

A Covalent Reporter of β -Lactamase Activity for Fluorescent Imaging and Rapid Screening of Antibiotic-Resistant Bacteria

Qing Shao,^[a] Yan Zheng,^[b] Xueming Dong,^[c] Kai Tang,^[c] Xiaomei Yan,^{*,[b]} and Bengang Xing^{*,[a]}

Abstract: Bacterial resistance to antibiotics poses a great clinical challenge in fighting serious infectious diseases due to complicated resistant mechanisms and time-consuming testing methods. Chemical reaction-directed covalent labeling of resistance-associated bacterial proteins in the context of a complicated environment offers great opportunity for the in-depth understanding of the biological basis conferring drug resistance, and for the development of effective diagnostic approaches. In the present study, three fluorogenic reagents LRBL1–3 for resistant bacteria

labeling have been designed and prepared on the basis of fluorescence resonance energy transfer (FRET). The hydrolyzed probes could act as reactive electrophiles to attach the enzyme, β -lactamase, and thus facilitated the covalent labeling of drug resistant bacterial strains. SDS electrophoresis and MALDI-TOF mass spectrometry characterization confirmed that these

probes were sensitive and specific to β -lactamase and could therefore serve for covalent and localized fluorescence labeling of the enzyme structure. Moreover, this β -lactamase-induced covalent labeling provides quantitative analysis of the resistant bacterial population (down to 5%) by high resolution flow cytometry, and allows single-cell detection and direct observation of bacterial enzyme activity in resistant pathogenic species. This approach offers great promise for clinical investigations and microbiological research.

Keywords: β -lactamase • cell cytometry • covalent labeling • FRET • fluorescent probes

Introduction

The remarkable increase of bacterial drug resistance has been considered among the highest concerns towards human healthcare, both within hospitals and in the community. The growing prevalence of antibiotic resistance calls for rapid detection of resistant strains and systematic understanding of the biological basis conferring bacterial drug resistance. One most common method for the study of bacterial susceptibility is the growth of cultures in antibiotic containing media.^[1] Although well-established, this method is labor intensive and time-consuming, and cannot offer direct

observation of intrinsic resistance in individual living cells.^[1] Optical imaging techniques enable rapid, direct and sensitive visualization of biological events at single-cell resolution, and thus have become powerful tools in monitoring subcellular protein dynamics and analysis of pathogen–host interactions.^[2] However, imaging of antibiotic resistance has been limited mainly by the lack of appropriate reagents to report the resistance and to produce essential signal contrast for reliable analysis. Incorporation of green fluorescent protein (GFP) or its color variants has allowed cellular detection of resistant genes^[3] and whole-body imaging of antibiotic response to bacterial infections,^[4] but the laboratory strains expressing foreign genes are not identical to native bacterial samples. The large size of such fluorescent tags (~27 kDa) may potentially disturb protein function or levels.^[5] Approaches to imaging native bacterial strains and studying their drug resistance usually involve staining with fluorescently labeled affinity groups, such as metal complexes, bacteria-binding peptides, antibodies and bacteriophages.^[6] However, most of these modifications are associated with noncovalent interactions and have inadequate specificity to pathogens with potent resistance.^[6f] Direct observation of resistant bacteria can be achieved by fluorescent antibiotic derivatives on the basis of their different activities towards antibiotic resistant and susceptible strains.^[7] But the intrinsic affinity between these fluorescent drug conjugates and bacteria may present concerns for specificity. To avoid nonspecific binding or adsorption thus requires more powerful designs to effectively report antibiotic resistance.

[a] Q. Shao, Prof. B. Xing
Division of Chemistry and Biological Chemistry
School of Physical and Mathematical Sciences
Nanyang Technological University (Singapore)
E-mail: bengang@ntu.edu.sg

[b] Y. Zheng, Prof. X. Yan
The Key Laboratory of Analytical Sciences
The Key Laboratory for Chemical Biology of Fujian Province
Department of Chemical Biology
College of Chemistry and Chemical Engineering
Xiamen University, Xiamen, Fujian, 361005 (P. R. China)
E-mail: xmyan@xmu.edu.cn

[c] X. Dong, Prof. K. Tang
School of Biological Sciences
Nanyang Technological University (Singapore)

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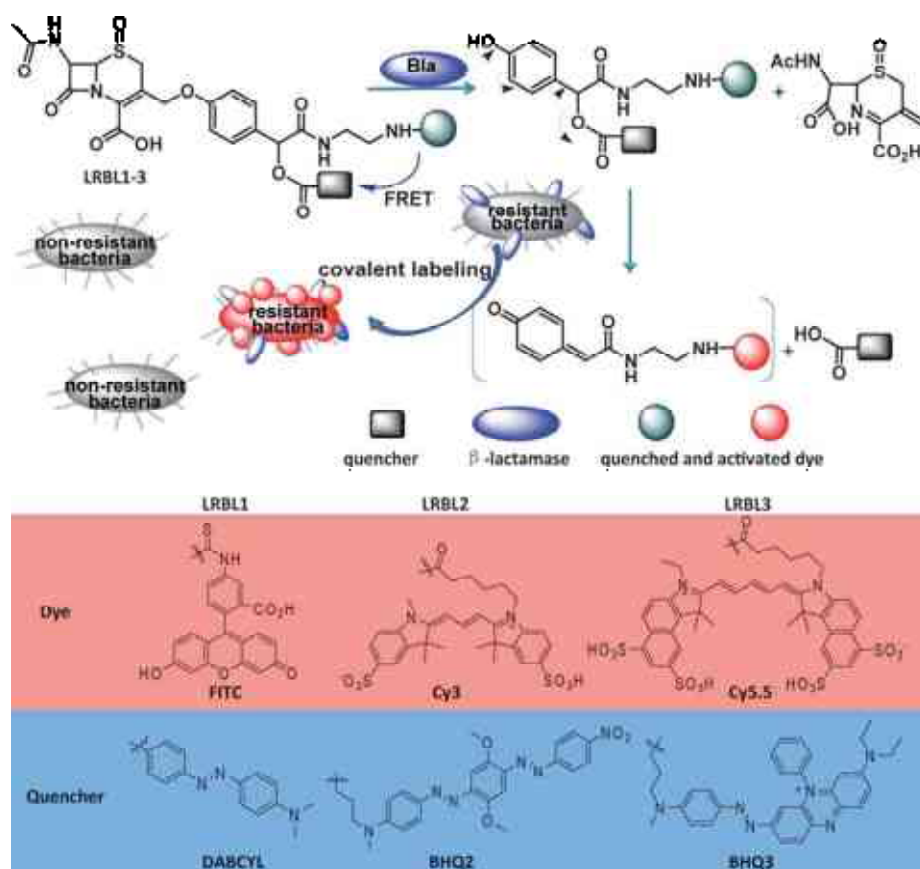
Recently, reporter enzyme fluorescence (REF) has been successfully employed to non-invasively image resistant bacterial infections in vitro and in vivo with tunable emission enhancement.^[8] In these designs, fluorescent turn-on probes can be efficiently activated by the resistance-associated β -lactamase (Bla), a naturally occurring bacterial enzyme that destroys penicillin and cephalosporin antibiotics. Targeting Bla presents unique advantages, as these enzymes are exclusively produced by antibiotic-resistant bacteria, which ensures labeling specificity with minimum interference from endogenous counterparts in mammalian cells. In addition, as a key element to deal with bacterial resistance and treatment of bacterial infections, the crystal structure and molecular mechanism of one commonly encountered β -lactamase, *Escherichia coli* TEM-1, a plasmid mediated bacterial enzyme, has been well investigated.^[9] These understandings provide great opportunity for systematic profiling of enzyme activation, and thus significantly facilitate the development of new imaging substrates with improved enzymatic reactivities. Currently, several imaging probes have been designed to readily identify the recombinant Bla reporters in mammalian cells or endogenous Bla in bacterial pathogens.^[10] Most of them employ sensitive enzyme substrates with well-suited fluorescence resonance energy transfer (FRET) pairs.^[8,10a-c] Upon enzyme hydrolysis, the intense on-off fluorescence switch or emission shift can provide essential signals for investigating enzyme activity or quantitatively imaging antibiotic-resistant pathogens in living animals with high sensitivity and specificity.^[8a,b] Although all of these prior studies have been significant and successful in vitro and in vivo, a covalent labeling capability toward the straightforward and in situ recognition of endogenous bacterial Bla molecules that prevents probe diffusion in living tissues will still be greatly appreciated;^[11] relevant investigations have not been fully exploited so far.

Inspired by previous successful studies, we describe the rational design of novel optical probes for detecting Bla and covalent fluorescent labeling of antibiotic-resistant bacteria. More importantly, unlike the traditionally used fluorescence or culture methods to determine the susceptibility of microbes, this β -lactamase-responsive bacterial labeling (LRBL) approach may greatly

reduce the background by minimizing the diffusion of the activated probes in living tissues, and thus shows the feasibility to perform reliable and rapid observation and differentiation of clinical resistance, especially for the systematic investigation of individual cells in a mixed population.

Results and Discussion

Scheme 1 illustrates the rational design of LRBL reagents. The labile *p*-hydroxybenzylic esters bearing FRET-quenched fluorescent tags were connected to the Bla-sensitive cephalosporin structure, at which point the sulfide bond was oxidized to sulfoxide for improved stability. Upon Bla hydrolysis, the released *p*-hydroxybenzylic derivatives underwent 1,6-elimination to generate fluorescent quinone-methide intermediates for the localized covalent labeling. Compared with conventional activity-based probes (ABPs) that directly interact with catalytic amino acid residues in the enzyme structure,^[12] formation of the quinone-methide nucleophilic traps requires enzymatic cleavage of a precursor and successive spontaneous elimination.^[13] In the process of these cascade reactions for effective covalent fluorescence attachment, the activated probes may not work as suicide inhibitors, thus largely maintain the enzyme activities to enhance



Scheme 1. Reaction process of enzyme-responsive fluorescent probes (LRBL1–3) for covalent labeling of antibiotic-resistant bacteria.

the fluorescent signals for single cell observation. In this study, three fluorescent molecules with different characteristics were used, including fluorescein, water-soluble Cy3 and near-infrared Cy5.5, to provide tunable emission properties, which could greatly facilitate the microscopic imaging and high-sensitivity flow cytometry (HSFCM) analysis. To maximize the signal-to-noise ratio, the selected fluorophores were prequenched by DABCYL, BHQ2 and BHQ3 moieties, respectively, and the labeling process was accompanied with recovered fluorescence that was employed for direct observation and screening of antibiotic-resistant bacterial strains. These probes were synthesized, purified by reverse phase HPLC and further characterized by NMR and mass spectrometry (see the Supporting Information).

Firstly, the enzymatic hydrolysis of LRBL1–3 was studied by measuring the changes in their fluorescence emission upon the addition of TEM-1 Bla in PBS buffer (0.1 M, pH 7.2). As shown in Figure 1, the probes were almost non-

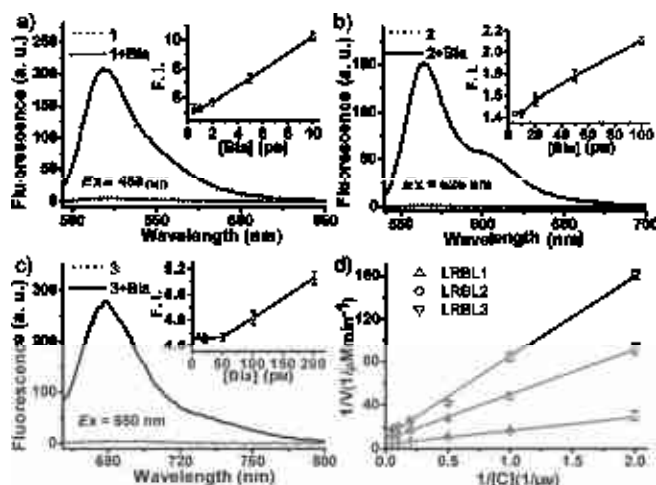


Figure 1. a, c) Fluorescence emission of LRBL1–3 (10 μ M) in the absence or presence of Bla hydrolysis (PBS, pH 7.2) with excitation at 488, 525 and 650 nm, respectively. Insets: detection limit of TEM-1 Bla. d) Enzyme kinetics of LRBL reagents to TEM-1 Bla.

fluorescent due to efficient FRET quenching. After incubation with TEM-1 Bla at 37°C for 30 min, intense fluorescence enhancement (38-, 110- and 80-fold for LRBL1, -2 and -3, respectively) was observed at a maximum wavelength of 518, 565 and 678 nm, respectively, corresponding to the connected fluorophores. These results indicated that enzyme hydrolysis would break FRET status by releasing the individual fluorescence quenchers. Enzyme kinetics of these probes to TEM-1 Bla hydrolysis were further studied in PBS at 37°C. The catalytic constants ($k_{\text{cat}} = 4.92, 2.16, 1.49 \text{ min}^{-1}$) and Michaelis constants ($K_M = 3.08, 4.28, 5.24 \text{ }\mu\text{M}$) for LRBL1, -2 and -3 were determined, respectively, by weighted least-squares fit of a double reciprocal plot of the hydrolysis rate versus probes concentration (Figure 1d). The catalytic efficiencies (k_{cat}/K_M) were calculated to be $2.66, 0.84$ and $0.47 \times 10^4 \text{ M}^{-1} \text{ s}^{-1}$, respectively, compar-

able to the previously reported enzyme probes with oxidized cephalosporin cores.^[10d,f,g] Control experiments without Bla incubation showed that these probes were quite stable in PBS buffer, thus the enzyme-responsive fluorescence enhancement allowed reliable determination of Bla at concentrations as low as 1.5, 10 and 50 μM by LRBL1, -2 and -3, respectively (Figure 1, inset).

Upon fluorescence enhancement activated by TEM-1 Bla, the enzyme labeling profile was also studied by SDS-PAGE analysis and matrix-assisted laser desorption/ionization-time of flight (MALDI-TOF) mass spectrometry. Typically, gel electrophoresis was carried out by loading the incubation mixture consisting of Bla (1 μg) and probe (2 μM) on a 10% polyacrylamide/SDS gel and then subjected to 1 h of separation. Using a laser scanner, the labeled enzyme could be directly read out by the tagged fluorescence signals, clearly suggesting the formation of the reactive quinone-methide moiety for effective attachment to the enzyme structure (the labeling mechanism is illustrated in Figure S1 in the Supporting Information). As a control, competition assays pretreated with Bla inhibitor clavulanic acid (CA) left the protein unlabeled, indicating the stability of the probes under aqueous conditions and the labeling activity of the specific enzyme reaction (Figure 2). MALDI-TOF mass

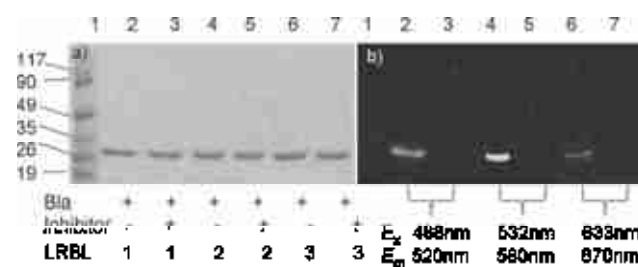


Figure 2. Labeling of TEM-1 Bla by LRBL1–3. SDS-PAGE analysis of Bla with probe incubation and imaged with: a) Coomassie blue staining, and b) fluorescence of merged green, yellow and red channels. Lane 1: molecular weight markers, in kDa; lane 2: Bla + LRBL1; lane 4: Bla + LRBL2; lane 6: Bla + LRBL3; lanes 3, 5 and 7: clavulanic acid inhibited Bla with LRBL1, -2 and -3, respectively.

spectrometric analysis of the fluorescently labeled samples provided further insight into the labeling profile of the enzyme structure. As shown in Figure 3, besides the peak from parent Bla enzyme (~ 28985 , Figure S2 in the Supporting Information), the molecular weight peaks of 29572, 29800 and 30081 with additional probe fragments were also observed in the MS spectrum, indicating the covalent attachment of the quinone-methide intermediates (M_W : 581, 813 and 1090 for LRBL1, LRBL2 + Na and LRBL3, respectively) into bacterial Bla. In the process of enzyme-responsive fluorescent labeling, two or more modifications in the enzyme sequence were also found in the MALDI-TOF analysis, implying the existence of more nucleophilic amino acids that may potentially contribute to the capture of the quinone-methide fragments after enzyme cleavage (see the Supporting Information).

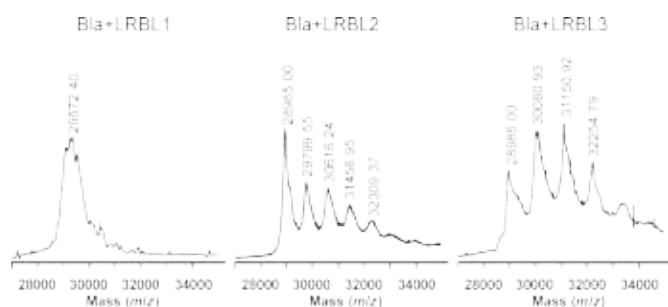


Figure 3. MALDI-TOF analysis of labeled TEM-1 Bla.

During the course of protein covalent labeling, the chemical modification may probably affect its biological functions if the targeted specific amino acid residues locate at the protein active site. To this end, the enzymatic activities of the labeled Bla were examined with the conventional hydrolysis assay using benzylpenicillin as substrate.^[14] Typically, TEM-1 Bla (0.5 μM) was incubated with LRBL probes (50 μM) for 30 min. Then the reaction mixture was added to benzylpenicillin (0.5 mM in PBS) with 1:200 dilution and the enzymatic hydrolysis profile of benzylpenicillin was monitored based on the absorbance change. As shown in Figure 4, compared to the hydrolysis activity with Bla alone, the similar absorbance change observed at 232 nm indicated that the enzyme activities were almost identical for the TEM-1 Bla samples that had been treated with different LRBL probes. As a control, the enzyme hydrolysis of benzylpenicillin was also performed in the presence of an efficient Bla inhibitor, clavulanic acid (50 μM). The insignificant absorbance change after incubation with Bla demonstrated that enzyme activity was effectively suppressed. It is plausible that although multiple enzyme modifications induced by quinone-methide fragments could occur, the covalent fluorescence labeling did not take place predominantly at the active site of the enzyme structure. Such unique behavior of catalytic formation and covalent labeling would ideally facilitate real-time imaging and direct screening of antibiotic-resistant bacteria.

In order to evaluate the specific covalent labeling of resistant bacteria, live cell imaging was performed with confocal microscopy. In a typical study, the Gram negative peni-

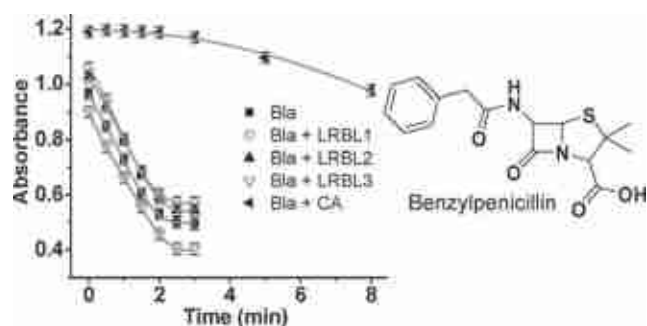


Figure 4. Activity of TEM-1 Bla after incubation with LRBL1–3. Enzymatic hydrolysis of benzylpenicillin based on absorbance decrease at 232 nm after incubation with LRBL probes or enzyme inhibitor.

cillin resistant *E. coli* JM109/pUC19 strain was selected as our target due to the higher production of class A Bla—a most prevalent plasmid-gene encoded bacterial enzyme mainly responsible for β -lactam antibiotic resistance in both clinic and community acquired infections.^[9] Meanwhile, the antibiotic susceptible *E. coli* JM109 strain that could not express Bla was used as a negative control. Cells were incubated with the probes (10 μM) and subjected to fluorescence imaging. As shown in Figure 5a, after incubation, strong fluorescence emission of LRBL1 (green), -2 (yellow) and -3 (red) was observed in the resistant JM109/pUC19 cells whereas there was no obvious fluorescence signal detected in the cells pretreated with enzyme inhibitor CA (Figure S3 in the Supporting Information) or in the negative control of *E. coli* JM109 strain. Similar bacterial imaging with LRBL1–3 was also conducted by using confocal laser scanning microscopy. Fluorescent signals from the various depths of the bacteria further proved that these LRBL probes could get into the cells and produce fluorescence upon Bla hydrolysis (Figure S4a–c in the Supporting Information). In addition, the bacterial lysates, upon PopCulture/lysozyme treatment, further indicated specific Bla hydrolysis and significant fluorescence enhancement in the resistant bacteria with minimum influence of other cellular proteins (Figure S5 in the Supporting Information). These results clearly demonstrated the native capability of the rationally designed enzyme-responsive probes to specifically detect endogenous Bla and label the resistant bacterial strains.

Moreover, the feasibility of labeling and screening the resistant strains was also exploited with a laboratory-built high-sensitivity flow cytometer (HSFCM). As a powerful tool for performing rapid analysis of individual cellular functions with excellent statistics in asynchronous cultures, flow cytometry has found extensive application in combination with effective detection reagents, including GFP, fluorescent antibodies, recombinant phages or specific dye molecules with good affinity to nucleic acids or bacterial membranes.^[15] With the covalent fluorescent labeling of bacterial enzyme provided by the LRBL reagents, HSFCM could facilitate a clear insight into population heterogeneity of bacterial strains in terms of antibiotic-resistance properties.

As a proof-of-concept, *E. coli* cells were incubated with LRBL1 (2 μM) after fixation and permeabilization. HSFCM green fluorescence signals from individual bacterial cells were collected at 520/35 nm under 488 nm excitation. Figure 5b and Figure S6 (in the Supporting Information) indicated that a strong fluorescence enhancement (more than 20-fold) can be observed in the resistant bacteria as compared to the negative control. The population of resistant strain JM109/pUC19 was completely separated from that of *E. coli* JM109 (Figure 5c), implying a high efficiency of enzyme-responsive labeling for drug resistant bacterial pathogens. The specificity of bacterial labeling was further confirmed by CA inhibition with an expected result of low fluorescence signal due to the potent inactivation of endogenous Bla (Figure 5b–c and Figure S6–c2 in the Supporting Information). The comparable scattering signals for *E. coli*

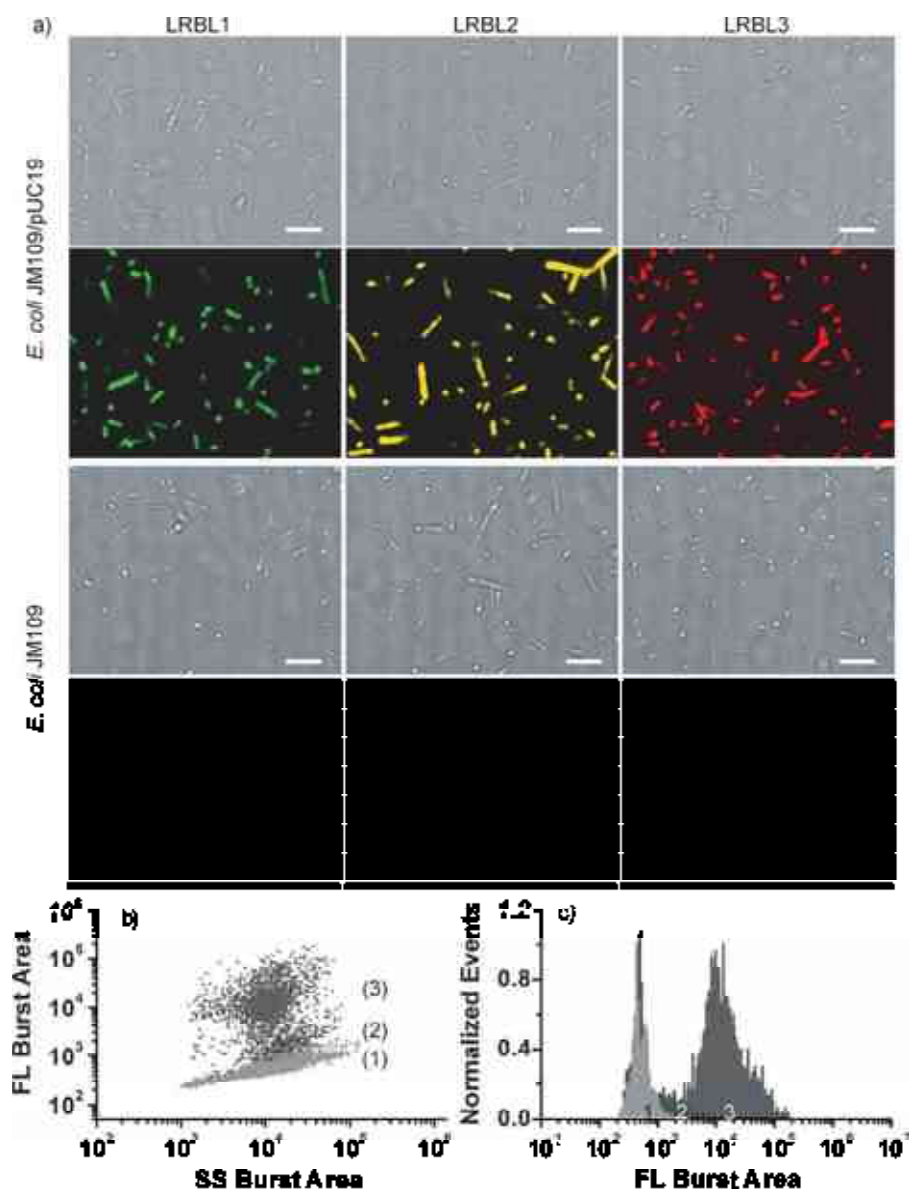


Figure 5. a) Fluorescent imaging of penicillin resistant *E. coli* JM109/pUC19 and penicillin susceptible *E. coli* JM109 incubated with LRBL1, -2 and -3 (10 μ M). Scale bar: 10 μ m. b), c) HSF-CM analysis of antibiotic-resistant bacteria with LRBL1 (2 μ M) labeling. Samples: *E. coli* JM109 (1), CA-treated *E. coli* JM109/pUC19 (2) and *E. coli* JM109/pUC19 (3). Concentration of bacteria was approximately 10⁸ cells mL⁻¹.

JM109 and *E. coli* JM109/pUC19 regardless of whether or not they were treated with CA inhibitor (Figure 5b) suggests that the specific enzyme-responsive covalent fluorescence labeling occurred in the target strains and such effective fluorescent labeling would not induce bacterial aggregation.

The potential interference of coexisting nonresistant bacteria in the detection of target strains was also investigated. A series of bacterial mixtures containing *E. coli* JM109 and JM109/pUC19 with a total number of 10⁸ cells mL⁻¹ but different percentages of JM109/pUC19 were prepared and incubated with LRBL1 (2 μ M). A clear separation of antibiotic resistant and susceptible cells provided the percentage of detected resistant bacteria at 6.6, 19.6, 40.5, 69.3 and 92.2%

(Figure 6), whereas the prevalence of JM109/pUC19 in the mixture was 5, 20, 50, 80 and 100%, correspondingly. Such excellent linear correlation of the ratios detected by FCM proved the labeling specificity to the antibiotic-resistant bacteria and the non-detection of LRBL probes of nonresistant strains. Quantitative analysis of antibiotic-resistant bacteria in a mixture could be easily demonstrated down to as low as 5%. These results made it clear that the inevitable multiple labeling of the quinone-methide probe did not necessarily mean a serious problem in the cellular context, as the large extent retention of the activated probes around the site of enzyme activity would significantly prevent the leakage from targeted cells and therefore greatly facilitate rapid cellular imaging and FCM studies.

As a comparison, similar enzyme activity and HSF-CM studies were also carried out by using a commercial fluorogenic Bla substrate (FluorocillinTM Green 495/525 reagent, Invitrogen). After the enzyme hydrolysis of this substrate in PBS (0.1 M, pH 7.2), a strong fluorescence enhancement could be detected, which was similar to the enzyme activities observed with LRBL1–3 (Figure S7d in the Supporting Information). However, owing to the fact that there was no covalent labeling

with FluorocillinTM Green 495/525 reagent, upon incubation with resistant *E. coli* JM109/pUC19 bacteria, the fluorescent products could not be retained within the strains properly, thus making FCM detection difficult (Figure S7 in the Supporting Information). These experiments clearly demonstrated the obvious advantage of the covalent attachment by our designed probes.

The general applicability of detecting and labeling antibiotic-resistant bacteria was further examined with pathogenic Gram positive *Bacillus cereus* and *Staphylococcus aureus*. These species are well-known to be involved in various serious infections, like brain abscesses and pneumonia, and represent a therapeutic challenge in terms of penicillin and cephalosporin resistance as a consequence of complicated

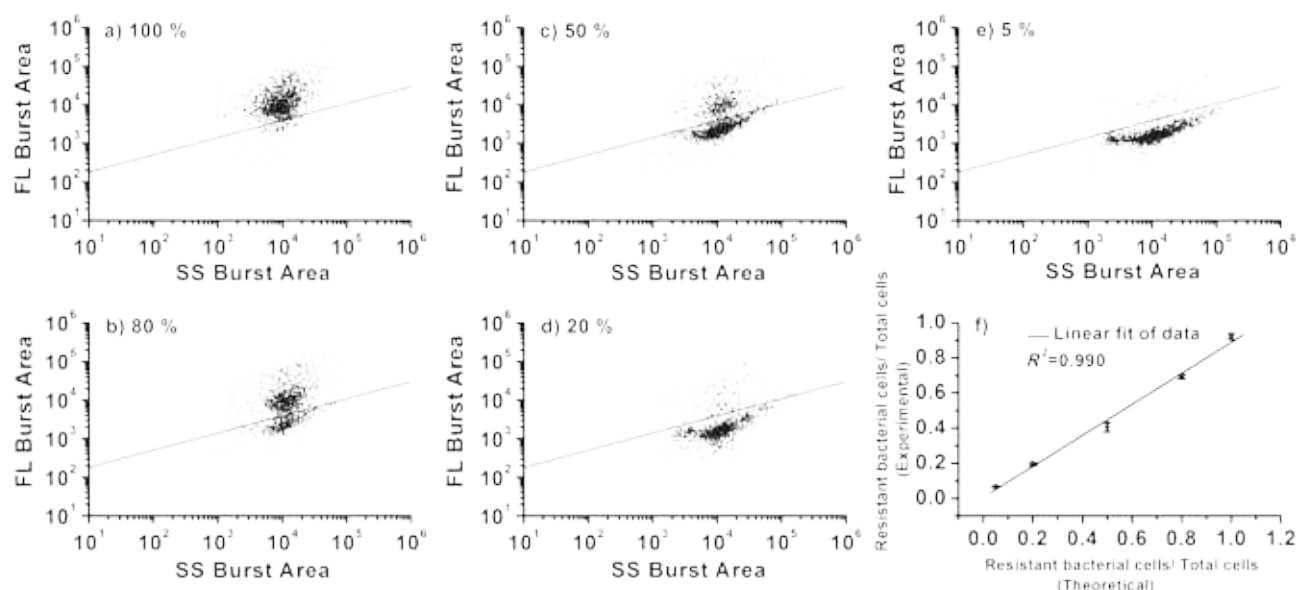


Figure 6. Differentiation of resistant *E. coli* JM109/pUC19 cells in bacterial mixtures. a)–e) FCM analysis of bacterial mixtures stained by LRBL1 (2 μM). The antibiotic-resistant and susceptible bacteria can be divided by the line. f) Linear correspondence between the theoretical and the FCM-measured percentages of antibiotic-resistant bacteria. Error bars indicate standard deviation ($n=3$).

mechanisms.^[16] In this work, three bacterial pathogens including one clinically isolated methicillin-resistant *S. aureus* (MRSA, ATCC BAA39), one penicillin resistant *B. cereus* 5/B (ATCC 13061), and one penicillin susceptible *S. aureus* (ATCC 29213) were selected as our main targets owing to their different extents of Bla production. Upon incubation of the probes (10 μM) with different bacterial cells, the fluorescent signals were monitored by confocal microscopy. As shown in Figure 7, after incubation, strong green, yellow and NIR fluorescence emissions could be clearly observed in the penicillin resistant MRSA and *B. cereus*. As a control, the signal detected for *S. aureus* was much weaker, which could be attributed to the low level Bla production in this strain.^[17] Bacterial imaging by confocal laser scanning measurements were also performed in penicillin resistant MRSA and *B. cereus* at various depths (Figure S4d,e in the Supporting Information); the results clearly indicated the effective cellular internalization of imaging probes and their specific labeling of Bla reporter enzyme in the antibiotic-resistant bacteria with resultant significant fluorescence enhancement.

Enzyme-responsive covalent labeling and bacterial screening in Gram positive strains were also monitored by HSFCM analysis. First, the penicillin resistant *B. cereus* was chosen to incubate with LRBL1 (2 μM).

The same bacterial strain without treatment with probe was used as the negative control. As expected, the FCM studies confirmed the efficient covalent labeling of *B. cereus* cells, and there was more than 40-fold fluorescence increase as compared to the background autofluorescence in the untreated bacteria (Figure 8, Figure S8 in the Supporting Information). Similar FCM analysis was also utilized to compare the Bla activities in drug susceptible *S. aureus* and clinically isolated MRSA. The effective bacterial labeling indicated that there was almost tenfold fluorescence enhancement observed in pathogenic MRSA over the control *S. aureus* strain (Figure S9 in the Supporting Information); this suggests the quantitative recognition of Bla activities between these two naturally existing bacterial pathogens, and is in

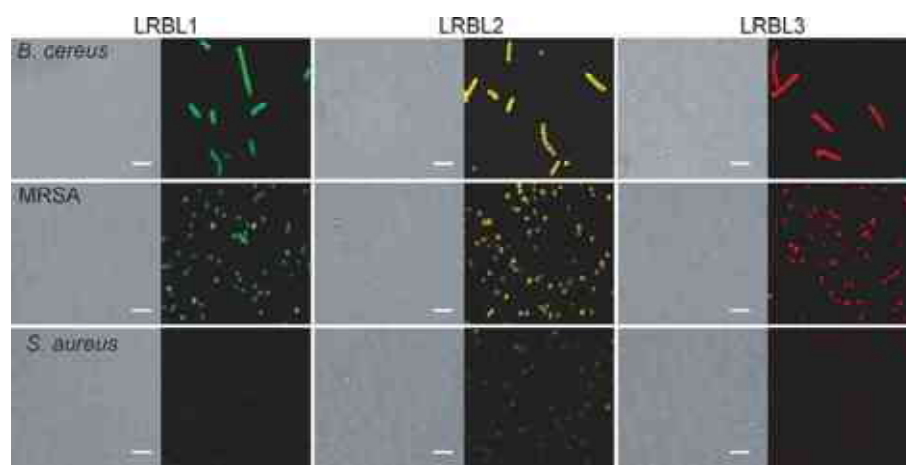


Figure 7. Fluorescence imaging of Gram positive *B. cereus*, MRSA and *S. aureus* after incubation with LRBL1, -2 and -3 (10 μM). Scale bar: 10 μm .

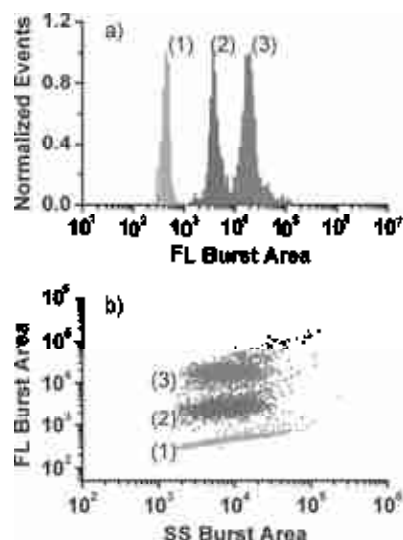


Figure 8. FCM analysis of resistance in Gram positive *B. cereus* by LRBL1 labeling. *B. cereus* (unstained, 1), 0.5 mM CA-treated *B. cereus* ($2 \mu\text{M}$ LRBL1 stained, 2), and *B. cereus* ($2 \mu\text{M}$ LRBL1 stained, 3). Concentration of *B. cereus* was approximately $10^8 \text{ cells mL}^{-1}$.

accord with the data obtained in previous fluorescence imaging measurements. In addition, FCM analysis showed that treatment of drug resistant bacterial strain (e.g., *B. cereus*) with Bla inhibitor could also result in an obvious decrease in fluorescence intensity (Figure 8). Considering the fact that the efficacy of bacterial enzyme inhibitors varies with different enzyme classes and different strains,^[18] fast reading of inhibitory efficiency through the enzyme-responsive bacterial covalent labeling may facilitate a simple and overall inspection of chemical–microbe interactions. This approach of enzyme-responsive bacterial labeling is therefore expected to find potential applications in clinical tests and new drug development to circumvent microbial resistance.

Conclusion

In summary, this work presents a set of fast and sensitive reagents for specific labeling and screening of antibiotic-resistant bacteria. By utilizing the endogenous production of β -lactamases in the resistant strains, three fluorescent quinone–methide intermediates could be activated; these serve as reactive electrophiles for the covalent labeling of drug-resistant bacteria. This specific β -lactamase responsive bacterial labeling, with significant fluorescence enhancement and tunable emission properties, allows single-cell detection and quantitative observation of resistant bacteria in a mixed sample, and thus provides a promising tool for clinical investigation and microbiological research. The Bla specificity and fluorescence activation can be applied to high-throughput enzyme inhibitor screening, either in new drug development or clinical diagnosis. Moreover, such covalent fluorescent labeling will provide a great opportunity for the direct

observation of biological pathways in bacterial growth and multiplication, real-time monitoring of bacterial infections and assessment of therapeutic efficacy in vitro and in vivo.

Experimental Section

Flow-cytometric analysis of antibiotic-resistant bacterial labeling: Live bacterial cells ($10^8 \text{ cells mL}^{-1}$) were incubated with LRBL1 ($10 \mu\text{M}$) in the dark for 60 min and washed with PBS. The fluorescence of the cells was detected by a laboratory-built high-sensitivity flow cytometer described previously.^[19] To study the labeling specificity, the bacterial cells were preincubated with Bla inhibitor clavulanic acid for 1 h, then stained with LRBL1 and subjected to FCM analysis. For the strains that were fixed and permeabilized, the cells were first fixed with PFA (1%) for 5 min and then incubated with permeating solution (25 mM Tris-HCl, 1.8% glucose, 10 mM EDTA, pH 7.4) for 20 min. After being washed with PBS, the treated cells were incubated with LRBL1 ($2 \mu\text{M}$) in the dark for 20 min, washed with PBS and subjected to FCM analysis. For comparison, the commercial Fluorocillin™ Green 495/525 β -lactamase substrate ($20 \mu\text{M}$) was incubated with the permeabilized *E. coli* JM109/pUC19 cells for 20 min. After being washed with PBS, the bacterial cells were subjected to FCM analysis.

Live cell imaging of labeled bacteria: An overnight bacterial suspension culture was diluted to $10^8 \text{ cells mL}^{-1}$ and incubated with LRBL probes ($10 \mu\text{M}$). After being washed with PBS, the bacteria were spotted on poly-L-lysine pretreated glass slides and covered with coverslips. Cell imaging tests were conducted with a Nikon Eclipse TE2000 Confocal Microscope.

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