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# Facile method to stain the bacterial cell surface for super-resolution fluorescence microscopy†

**Ian L. Gunsolus**[a], **Dehong Hu**[b], **Cosmin Mihai**[b], **Samuel E. Lohse**[c], **Chang-soo Lee**[d], **Marco D. Torelli**[d], **Robert J. Hamers**[d], **Catherine J. Murhpy**[c], **Galya Orr**[b], and **Christy L. Haynes**[a]

Christy L. Haynes: chaynes@umn.edu

[a]Department of Chemistry, University of Minnesota, 207 Pleasant St SE, Minneapolis, MN, 55455, USA

[b]Environmental Molecular Sciences Laboratory, Pacific Northwest National Laboratory, Richland, WA 99354, USA

[c]Department of Chemistry, University of Illinois at Urbana-Champaign, 600 South Mathews Avenue, Urbana, Illinois 61801, USA

[d]Department of Chemistry, University of Wisconsin, Madison, Wisconsin 53706, USA

## Abstract

A method to fluorescently stain the surfaces of both Gram-negative and Gram-positive bacterial cells compatible with super-resolution fluorescence microscopy is presented. This method utilizes a commercially-available fluorescent probe to label primary amines at the surface of the cell. We demonstrate eficient staining of two bacterial strains, the Gram-negative *Shewanella oneidensis* MR-1 and the Gram-positive *Bacillus subtilis* 168. Using structured illumination microscopy and stochastic optical reconstruction microscopy, which require high quantum yield or specialized dyes, we show that this staining method may be used to resolve the bacterial cell surface with sub-diffraction-limited resolution. We further use this method to identify localization patterns of nanomaterials, specifically cadmium selenide quantum dots, following interaction with bacterial cells.

## Introduction

Super-resolution fluorescence microscopy is becoming a popular method for addressing biological questions that require sub-diffraction-limited spatial resolution. Super-resolution fluorescence microscopy techniques include stochastic optical reconstruction microscopy (STORM), a type of localization microscopy[1] which requires photo-switching fluorescent dyes to achieve images with 10–30 nm spatial resolution, and structured illumination microscopy (SIM),[2] which requires dyes with high quantum yield and photo-stability to achieve images with 120–130 nm spatial resolution. An extension of traditional fluorescence microscopy, super-resolution fluorescence microscopy provides opportunities for imaging

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Correspondence to: Christy L. Haynes, chaynes@umn.edu.

intact live and hydrated cells using direct labeling of molecules and cellular structures with resolution that could previously be achieved only by electron microscopy, which requires sectioning of frozen or chemically fixed cells. Fluorescence labeling schemes are a central challenge in super-resolution fluorescence microscopy, requiring probes that have high quantum yield, excellent photostability, and in the case of STORM, dynamic fluorescence behavior (*e.g.* photoswitching or photoactivation). To date, two primary fluorescence labeling strategies have been utilized: genetically-encoded fluorescent proteins and small molecule fluorescent probes.[3] Small molecule probes provide several advantages over fluorescent proteins, including higher average quantum yields and increased labeling flexibility.[4] Continued development of suitable small molecule fluorescent probes, as well as methods for tagging cellular structures with these probes, are necessary to expand the scope of biological questions that can be addressed *via* super-resolution fluorescence microscopy.

A ripe area for the application of super-resolution fluorescence microscopy is microbiology, given that many features of microorganisms typically cannot be resolved by traditional fluorescence microscopy. Already, super-resolution microscopy has provided insight into fundamental bacterial cell biology, *e.g.* the mechanism of cell division and protein distribution and activity.[5] Here, we propose a new application of these techniques to probe the interface of bacterial cells with their extracellular environment. Our specific focus is the nanomaterial–cell interface, an area which has recently received growing attention, motivated by the potential applications of nanomaterials as antimicrobial agents and a desire to assess the potential for unintentional ecological consequences of nanomaterial release into the environment.[6] To date, researchers have relied heavily on electron microscopy to characterize both nanomaterial localization at the microbial cell membrane[7] and cellular penetration;[8] while electron microscopy provides unparalleled spatial resolution, it struggles to observe cells in their natural hydrated state. The ability of super-resolution microscopy to observe hydrated cells with nanometer resolution will provide insightful *in situ* characterization of cell–nanomaterial interactions.

Fluorescent labeling of the microorganism cell wall or surface is a necessary first step in this direction, and labeling methods have been presented in a handful of studies. Foster and coworkers monitored cell wall assembly in Gram-positive bacteria by conjugating a fluorescent vancomycin to the peptidoglycan layer at the cell surface,[9] while Moerner and coworkers labeled the Gram-negative *Caulobacter crescentus* using Cy3–Cy5 covalent heterodimers to target lysine residues at the cell surface.[10] Though these two examples are important, there is no precedent for a simple, fast, and generalizable method to label the bacterial cell wall or surface for super-resolution fluorescence microscopy.

Here, we present a labeling method for both Gram-negative and Gram-positive bacteria using a commercially available Alexa Fluor dye conjugate used commonly to label free proteins. A subset of the Alexa Fluor dyes are capable of photo-switching between dark, non-fluorescent, and bright, highly fluorescent states, and are among the limited number of fluorophores compatible with STORM or photoactivated localization microscopy (PALM).[11] The photo-switching phenomenon can be exploited to achieve images with nanometer resolution using STORM and PALM. Using this labeling strategy, which has

been employed in previous studies to label the bacterial cell surface for traditional fluorescence microscopy,[12–14] we achieve sub-diffraction limited spatial resolution of the cell wall of Gram-negative and Gram-positive bacteria using both SIM and STORM. We further use SIM to characterize the interface of the Gram-negative bacterium *S. oneidensis* with cadmium selenide quantum dots. This focus on the nanomaterial–prokaryote interface runs in parallel to studies of the engineered nanomaterial–eukaryotic cell interface, a topic which has received growing attention as the consumer product and biomedical applications of nanoparticles grow,[15] necessitating a greater understanding of cellular response to nanomaterial exposure and the material properties governing this interaction.[16]

## Experimental

### Bacterial culture preparation

**Shewanella oneidensis—**MR-1 was a giff from the lab of Jeff Gralnick at the University of Minnesota, and *Bacillus subtilis* 168 was purchased from the Bacillus Genetic Stock Center. Both were cultured on LB agar plates (BD Biosciences) from frozen stocks stored at −80 °C. These were incubated at 30 °C to achieve colony formation, and individual colonies were used to inoculate LB broth. Liquid cultures were incubated at 30 °C with 300 RPM shaking until stationary growth phase was reached as determined by optical density measurements.

### Cell staining

Cells were harvested at the stationary phase by centrifugation of 1 mL suspensions at 2000 rcf for 10 minutes. The resulting cell pellet was rinsed twice with calcium- and magnesium-free phosphate buffered saline (PBS), (Gibco, Life Technologies) without resuspending the cells. Cells were then resuspended in PBS at a cell density of $10^8$ cells per mL. (Note: staining was also successfully performed in 0.1 M bicarbonate buffer though all images presented herein were acquired from suspensions stained in PBS.) A 250 μL aliquot was removed, and then 9.4 μL of 0.5 mg $mL^{-1}$ Alexa Fluor 488 carboxylic acid succinimidyl ester (Life Technologies) in DMSO (Sigma Aldrich) was added. The resulting suspension was incubated in the dark for one hour at room temperature with frequent mixing. The suspension was then centrifuged at 2000 rcf for 10 minutes, the supernatant was discarded, and the cell pellet was resuspended in 500 μL of PBS.

### Preparation for imaging

To facilitate cell adherence to the glass surface, glass coverslip-bottom dishes were coated with poly-L-lysine by incubating the dishes with 500 μL of 0.1 mg $mL^{-1}$ poly-L-lysine (Sigma Aldrich) for four hours at room temperature, then aspirating the solution and drying overnight. The stained cell suspension was loaded onto the poly-L-lysine-coated dish and incubated for 30 minutes at room temperature to allow cell adherence. The suspension was then aspirated and replaced with 500 μL of 4% formaldehyde (Ted Pella, Redding CA) in PBS and incubated for 15 minutes at room temperature; then the solution was aspirated and replaced with PBS. SIM imaging was done in PBS. For STORM imaging, the suspension was aspirated and replaced with 100 mM 2-mercaptoethyl amine (Sigma Aldrich) in water.

### Preparation of nanomaterials

Cadmium selenide/zinc sulfide core–shell quantum dots were purchased from Life Technologies. According to the manufacturer's specifications, the principal nanoparticle size is approximately 10 nm (see ESI[†]) and the nanoparticle surface is functionalized with amino-poly(ethylene glycol) (Qdot 605: Life Technologies Q21501MP). The stock solution of quantum dots had a concentration of 8 μM.

### Cell exposure to nanomaterials

Cells were cultured in LB broth, harvested, and diluted to $10^8$ cells per mL in PBS as described above. Quantum dots, at a concentration of 250 nM, were added to the cells and incubated for 1.5 hours at 30 °C with 300 RPM shaking. Cell staining, as described above, was performed after nanoparticle exposure.

### Microscopy parameters

SIM imaging was performed on a Zeiss Elyra S1 microscope using a 100× magnification (NA = 1.46) oil-immersion objective. Excitation of Alexa Fluor 488 and cadmium selenide quantum dots was achieved using 488 nm and 561 nm laser excitation, respectively. Emission bandpass filters were 495–550 nm and 570–620 nm, respectively. The two excitation/emission color channels were recorded consecutively. Within each color channel, the raw data contained 5 rotations, 5 phases and 0.1 μm spacing *z* stack images. The super-resolution images were then reconstructed from raw images using ZEISS Efficient Navigation (ZEN) 2012 software to provide 2D and 3D projections.

STORM imaging was performed on a Zeiss Axio Observer inverted microscope using a 100× magnification (NA = 1.45) oil-immersion objective and an additional 1.6× magnification in the emission pathway of the microscope. Excitation of Alexa Fluor 488 was achieved using 473 nm laser excitation at 500 W $cm^{-2}$ and an exposure time of 0.5 seconds. The emission was acquired using a 500–550 nm bandpass filter and an electron multiplying CCD camera (iXon 897, Andor). Thousands of raw single molecule fluorescence images were acquired and processed, and STORM images were reconstructed using in-house software written in MATLAB.

Confocal fluorescence imaging was performed on a Zeiss LSM 710 laser scanning confocal microscope using a 100× magnification (NA = 1.46) oil objective. Excitation of Alexa Fluor 488 was achieved using 488 nm excitation, and the emission was acquired in the 499–592 nm spectral window. Optical sections were taken at 1 μm thickness, and images were processed in ZEN 2012 software.

## Results and discussion

The cell surfaces of both Gram-positive *Bacillus subtilis* 168 and Gram-negative *Shewanella oneidensis* MR-1 were labeled using an amine-reactive fluorescent probe (Alexa Fluor 488 carboxylic acid, succinimidyl ester) that binds to primary amines found in outer-membrane

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proteins, peptide residues in the surface peptidoglycan layer of Gram-positive bacteria, and lipopolysaccharides on the surface of Gram-negative bacteria (present in *e.g. o*-phosphorylethanolamine).[17] By utilizing an unmodified, commercially-available fluorescent probe and requiring minimal sample preparation, this method offers a simple alternative to existing super-resolution fluorescence microscopy-compatible fluorescence labeling methods and is compatible with both SIM and STORM. This method of live-cell labeling is non-trivial: labeling efficiency depends greatly on the reaction conditions, especially protein concentration, as specified by the manufacturer.

Successful labeling of Gram-positive cells is demonstrated in Fig. 1, which compares the resolution obtained using laser-scanning confocal microscopy (left) and SIM (right). While confocal microscopy resolves individual cells, it is less capable than SIM of resolving the cell surface from the cell interior.

Gram-negative bacteria were also successfully labeled and imaged with SIM using identical sample preparation and imaging conditions (Fig. 2 left). One major difference between using this method to perform SIM and STORM is that a thiol-containing buffer, 2-mercaptoethyl amine in this case, must be used to achieve STORM because this allows photoswitching of the Alexa Fluor 488.[18] Without a thiol group present, Alexa Fluor 488 fluoresces continuously upon irradiation as it cycles primarily between the singlet ground and excited states. In the presence of a thiol, the triplet state of the excited fluorophore can react to form a radical anion, resulting in a dark state that is stable on the millisecond to second time-scale. The fluorescent state is recovered through oxidation by molecular oxygen. Unlike SIM, which is, in principle, compatible with any fluorophore, STORM requires fluorophores capable of cycling repeatedly between dark and bright states.[19] This requirement places a strict limitation on the fluorophores available for STORM and makes this facile approach especially powerful. Using the approaches described in Thompson *et al.* and Mortensen *et al.*,[20,21] we estimate the localization precision achieved in STORM images to be 30–40 nm (see ESI†), compared with the resolution in SIM images, which is 120–130 nm in the lateral dimension, according to the manufacturer's specifications.

The imaging scheme presented here expands the range of biological systems that may be imaged by super-resolution fluorescence microscopy by providing a simple method to fluorescently label either Gram-positive or Gram-negative bacteria. This approach has the potential to facilitate a wide range of biological fluorescence imaging studies because it targets primary amines, a ubiquitous biological functional group, using a bright, stable, and commercially-available fluorophore.

To demonstrate the potential utility of this imaging scheme, we characterized the localization pattern of an engineered nanomaterial (cadmium selenide quantum dots) with bacterial cells. Such characterization is of great importance to understanding and exerting control over the engineered nanomaterial–biological interaction, and is motivated by the increasing production of nanomaterials and the corresponding increase in the likelihood of their entry into natural environments;[22] given the fundamental role of bacteria in the ecosystem, the consequences of their interactions with engineered nanomaterials (*e.g.* to cell viability and function) may be significant to ecosystem health.[6] Electron microscopy (EM)

is the current standard for qualitative analysis of bacterial cell–engineered nanomaterial interactions, but requires significant sample preparation that often precludes imaging in the natural hydrated state, has limited options for cell labeling, and is not compatible with low atomic mass nanoparticles (*e.g.* carbon-based materials). Super-resolution fluorescence microscopy can be used to observe live, intact, and hydrated cells with greater resolving power than traditional fluorescence microscopy and greater flexibility in terms of sample preparation and labeling than EM. As a result, super-resolution fluorescence microscopy will give significant insight into nanomaterial–cell interactions.

Herein, the utility of super-resolution fluorescence microscopy for characterizing nanomaterial–cell interactions was demonstrated by imaging *Shewanella oneidensis* MR-1 after 1.5 hour interaction with fluorescent cadmium selenide/ zinc sulfide core–shell quantum dots. A representative image from this analysis is shown in Fig. 3. Clusters of quantum dots of approximately 10–15 nm diameter (resolved by transmission electron microscopy, see ESI[†]) with amino-poly(ethylene glycol) surface functionalization associate with the cell surface with, at best, partial penetration into the cell membrane. Lack of nanoparticle internalization may be attributed to the rigid cell structure of Gram negative bacteria and lack of endocytosis pathways for species that cannot diffuse through outer-membrane porins. The role of outer-membrane components in preventing nanomaterial permeation of the cell membrane will be evaluated in future work.

Traditional laser-scanning confocal microscopy provides insufficient resolution to definitively identify the location of cell-bound quantum dots (inside the cell *vs.* on the cell surface *vs.* embedded in the extracellular polymeric matrix). A representative image is shown in Fig. 4. Comparison of Fig. 3 and 4 demonstrates the power of super-resolution fluorescence microscopy for characterizing the nanomaterial–biological interface in the natural hydrated state.

## Conclusions

By targeting primary amines on the cell surface, the method presented here provides a facile means to label the surface of both Gram-positive and Gram-negative bacteria and is compatible with dyes required for super-resolution fluorescence microscopy. Requiring less than two hours of sample preparation time and using only commercially-available materials, this method will make super-resolution fluorescence microscopy more available to researchers lacking experience in designing and preparing novel fluorophores and tagging approaches. Using this method, we have observed quantum dot localization at the membrane of hydrated, Gram-negative bacterial cells without membrane penetration. This method will facilitate the application of super-resolution fluorescence microscopy to address new questions in microbiology, for example, assessment of structural changes under environmental stress or interactions with nanomaterials at the cell membrane of hydrated cells.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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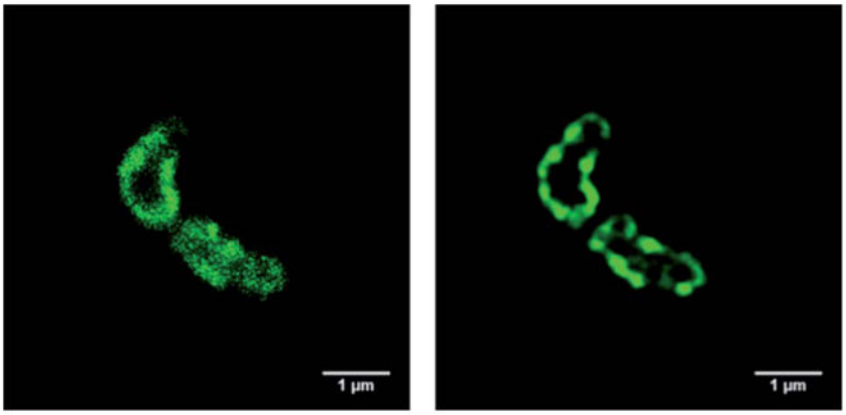


**Fig. 1.**

Confocal (left) and SIM (right) images of stained *Bacillus subtilis* 168 adhered to a poly-L-lysine-coated glass surface and immersed in PBS. A single optical section from a *z*-stack is shown in both images.

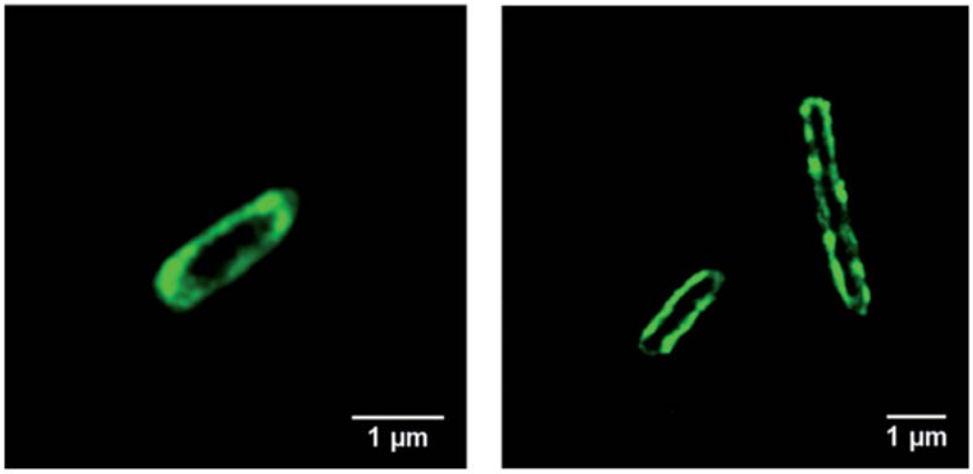


**Fig. 2.**

SIM (left) and STORM (right) images of stained *Shewanella oneidensis* MR-1 adhered to a poly-L-lysine-coated glass surface and immersed in PBS for SIM or 2-mercaptoethyl amine for STORM.

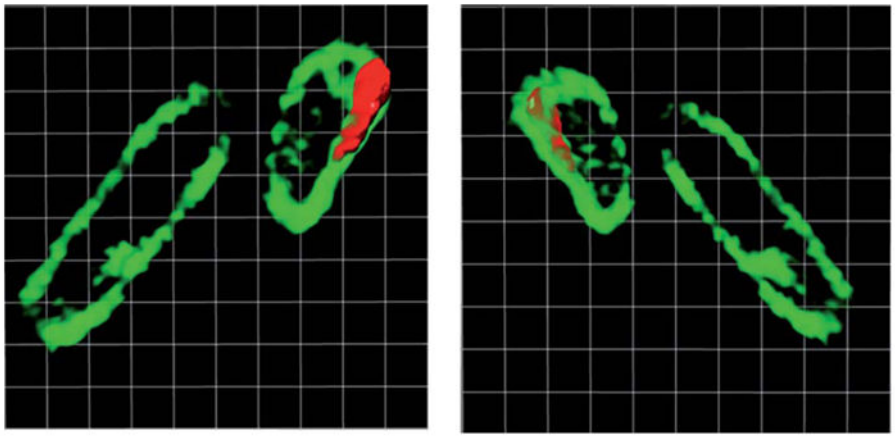

**Fig. 3.**

Left: SIM image of *Shewanella oneidensis* MR-1 with associated amino-PEG quantum dots (quantum dots in red and bacterial cell wall in green). Colocalization of the quantum dots and the bacterial cell wall is evident, and quantum dot internalization is not observed. Right: view upon 180° rotation along the vertical axis in the image plane. Each square has side length 0.42 μm.

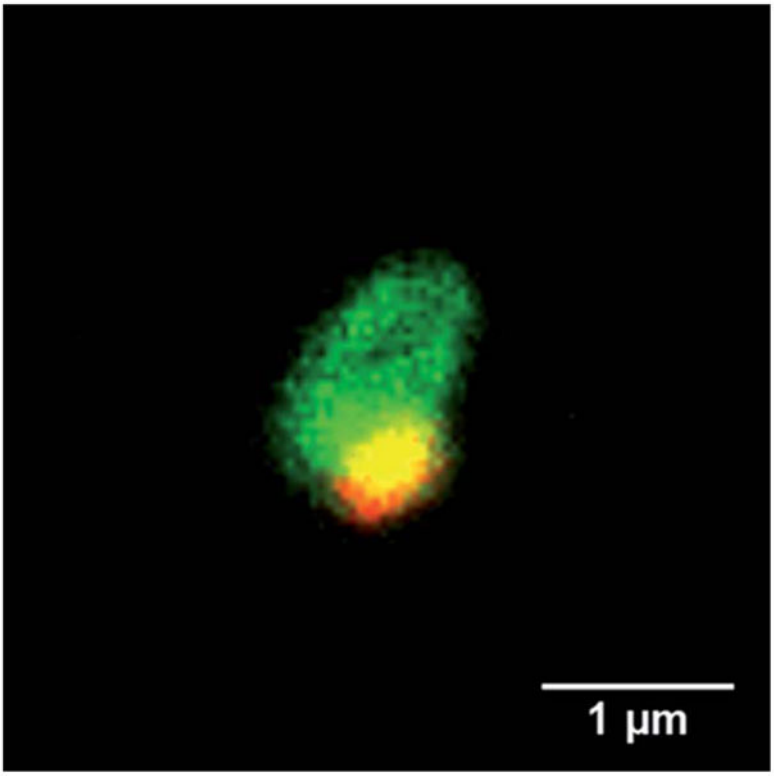


**Fig. 4.**

Laser-scanning confocal microscopy image of *Shewanella oneidensis* MR-1 with associated amino-PEG quantum dots (quantum dots in red and bacterial cell wall in green).