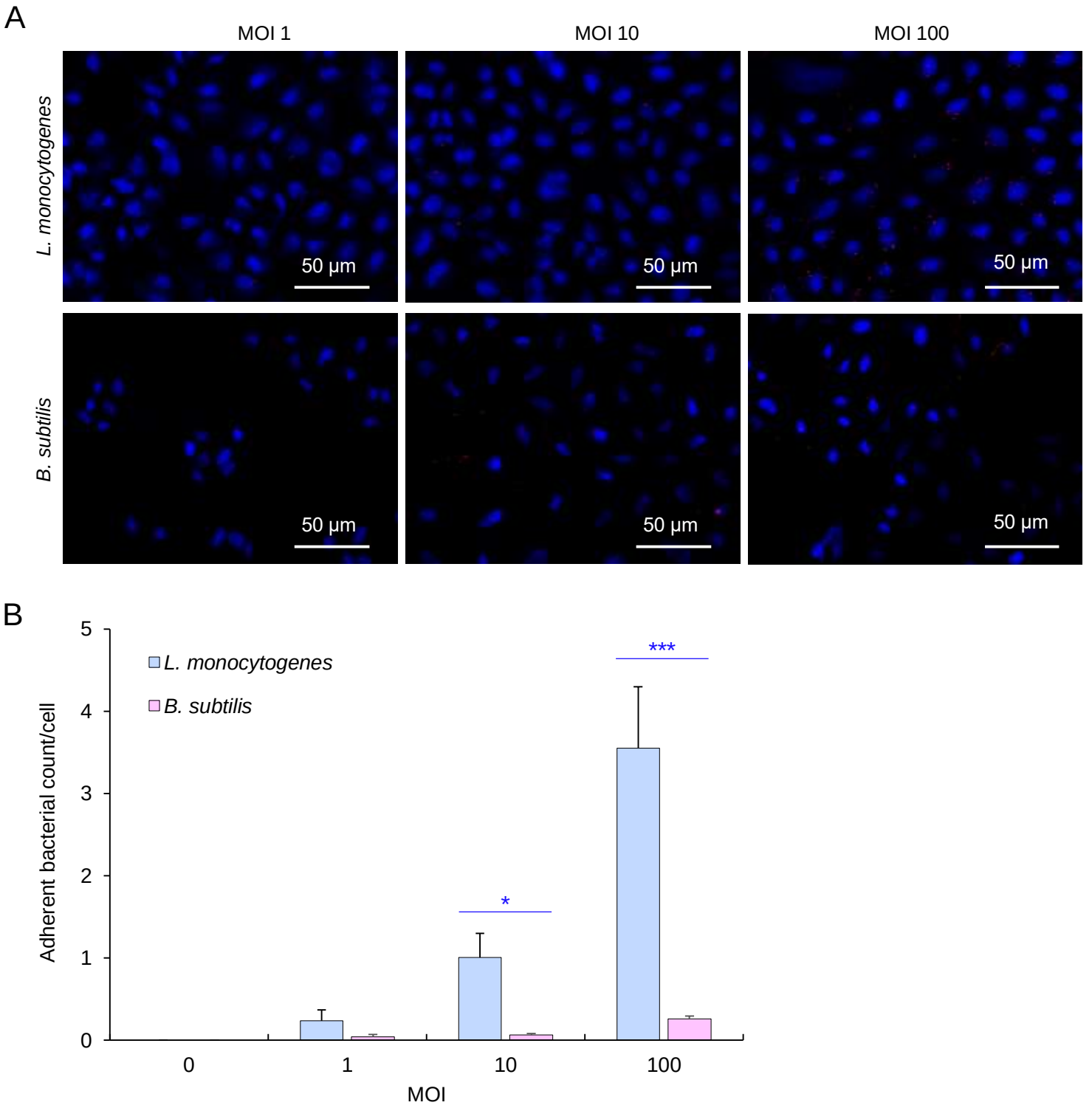

Figure 3

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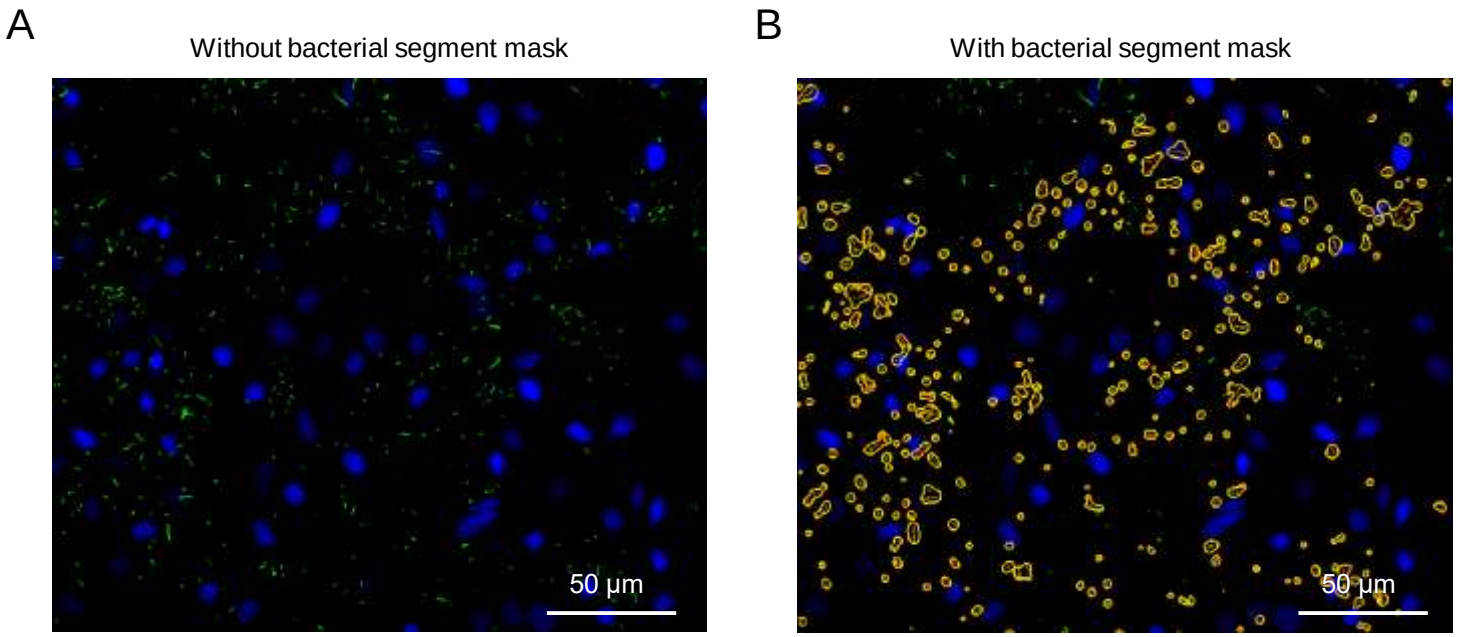

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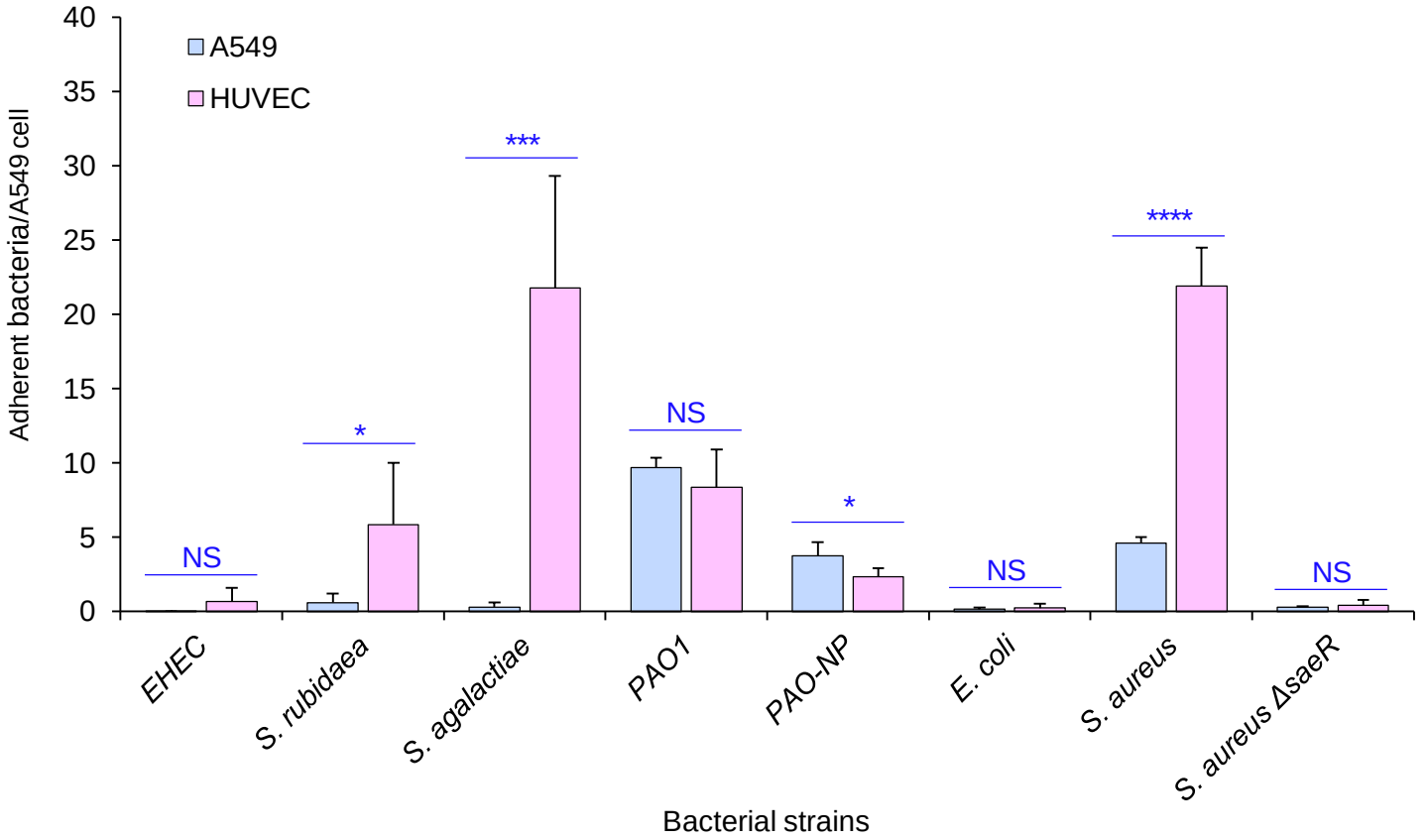

Table 1

	Image 1	Image 2	Image 3	Image 4	Image 5	Image 6	Image 7	Image 8	Image 9
Host Sum Count	99	34	80	37	42	49	29	29	48
Bacterial Spots ratio	5.37	5.4	3.6	7.8	9.1	5.5	9.2	9.2	7.1
Bacterial Sum Spots	532	182	289	289	383	270	267	267	341
Host Count [bacterial adherent]	93	34	80	37	42	49	29	29	48

Note: Representative images from A549 cells infected by *Pseudomonas aeruginosa* strain PAO1 at an MOI of 100. Host sum count and bacterial sum spots represent the total counts of host nuclei and total adherent bacterial counts recognized by the system, respectively. Bacterial spots ratio represents the average adherent bacterial count per host cell, derived from the bacterial sum spots/ host sum count.

Table 2.

Host No.	Host Size (μm)	Host Area ($μm^2$)	Host Fluorescence Intensity	Bacterial Area ($μm^2$)	Bacterial Florescence Intensity	Bacterial Spot Count
1	12.8	128	16078	57	8343	4
2	14.4	162	19511	45.6	7053	5
3	16	199	17258	17.5	6840	1
4	13.1	132	13025	36.9	6716	2
5	12.5	122	14289	19.3	7287	1
6	8.3	51.9	2017	4.97	7849	1
7	14.2	155	14880	59.7	7288	8
8	14.6	167	13687	202	6881	14
9	15.8	194	25031	71.2	7509	7
10	11.7	105	14658	41.7	6892	4
11	13.1	130	12052	98.3	6500	6
12	11.3	101	14445	64.3	7029	5
13	10.7	88.9	8069	8.6	6802	1
14	12.3	115	16037	217	7278	15
15	12.5	120	14471	102	6706	11
16	15.6	187	18932	178	6745	18
17	14	130	13538	104	6965	10
18	11.3	99.4	18635	57.8	6336	4
19	12	110	18909	58.1	5348	2
20	12.9	130	13921	34.7	6569	4
21	11.3	99.4	8127	197	8461	13
22	13.7	147	7974	99	6176	7
23	12.3	116	16974	88.2	7260	8
24	12.6	124	6898	81	7299	9
25	12.3	119	14606	41.3	7401	4
26	12.4	118	15595	59	7134	6
27	12.6	124	13570	154	8883	11
28	13.5	136	15426	79.3	6995	8
29	10.3	82.9	5184	65.1	6730	3
30	13	131	18195	187	7191	17
31	13.5	140	11748	10.9	7705	2
32	12.3	120	7494	48.3	9583	2
33	13.8	149	8432	102	8338	7
34	10.2	79.2	7894	177	7686	15
35	11.8	109	11389	0	0	0
36	15.5	188	12170	8.39	9723	2
37	12.2	109	5789	40.9	7326	5
38	13.3	137	12358	14.8	6633	1
39	12.5	115	7983	0	0	0

40	11.8	109	12207	51.6	8850	7
41	13.3	136	14668	221	11525	9
42	13.5	142	18554	47.8	8093	5
43	11.9	111	14177	40	7486	3
44	12.6	122	19205	69.2	5712	3
45	13.3	137	17884	115	6393	13
46	12.9	129	13718	36	7073	3
47	13.1	130	14150	67.5	8297	4
48	14.7	165	10998	0	0	0
49	11.5	104	15798	75.5	7859	7
50	15.3	181	23793	73.1	6452	7
51	12.9	131	18124	91.6	6795	9
52	11.9	109	16710	97.4	7150	8
53	11.5	102	17267	74.4	6532	6
54	13.8	132	13483	48.9	6139	4
55	14.1	154	16448	1.76	20295	1
56	14.5	161	15834	13.6	6759	1
57	14.3	156	19581	29.6	7245	2
58	14	149	19724	44.1	7319	5
59	12.8	129	10605	16.4	8000	2
60	11.6	104	16093	149	8765	10
61	13.9	152	9107	15.9	8693	3
62	13.9	150	20148	73.8	7315	9
63	12.5	121	21649	9.64	8451	2
64	12.9	122	15111	54	7630	5
65	11.6	93.8	4812	95.5	7035	5
66	14.1	142	7915	9.95	6435	1
67	11.3	100	2053	7.88	6745	1
68	13.3	138	13223	26	8379	4
69	12	112	7287	0	0	0
70	12.5	121	11467	29.5	6768	4
71	13.3	139	18398	4.97	8918	1
72	11.9	106	11840	0	0	0
73	11.4	101	7263	112	7888	8
74	21.5	324	16766	87.2	7501	6
75	10.9	86.5	9430	98.6	6827	10
76	13.5	137	18207	19.6	6926	2
77	13.1	133	14261	19	7661	3
78	14.2	154	15608	94.3	7783	7
79	12.9	129	15776	114	7894	7
80	10.4	84.1	3532	12	7468	1
81	11.6	105	17831	9.01	9213	2
82	12.8	128	17133	0.311	908	0

83	11.1	96.2	7674	20.6	7872	2
84	15.5	173	3949	60.5	7278	8
85	11.9	109	18068	120	6760	11
86	11.4	102	15633	42	7633	6
87	12.2	115	15954	25.9	7788	1
88	12.5	115	7372	29.6	6600	3
89	12.6	121	12940	65.4	6527	5
90	14.1	155	8905	11.5	6047	1
91	15.4	185	13110	19.9	7088	3
92	11.9	109	17251	51.6	7431	7
93	13.4	134	8303	36.4	8159	3
94	15.4	175	20660	115	7260	8
95	13.5	141	5659	54.5	7029	4
96	11.4	98.9	15924	31.1	8138	4
97	11.6	101	12086	22.9	8324	2
98	11.6	101	16619	231	7340	23
99	12.4	118	13818	122	7311	8

Note: A representative image captured from A549 cells infected by *Pseudomonas aeruginosa* strain PAO1 at an MOI of 100. Host size and area represent the average diameters and areas of each stained host nuclei. Host fluorescence intensity represents the average intensity of the stained nuclei. Bacterial spot count and area represent the bacteria which are adherent to each host cell and their total areas. Bacterial fluorescence intensity represents the average intensity of all adherent bacteria.

Editorial comments:

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues. The manuscript was formatted to fit the journal standard.
Responses: The issue has been solved in the revised manuscript.

2. Please revise the text to avoid the use of any personal pronouns (e.g., "we", "you", "our" etc.).
Responses: The issue has been addressed in the revised manuscript.

3. Line 96/121: Please specify the volume of PBS used.
Responses: The volume of PBS used has been provided in the revised manuscript in Line 104/130.

4. Line 97: Vessels? Please specify the type of containers used.
Responses: The information of the used containers has been provided in the revised manuscript in line 105.

5. Line 108/111: Please specify the volume of TSB.
Responses: The volume of TSB has been included in the revised manuscript in line 120.

6. Line 136: Please specify the volume of F-12K medium used for resuspending bacteria.
Responses: The volume of F-12K medium used for resuspending bacteria has been included in the revised manuscript in line 155.

7. Line 144/151: Please mention how the washing step is performed. How much volume? What is the duration of each wash?
Responses: The required information has been included in the revised manuscript in line 167-169.

8. Line 164-167: Please include the details of magnification used to capture fluorescent images.
Responses: The details of magnification used to capture fluorescent images have been included in the revised manuscript in line 189.

Reviewer #1:

Manuscript Summary:
In this manuscript authors use a method based on fluorescence microscopy imaging and subsequent image processing and statistical analysis for quantifying the number of adhering bacteria onto host cells. For proof of principle they use different bacterial species and test their protocol by measuring the adherence of bacteria onto different host cell types.

Major Concerns:
1. Nowhere in the manuscript are images of the resulting segmentation of the objects (i.e. the bacteria) provided. Given that individual bacteria in many cases touch each other, it seems to me extremely hard at least for the higher MOIs to believe that bacteria are properly segmented and counted. E.g. look at Figure 1A first row for S. aureus MOI 10 to 100. The bacteria are clumped together, the segmentation result is not shown and as a result I don't trust much the blue boxplots in Figure 1D. So either the MOI needs to be very low to properly segment and count objects, or the fluorescence level of the bacteria needs to be used as a proxy of the number of the bacteria present per host cell, or another algorithm needs to be used to segment the objects at these MOIs. For example, one can use seed points to do the segmentation of bacteria or use deep learning approaches (e.g. see https://www.nature.com/articles/s41467-020-19866-8).

Responses: We appreciate the comments by this reviewer. To thoroughly address the concerns, we performed additional experiments and have provided convincing images of bacterial infection and segmentation (**Figure 3**). In the revised manuscript, the fluorescence intensity of bacteria was adjusted to make the image look clearer and to minimize the possibility of misreading in bacterial segmentation. Please note that the goal of this method is majorly targeting to rapidly detect the potential host-bacteria adherence, not to provide a precise count of adherent bacteria on each host cell. MOI of 100 used in this protocol is just to demonstrate the multi-dimensional difference between the positive- and negative-adherence bacteria, which is not a necessary condition in most of studies. Therefore, although there are a few inevitable mis-readings from the aggregation-featured bacteria in the condition of higher MOI (≥100), the final evaluation outcome of bacterial adherence is not affected.

2. In Table 1 more explanation is needed to understand what each row represents. By host sum count do you mean total number of nuclei in an image? What is bacteria spots ratio, is it number of bacteria per host cell? What is bacteria sum spots, total number of bacteria in the image? What is host count? It would be convincing to actually show one of these images and the segmentation to actually trust that it works out well rather than just show numbers.

Responses: More details have been included in the revised manuscript and Tables 1. Example images of how bacteria were segmented have also been shown in the revised Figure 3. The statistical analysis results from the representative images were also shown in table1 (Image 1) and Table 2. For table 1, in each image, host sum count and bacterial sum spots represent the total counts of host nuclei and total counts of host-adherence bacteria matching to the selection parameters, respectively. Bacterial spots ratio represents the average adherent-bacterial count per host cell which derives from the bacterial sum spots/ host sum count. The detailed information has also been included in the revised Table 1 and Table 2. We hope that the detailed explanation can be adequately address the reviewer's questions.

3. Table 2 is without any units at all. How is host size different from host area? Is host area in um^2? What is host fluorescence intensity and why do we care about it? Also given that the number of cells are determined based on the nucleus fluorescence how is the area of a host cell determined (since the cells are not marked in their periphery)? Are you using Voronoi tessellation? All that needs to be at least documented for one to be able to use such a protocol.

Responses: The required units have been included in the revised Table 2. In Table 2, host size represents the average of lengths and widths of a stained host cell, and host area (μm^2) is a specific field area of that stained host cells. Fluorescence intensity is an additional indictor to present the quality of images if the staining of host cells and bacterial cells is consistent. Here, we used the nuclei staining to just count host cells, while host area was roughly estimated by expanding 15 μm from stained nuclei center based on the known information of the diameter of A549 cell is 10.59–14.93 μm to rapidly determine the host-bacteria adherence. The host areas can work as the secondary evidence to simply monitor host cell healthy state. Therefore, it doesn't affect the outcomes in estimating adherent bacterial counts. In addition, if the precise cell size is required for some experiments or cell types, this protocol is also compatible with cytoskeleton staining to delineate cell boundary instead of nuclei staining.

Minor Concerns:
No minor concerns.

Reviewer #2:
Manuscript Summary:
The authors have taken into account my remarks and I am therefore satisfied with their replies and the changes made. I therefore fully support the publication of the revised version of the manuscript.

Responses: We very much appreciate the comments by reviewer #2.