Metal-Enhanced Fluorescence to Quantify Bacterial Adhesion

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Bacterial adhesion, the first step of biofilm formation, is of fundamental significance for multiple industries (e.g., petroleum recovery, food processing, drinking water, medicine and healthcare, shipping, or pulp and paper production) due to the huge economic costs associated with biofilm formation. Assays to monitor bacterial adhesion are the key to elucidate mechanisms of colonization and biofilm formation. However, the existing microscopy tools typically used to monitor bacterial adhesion are based on observations of bacterial colony formation. In particular, the current optical toolboxes qualitatively define cell adhesion as a simply physically stable association. They neither allow for studying the three-dimensional distribution of bacteria associated with a surface nor for molecular-level analysis of the bacterial interactions that mediate close contact. While some effort has been made in recent years to monitor the adhesion of bacteria populations in real-time using surface plasmon resonance, those techniques do not allow for individual cells to be tracked and provide no quantitative information regarding contact distance so far.[1] Here, we demonstrate the possibility of obtaining precise information on the three-dimensional distribution of bacteria coming into contact with a surface and propose this new optical technique as a method to monitor and probe individual bacterial adhesion.

In our experimental approach (**Figure 1**a), a flat gold surface of 35 nm thickness was used as the basic substrate for cell adhesion and was prepared by a standard electron beam lithographic technique. Previous reports showed that field enhancement is maximal for 30–60-nm-thick gold layers, and the thickness (35 nm) used in our studies was in this range.[2] The morphologies of the glass substrate and gold surfaces were investigated using atomic force microscopy (AFM), and the root-mean-square (RMS) roughness of the gold-coated surface was similar to the one of the glass substrate (Figure S1 in the Supporting Information). The membranes of *Pseudomonas aeruginosa* strains (PA14 wild type, PA14 *pilB*, or PA14 *flgK*::Tn5 Tet) were labeled with FM4-64, a red fluorescent dye, as it provided the most uniform and even staining of the various dyes we examined (Table S1 in the Supporting Information).[3] Wild-type PA14 in close contact with a gold substrate showed a strong increase of FM4-64 fluorescence compared to cells in contact with the adjacent glass substrate (Figure 1b and demonstration movies in the Supporting Information). The fluorescence enhancement was hypothesized to mirror the distance between the cell and the gold surface and to provide a tool to quantitatively monitor bacterial proximity to surfaces. The behavior of individual bacteria on gold surfaces was analyzed next. First, the residence time of the bacteria, defined by the length of time the bacteria exhibited enhanced fluorescence, was analyzed, and a strong correlation was noted between the fluorescence intensity of the wild-type cells at the initial surface contact and their subsequent residence time (Figure 1c). In cases where the fluorescence intensity was increased about three times, cells showed a long-term stable positioning on the substrate. Based on this analysis, a more than threefold increase of fluorescence intensity was chosen as an indicator for sufficiently close contact to mediate stable cell adhesion.

The fluorescence enhancement noted in these cell-attachment studies can arise from at least two mechanisms: surface plasmon resonance[4] and a mirror effect.[6] Excellent overlap of the dye absorption/emission spectrum with the scattering spectrum of the gold surface is required for effective surface plasmon resonance,[5] and FM4-64 shows a broad absorption and emission spectrum, which provides a good overlap with the gold scattering spectrum (Figure S2 in the Supporting Information). Importantly, the degree of metal–fluorophore interactions strongly depends on the distance between the dye and the surface, providing a nanoscale ruler. The other possible mechanism involves a mirror effect on the metal surface by the Raman-scattered field, as a molecule near a perfect conducting metal surface is illuminated by both a direct and a reflected field, yielding an up to fourfold increase of the original intensity.[6] The fluorescence enhancement in these cell adhesion experiments is likely a result of both mechanisms.

The terms "adhesion" or "close contact" in bacterial adhesion studies are often used in a qualitative sense, as methods to quantitatively define adhesion are very limited. The method described in the present study, which provides a more molecular-level insight of the contact between a cell and a surface, is suitable to yield a precise quantitative relationship. To determine the specific distance (*z*-direction) between the bacteria and the surface, a correlation between the fluorescence intensity of the dye and its distance from the surface was first established. The distance between the FM4-64 dye and the gold surface

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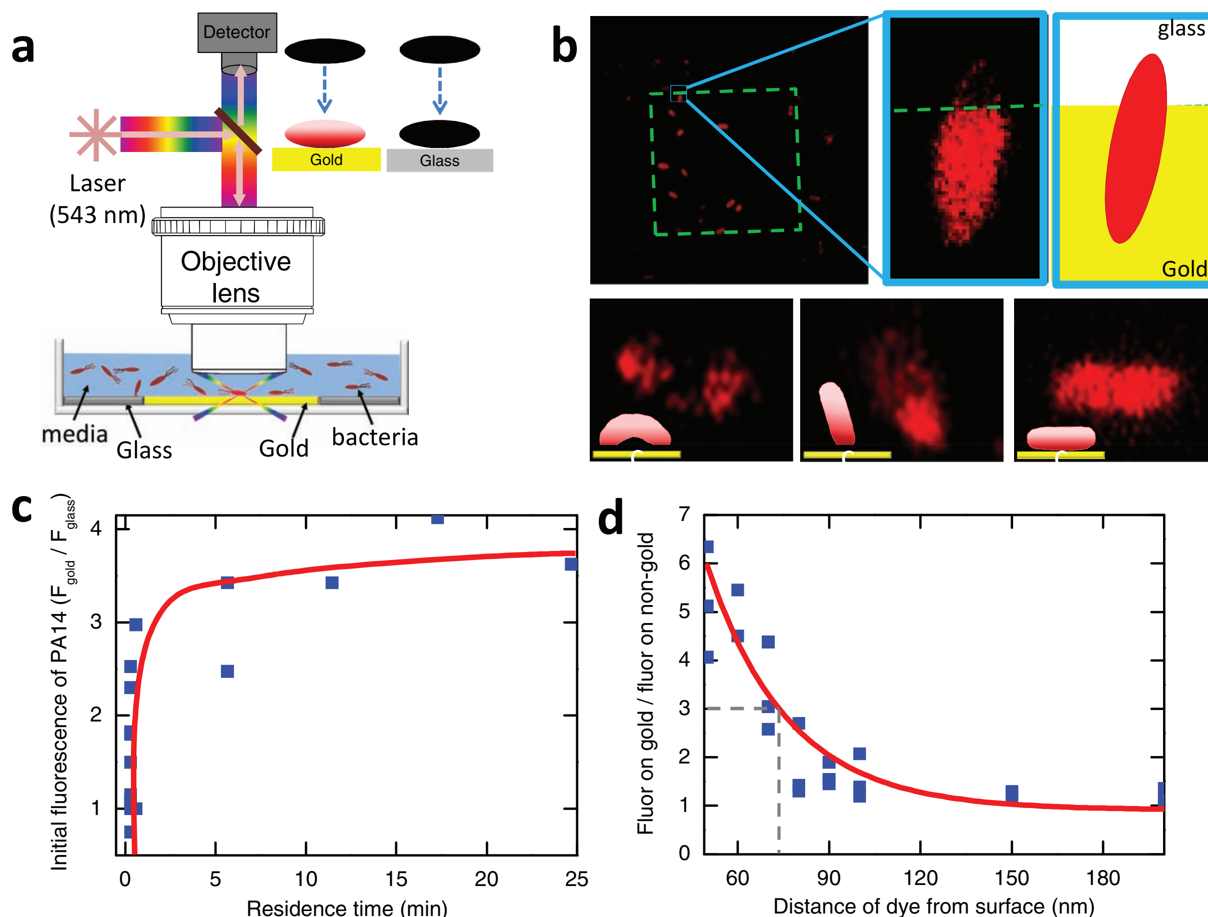

Figure 1. a) Schematic diagram of a metal-enhanced fluorescence technique for a living cell adhesion assay. A gold substrate prepared by e-beam lithography and metal-deposition was placed on a plastic dish. Cells were labeled with FM4-64 dye and suspended in media. Time-lapse fluorescence images were obtained by excitation with a 543 nm laser and analyzing emission at 560 nm long band pass filter (scanning time: 3.93 s, interval: 2 s). A water-immersion lens (100×/1.0) was used in this study. b) Representative images of fluorescence enhancement upon cell attachment to gold surfaces. The dotted green line indicates the edges of the gold layer. A magnified image of a cell sitting on the edge of the gold shows the enhanced fluorescence on the gold side. Images at the bottom show various modes of adhesion to the substrate and the corresponding fluorescence images. c) Initial fluorescence intensity of labeled cells versus residence time on the surface. Each spot represents an individual bacterium. d) Measured intensity profile of fluorescence as thickness of silica between the gold and fluorophore is altered. The *y*-axis represents the relative fluorescence on gold, compared to that on glass.

was controlled by depositing a silica layer of varying thickness onto the gold using electron-beam evaporation (Figure S3 in the Supporting Information). After deposition of silica layers with controlled thicknesses, the silica layer was reacted with dodecyltriethoxysilane to render the surface hydrophobic prior to the adsorption of FM4-64. The hydrophobic alkyl monolayer is required because the fluorescence intensity of FM4-64 is strongest in hydrophobic sites such as cell membranes, and the alkyl monomer similarly leads to enhanced FM4-64 fluorescence. A calibration curve of fluorescence intensity versus silica thickness was then obtained (Figure 1d). Fluorescence enhancement was not observed at large distances between dye and surface (>100 nm). However, the fluorescence intensity abruptly increased and showed a maximum at ≈40 nm separation. The fluorescence subsequently decreased with closer contact, likely due to Förster type energy transfer at very close contact (<10 nm).[2] Although the intensity normal to the gold surface obtained in this experiment decreases for distances less than 40 nm to the gold surface, the bacterial cells being examined are much larger (sizes about a few hundred nanometers); this leaves the bigger part of the cell body at 40 nm (leading to a maximal signal) or larger distances from the surface even if some part of the cell gets closer to the gold surface than 40 nm. Therefore, the calibration curve can be utilized to calculate the minimum distance (using the measured maximum fluorescence of the cell) of a cell from the surface. Strikingly, the fluorescence enhancement that correlated with stable residence (Figure 1c) corresponded to a distance of <75 nm. These results lead us to propose a quantitative definition of bacterial adhesion to a surface: a bacterium separated from an adhesion substrate by less than 75 nm has formed a stable adhesion to the surface.

As fluorescence enhancement upon close bacterial contact to gold substrates allows to follow adhesion readily in a real-time, non-destructive manner, the utility of this technique and the resulting definition for monitoring the adhesion of various types of bacterial strain was investigated. Three different strains were examined; a wild type (PA14 wild type), a pili knockout (PA14 *pilB*), and a flagella knockout (PA14 *flgK*::Tn5 Tet).

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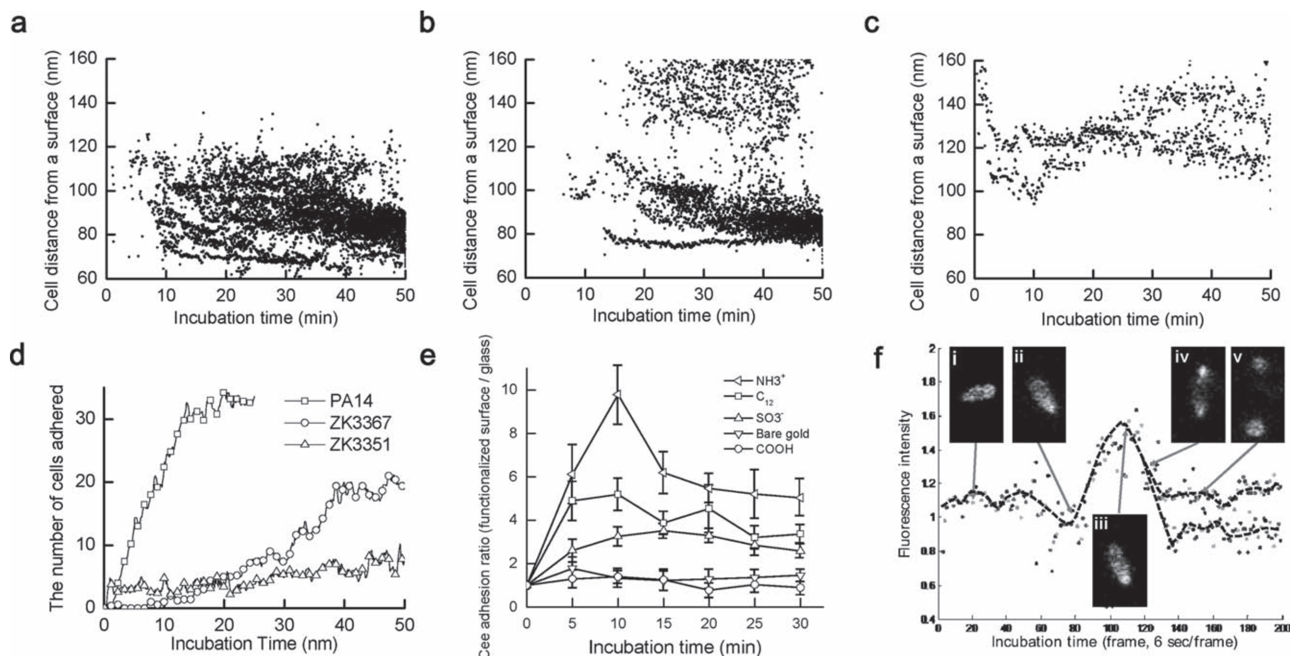

Figure 2. Distance profiles of the adhered wild-type cells (PA14) (a), ZK3367, a flagella knockout (PA14 *flgK*::Tn5 Tet) (b), and ZK3351, a pili knockout (*pilB*) (c) as a function of time after plating. For these data, a series of time-lapse images was taken for 50 min immediately after cell seeding (scanning time: 3.93 s, interval: 2 s). Data were plotted from single experiments and repeating the experiment several times showed reproducibility. Each spot represents an individual bacterium. The cell distance was calculated using the equation obtained from the fitted calibration curve. The same concentration of cells was incubated in all experiments [0.1 O.D. (optical density) at 600 nm]. The mean fluorescence from each cell was used to calculate the distances. d) The number of adherent cells (showing enhanced fluorescence) as a function of time. e) Surface functionality effects on bacterial adhesion. Gold surfaces were modified by standard thiol chemistry prior to use. The number of cells showing fluorescence enhancement were quantified as a function of time. Values represent mean and standard deviation. f) Tracking a single cell upon division into two daughter cells. All dots originate from the same cell over time until after cell division, as two cells were then tracked. The lines represent fluorescence intensity (smoothening) for each cell over time. The optical density at 600 nm was measured for each experiment and bacterial concentrations were adjusted to give the same optical densities for each experiment and each phenotype (O.D. = 0.1).

Flagella and pili are surface organelles that are required for swimming and twitching motility, respectively.[3,7] Both processes are involved in mature biofilm formation. For this analysis, only bacterial cells that showed a stable enhanced fluorescence, indicative of minimum movement (stationary state) were taken into account. Cells showing a transient enhanced fluorescence represented cells randomly moving in and out of close contact (mobile state) were excluded from analysis. Processing of time-lapse images was performed via cell imaging software followed by conversion of the fluorescence intensity into the distance from the surface using the calibration curve (Figure 1d, details provided in the Supporting Information). Wild-type PA14 cells approaching the gold surface began to show a strongly enhanced fluorescence only a few seconds after cell seeding (**Figure 2**a). Over time, a large number of cells made close contact (<100 nm) to the surface and formed a stable association with the substrate. Flagella mutant cells (PA14 *flgK*:: Tn5 Tet) showed very little swimming motility due to the absence of flagella (not shown). They approached the surface by gravitational sedimentation, which led to a 10–15 min time delay for surface contact (Figure 2b). However, once these cells approached the surface, they attached firmly, as indicated by a noticeable enhancement of fluorescence intensity that is comparable to that of PA14. Pili mutants (PA14 *pilB*) showed active swimming motility in media, comparable to the motility of wild-type PA14 (not shown), but they did not show a prominent fluorescence enhancement, indicating they do not adhere to the surface (Figure 2c). Instead, they showed an unstable and transient association with the surface (around 120 nm away from the surface) with little enhancement of emission. This result is in good agreement with previous findings, which indicate that type IV pilus retraction is a predominant driving force for the adhesion of *P. aeruginosa* on surfaces.[1b,8] The number of cells that met our definition of adhesion was quantified (Figure 2d). This analysis demonstrated a noticeable difference between the strains, suggesting this new tool is useful to image and quantify cell adhesion in an absolute, real-time and non-destructive manner.

The gold surfaces can be modified with various chemical functionalities of interest to investigate the effect of surface chemistry on the adhesion of cells. For this purpose, the gold surface was altered with different functionalities, including amine, carboxylic, sulfonate, and alkyl chains; again only cells showing fluorescence enhancement indicating close contact were counted (Figure 2e). Qualitatively, the number of cells adhering to gold, as measured by fluorescence enhancement and the new definition of adhesion, was similar to that on a glass surface (the stationary cells were counted using cell imaging software). The surfaces of PA14 are negatively charged because of the presence of membrane proteins and other wall and cell membrane components that contain phosphate

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or carboxylic and other acid groups. PA14 would be expected to adhere preferredly to hydrophobic and positively charged surfaces, and the gold surface modified with positively charged amines showed the highest ratio of adhesion.[9] When compared with a plain gold surface, the degree of bacterial adhesion to the amine gold surface was about 6.3 times higher. A hydrophobic dodecyl-modified gold surface also showed a high degree of cell adhesion (3.4× compared to a plain gold surface). Even though sulfonic acids are strong acids, the corresponding sulfonates are weak bases, and a positive counterion acting as a Lewis acid likely provided a favorable adhesion substrate for cells (2.7× compared to a plain gold surface). Hydrophilic and negatively charged carboxylic surfaces suppressed cell attachment, compared to an amine or an alkyl surface and even the bare gold surface, likely due to the electrostatic repulsion between the cells and carboxylic groups. This technique can also be utilized to rapidly and easily quantify cell adhesion as the composition of the culture medium is varied (Figure S4 in the Supporting Information).

Finally, individual cells in the process of proliferation could be tracked over time with this assay (Figure 2f). The cell fluorescence was initially high, indicating that the cell was "sitting" in a fairly flat manner on the surface (stage i). Before dividing, the fluorescence first slightly decreased, suggesting that the "sitting posture" changed to a one-point contact (stage ii). However, close cell contact on the surface subsequently resumed, as indicated by an even higher fluorescence intensity at one end of the cell (stage iii). After a short time, both sides of the cell showed intense fluorescence, indicating that both ends were in close proximity to the surface, and this change corresponded with the cell dividing into two daughter cells that could be individually tracked, and demonstrated different fluorescence intensities (stage iv–v).

In summary, a fast and facile optical method to monitor and probe three-dimensional bacterial interactions with a surface by metal-enhanced fluorescence was developed in this study, and a quantitative definition of bacterial adhesion is proposed. A gold surface with a roughness comparable to a glass surface (control) was prepared by electron-beam lithography and deposition technique. The gold surface provides a cell-adhesion efficiency comparable to a glass substrate and allows for facile introduction of various functionalities by simple chemistry. Other types of metal-coated thin films showing fluorescence enhancement potentially can also be used with this assay. A prominent enhancement of fluorescence intensity was observed by electronic coupling of the gold surface and fluorescence-labeled cells upon close contact to the gold surface. This technique provides a powerful new tool to investigate cell behavior as they approach surfaces and may provide a tool to determine thermodynamic parameters for cell binding in future studies. Furthermore, this method is applicable to study a variety of cell–substrate chemistry or cell–cell interactions and may potentially be applied to studies of mammalian cell adhesion.

Experimental Section

Details on the preparation of gold substrate, strains and growth conditions, bacteria staining, further discussion of cell staining, microscopy, media effects on adhesion behavior of PA14 on gold surfaces, imaging processing of fluorescently-enhanced cells, gold surface modification, calibration curve, and a movie file of fluorescence enhancement upon cell adhesion can be found in the Supporting Information.

Supporting Information

Supporting Information is available from the Wiley Online Library or from the author.

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[1] a) A. T. Jenkins, R. H. ffrench-constant, A. Buckling, D. J. Clarke, K. Jarvis, *Biotechnol. Prog.* **2004**, *20*, 1233; b) A. T. A. Jenkins, A. Buckling, M. McGhee, R. H. ffrench-Constant, *J. Roy. Soc. Interface* **2005**, *2*, 255; c) C. Garcia-Aljaro, X. Munoz-Berbel, A. T. Jenkins, A. R. Blanch, F. X. Munoz, *Appl. Environ. Microbiol.* **2008**, *74*, 4054.
[2] *Handbook of Surface Plasmon Resonance*, (Eds: R. B. M. Schasfoort, A. J. Tudos), Royal Society of Chemistry, Cambridge, UK **2008**, Ch. 1, 2, 3, and 9.
[3] G. A. O'Toole, R. Kolter, *Mol. Microbiol.* **1998**, *30*, 295.
[4] a) C. D. Geddes, J. R. Lakowicz, *Fluorescence* **2002**, *12*, 121; b) K. Aslan, I. Gryczynski, J. Malicka, E. Matveeva, J. R. Lakowicz, C. D. Geddes, *Curr. Opin. Biotech.* **2005**, *16*, 55; c) P. P. Pompa, L. Martiradonna, A. Della Torre, F. Della Sala, L. Manna, M. De Vittorio, F. Calabi, R. Cingolani, R. Rinaldi, *Nat. Nanotechnol.* **2006**, *1*, 126.
[5] Y. Chen, K. Munechika, D. S. Ginger, *Nano Lett.* **2007**, *7*, 690.
[6] M. Moskovits, *Rev. Mod. Phys.* **1985**, *57*, 783.
[7] J. S. Mattick, *Annu. Rev. Microbiol.* **2002**, *56*, 289.
[8] a) P. Doig, T. Todd, P. A. Sastry, K. K. Lee, R. S. Hodges, W. Paranchych, R. T. Irvin, *Infect. Immun.* **1988**, *56*, 1641; b) M. Klausen, A. Heydorn, P. Ragas, L. Lambertsen, A. Aaes-Jorgensen, S. Molin, T. Tolker-Nielsen, *Mol. Microbiol.* **2003**, *48*, 1511.
[9] M. Fletcher, *Bacterial Adhesion: Molecular and Ecological Diversity*; Wiley, New York, USA **1996**.

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