

Supporting information

Optical Tracking of Phagocytosis with an Activatable  
Profluorophore Metabolically Incorporated into Bacterial  
Peptidoglycan

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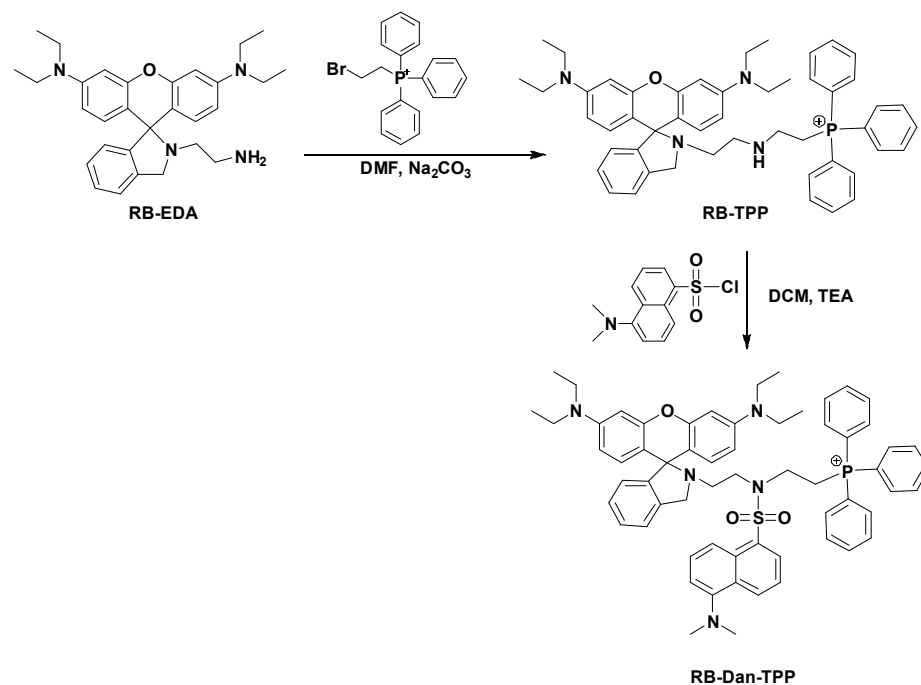
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## Experimental

### Material and methods

LysoTracker Blue DND 22 was purchased from Invitrogen. BFA was purchased from Sigma. Rhodamine X was obtained from Bioluminor, Xiamen, China. (2-Bromoethyl) triphenylphosphonium bromide was purchased from J&K Scientific L. FITC-d-Lys was synthesized following a reported procedure.<sup>[1]</sup> Rhodamine B-deoxylactam-ethylenediamine was prepared as previously described.<sup>[2]</sup> All other reagents were purchased from Alfa Aesar. Column chromatography was performed on silica gel (100-200 mesh). NMR spectra were recorded on a Bruker instrument using tetramethyl silane as the internal reference. Mass analysis was performed in Bruker En Apex ultra 7.0T FT-MS. Fluorescence spectra and UV-vis absorption spectra were recorded on a spectrofluorometer (SpectraMax M5, Molecular Device). Confocal fluorescence microscopy images were obtained on Leica SP5 using the following filters:  $\lambda_{ex}@543$  nm and  $\lambda_{em}@565-625$  nm for RB and ROX signal;  $\lambda_{ex}@488$  nm and  $\lambda_{em}@500-530$  nm for FITC signal.  $\lambda_{ex}@405$  nm and  $\lambda_{em}@410-480$  nm for LysoTracker blue and Fluorescence images were merged using Adobe Photoshop CC 2014. Flow cytometric data were obtained on Beckman Coulter. The fluorescence emission intensity of ROX was recorded by filter FL2 (565-625 nm). 10000 cells were analyzed and the data were processed by Origin 8.0. Raw 264.7 cells were obtained from American Type Culture Collection and grown at 37 °C under 5% CO<sub>2</sub> in Dulbecco's Modified Eagle Medium (DMEM, Gibco; Invitrogen) supplemented with 10% fetal bovine serum.

### Synthesis of RB-Dan-TPP

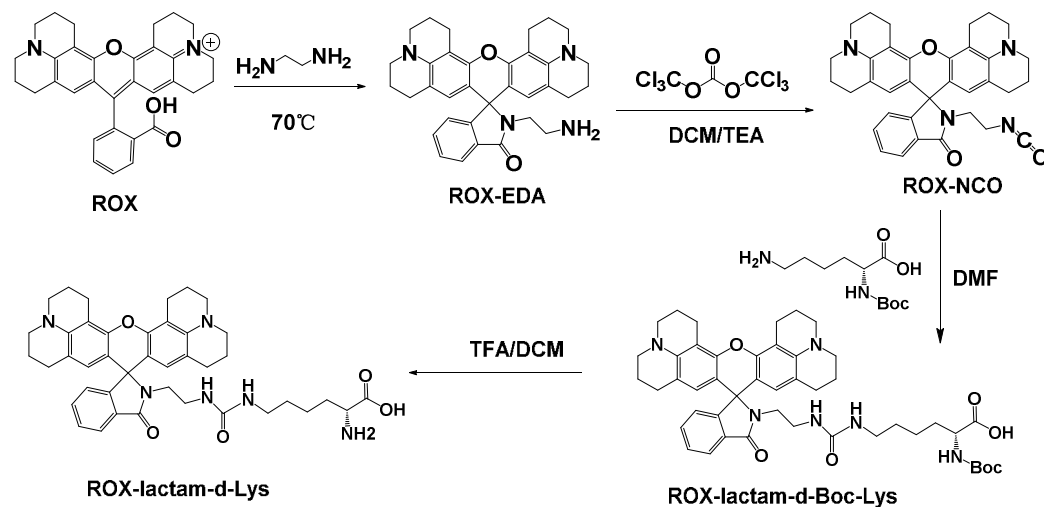


### Scheme S1. Synthesis of RB-Dan-TPP

To the solution of RB-EDA (10 g) in N,N-dimethylformamide (DMF, 50 ml) were added (2-bromoethyl)triphenyl phosphonium (9.5 g) and sodium carbonate (4.5 g). The mixture was stirred for 1 h at room temperature and then diluted with dichloromethane (250 ml). The mixture was washed with H<sub>2</sub>O (150 ml) for three times. The organic layer was dried over anhydrous sodium sulfate, and concentrated *in vacuo*. The residue was purified by flash column chromatography on silica gel (CH<sub>2</sub>Cl<sub>2</sub>:hexane = 1:2) to afford crude RB-TPP (7.3 g, 45%). To the solution of RB-TPP (3 g) in dichloromethane (20 ml) was added dropwise dansyl chloride (3.2 g) dissolved in dichloromethane (10 ml) and triethylamine (1 ml). The solution was stirred for 30 min at room temperature and then concentrated *in vacuo*. The residue was purified by flash column

chromatography on silica gel (CH<sub>2</sub>Cl<sub>2</sub>:hexane = 1 : 1) to afford RB-Dan-TPP (2.5 g, 77%). <sup>1</sup>H-NMR (500 MHz, CDCl<sub>3</sub>) δ: 8.42 (d, J = 8.5 Hz, 1H), 8.09 (d, J = 8.6 Hz, 1H), 7.81 (d, J = 7.2 Hz, 1H), 7.73 (t, J = 6.9 Hz, 4H), 7.64 (t, J = 6.4 Hz, 4H), 7.62–7.58 (m, 7H), 7.41–7.33 (m, 4H), 7.17 (t, J = 7.4 Hz, 1H), 7.10 (d, J = 7.5 Hz, 1H), 6.77 (d, J = 7.5 Hz, 1H), 6.25 (d, J = 9.1 Hz, 4H), 5.98 (dd, J = 8.8, 2.4 Hz, 2H), 3.70 (t, J = 3.5 Hz, 2H), 3.57 (t, J = 5.0 Hz, 2H), 3.47 (s, 2H), 3.24 (q, J = 7.0 Hz, 8H), 2.83 (s, 8H), 2.40 (t, J = 5.2 Hz, 3H), 2.21 (t, J = 7.7, 1H), 1.10 (t, J = 7.0 Hz, 12H); <sup>13</sup>C-NMR (126 MHz, CDCl<sub>3</sub>) δ: 153.20, 151.71, 148.04, 135.14, 133.62, 130.60, 130.06, 128.16, 127.47, 126.96, 126.74, 126.50, 125.56, 124.41, 123.50, 122.51, 119.23, 117.97, 117.28, 115.19, 110.90, 107.43, 97.92, 55.94, 46.92, 45.43, 44.30, 29.71, 22.27, 12.69; MS (ESI) calculated for C<sub>62</sub>H<sub>68</sub>N<sub>5</sub>O<sub>3</sub>PS<sup>+</sup> (M + H<sup>+</sup>) 994.48; found 994.40.

### Synthesis of ROX-lactam-d-Lys



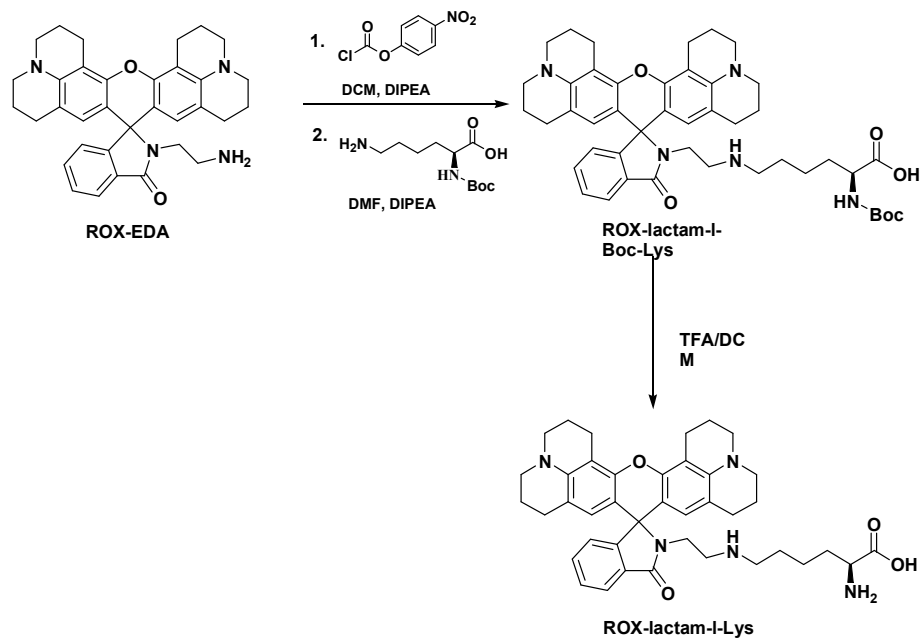
### Scheme S2. Synthesis of ROX-lactam-d-Lys

Rhodamine ROX (3 g) was added to a flask containing ethylenediamine (20 ml). The mixture was heated at 70 °C for 1 h and the solution was concentrated *in vacuo*, the residue was purified by flash column chromatography on silica gel (EtOAc:hexanes=5:1) to give ROX-EDA as a pale yellow solid (3.1 g, 95%). <sup>1</sup>H-NMR (500 MHz, CDCl<sub>3</sub>) δ 8.06–7.82 (m, 1H), 7.45 (dd, J = 5.4, 3.0 Hz, 2H), 7.16–7.02 (m, 1H), 6.01 (s, 2H), 3.19 (dd, J = 10.7, 3.7 Hz, 2H), 3.18–3.15 (m, 3H), 3.13–3.09 (m, 3H), 2.96–2.84 (m, 4H), 2.56–2.47 (m, 4H), 2.46–2.42 (m, 2H), 2.06 (d, J = 4.0 Hz, 4H), 1.88 (dd, J = 10.5, 5.2 Hz, 6H); <sup>13</sup>C-NMR (126 MHz, CDCl<sub>3</sub>) δ 168.82, 153.87, 148.23, 143.57, 132.39, 131.04, 127.86, 124.51, 123.98, 122.65, 117.36, 107.64, 105.61, 65.79, 49.91, 49.46, 43.75, 40.66, 27.15, 21.99, 21.49, 21.21; MS (ESI) calculated for C<sub>34</sub>H<sub>36</sub>N<sub>4</sub>O<sub>2</sub> (M+H<sup>+</sup>) 534.28; found 534.70.

ROX-EDA (600 mg) was dissolved in dichloromethane (5 ml). The solution was added dropwise to a flask containing triphosgene (670 mg) and dichloromethane (5 ml) on ice followed by addition of triethylamine (1 ml). The mixture was further stirred at room temperature for 30 min. The solvent was removed by rotary evaporation. The resultant ROX-NCO was dissolved in DMF (10 ml) and then added into a flask containing triethylamine (1 ml) and Boc-D-Lys-OH (832 mg) dissolved in DMF (10 ml). The resultant crude ROX-lactam-d-Boc-Lysine added to a solution of trifluoroacetic acid (3 ml) in dichloromethane (3 ml). After stirred at room temperature for 3 h, the solution was concentrated *in vacuo* and the residue was purified by flash column chromatography on silica gel (EtOAc:hexane = 10:1) to afford ROX-lactam-d-Lys (500 mg) in 63% overall yield. <sup>1</sup>H-NMR (400 MHz, CDCl<sub>3</sub>) δ 7.84–7.79 (m, 1H), 7.35 (t, 2H), 7.01 (dd, J = 5.6, 2.6 Hz, 1H), 5.98 (d, J = 3.9 Hz, 2H), 3.18 (m, 2H), 3.10 (m, 3H), 3.03 (m, 4H), 2.88 (m, J = 4.6 Hz, 5H), 2.46 (t, J = 6.8 Hz, 2H), 2.40 (t, J = 7.0 Hz, 2H), 2.03–1.97 (m, 4H), 1.79 (dd, J = 11.1, 5.4 Hz, 6H), 1.45–1.35 (m, 4H), 1.01 (t, J = 7.0 Hz, 2H), 0.89 (t, J = 6.8 Hz, 2H); <sup>13</sup>C-NMR (126 MHz, CDCl<sub>3</sub>): δ 174.47, 169.02, 159.11, 148.11, 143.62, 124.29, 117.47, 107.74, 105.01, 66.20, 49.88, 49.43, 45.73, 31.93, 29.70, 29.37, 27.11, 22.70, 21.94,

21.45, 21.24; MS (ESI) calculated for C<sub>41</sub>H<sub>48</sub>N<sub>6</sub>O<sub>5</sub> (M+H<sup>+</sup>) 705.37 found 705.50.

### Synthesis of ROX-lactam-L-Lys

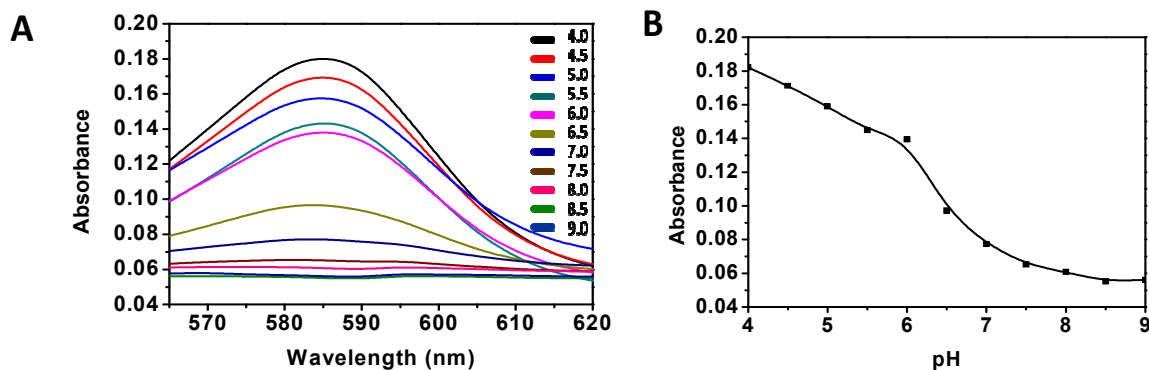


### Scheme S2. Synthesis of ROX-lactam-L-Lys

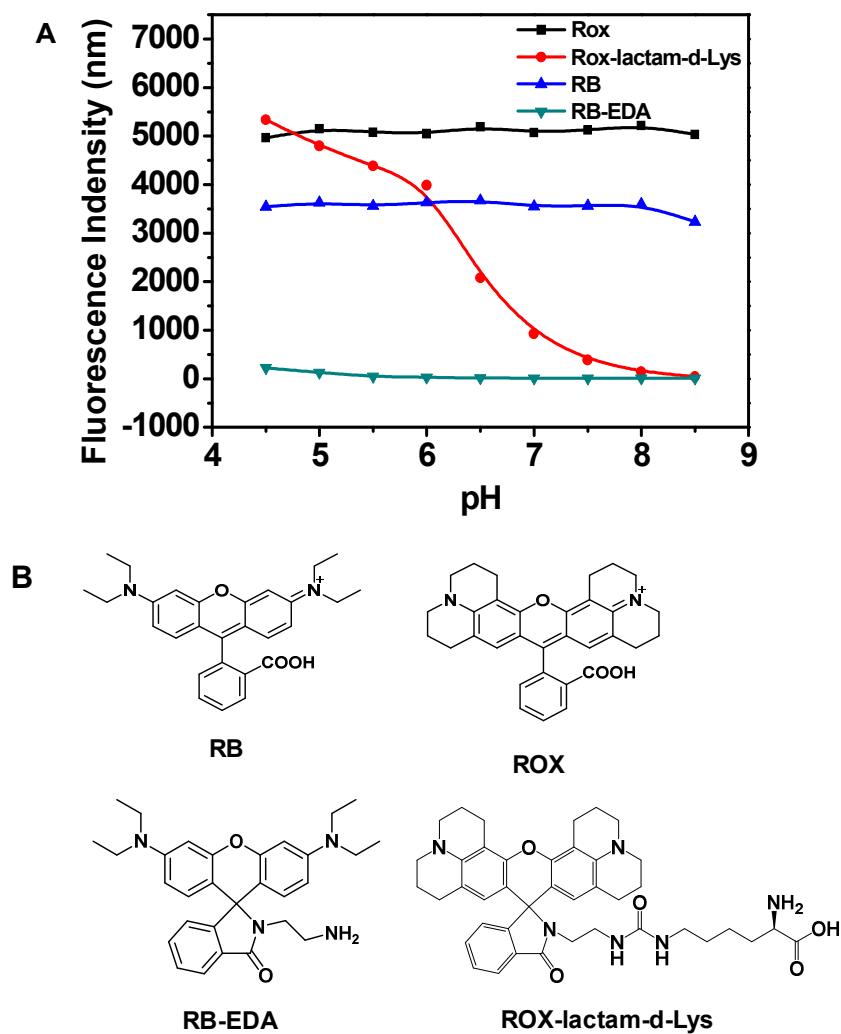
ROX-EDA (300 mg) dissolved in dichloromethane (5 ml) was added dropwise to a flask containing 4-Nitrophenyl chloroformate (340 mg) and dichloromethane (5 ml) followed by addition of N,N-Diisopropylethylamine (1 ml). The mixture was further stirred at room temperature for 30 min. The solvent was removed by rotary evaporation. The resultant was dissolved in DMF (10 ml) and then added into a flask containing DIPEA (1 ml) and Boc-L-Lys-OH (420 mg) dissolved in DMF (5 ml). After stirred at room temperature for 1 h, the solution was concentrated *in vacuo* and the residue was purified by flash column chromatography on silica gel (EtOAc:hexane: 20:1) to afford ROX-lactam-l-Boc-Lys (400 mg) which was added to a solution of trifluoroacetic acid (3 ml) in dichloromethane (3 ml). After stirred at room temperature for 3 h, the solution was concentrated *in vacuo* and the residue was purified by flash column chromatography on silica gel (EtOAc:hexane: 10:1) to afford ROX-lactam-d-Lys (300 mg) in 85% yield. <sup>1</sup>H NMR (500 MHz, CDCl<sub>3</sub>) δ: 7.80 (d, *J* = 6.3 Hz, 1H), 7.37 – 7.29 (m, 2H), 7.00 (d, *J* = 6.4 Hz, 1H), 5.98 (d, *J* = 6.4 Hz, 2H), 3.66 (s, 1H), 3.19 (br s, 1H), 3.09 (s, 3H), 3.05 – 2.98 (m, 5H), 2.90 – 2.77 (m, 7H), 2.45 (t, *J* = 6.8 Hz, 2H), 2.37 (t, *J* = 7.0 Hz, 2H), 2.05 – 1.93 (m, 4H), 1.78 (td, *J* = 13.0, 6.7 Hz, 6H), 1.39 (d, *J* = 14.7 Hz, 4H), 1.25 (s, 1H), 1.16 (t, *J* = 7.3 Hz, 2H); <sup>13</sup>C NMR (126 MHz, CDCl<sub>3</sub>) δ: 169.20, 159.08, 154.23, 148.10, 143.62, 130.30, 124.34, 117.50, 107.72, 105.03, 66.21, 49.89, 49.43, 45.72, 30.71, 27.10, 21.94, 21.46, 21.23; MS (ESI) calculated for C<sub>41</sub>H<sub>48</sub>N<sub>6</sub>O<sub>5</sub> (M+H<sup>+</sup>) 705.37 found 705.30.

### pH titration of ROX-lactam-d-Lys

Aliquots of the stock solution of ROX-lactam-d-Lys, ROX, rhodamine B (RB), rhodamine B-lactam-ethylenediamine (RB-EDA) in DMF were respectively added to sodium phosphate buffer (100 mM, 1 ml) of various pH to a final concentration of 1 μM. Fluorescence emission of the solutions was recorded as a function of buffer pH using *lex@560* nm for RB and RB-EDA, *lex@585* nm for ROX and ROX-lactam-d-Lys.



**Figure S1.** UV-vis-NIR absorption spectra of ROX-lactam-d-Lys in sodium phosphate buffer (100 mM, 1ml) of pH 4.0-9.0 (A). The absorbance@585 nm was plotted as a function of buffer pH (B).



**Figure S2.** pH titration of RB, RB-EDA, ROX and ROX-lactam-d-Lys (1  $\mu$ M) in buffer of pH 4.0-9.0 (A). The structure of RB, ROX, RB-EDA, and ROX-lactam-d-Lys (B).

**Acidic pH dependent fluorescence of RB-TPP loaded bacteria**

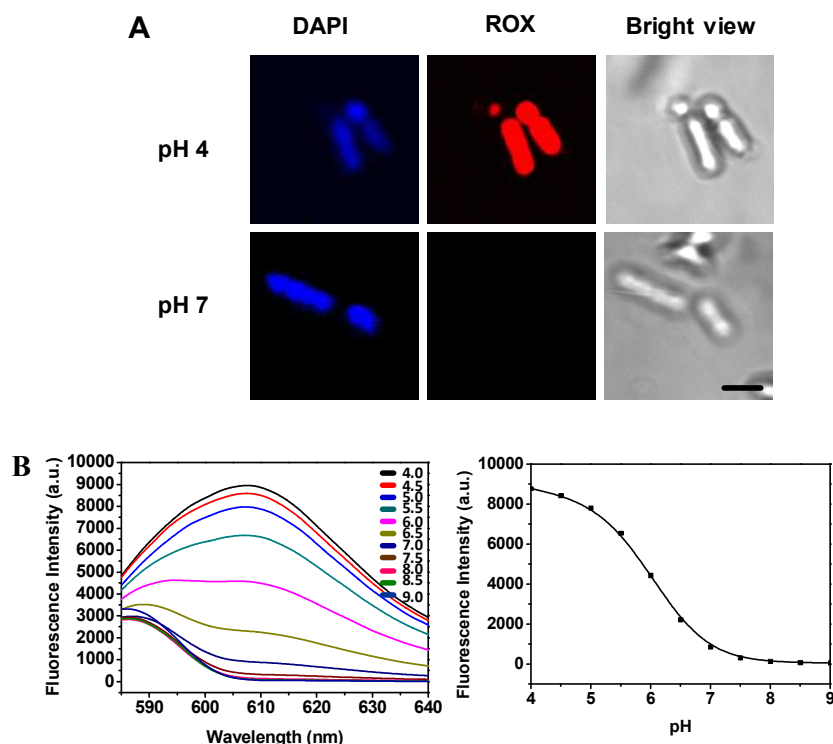
*E. coli* were grown at 37 °C in LB medium to OD<sub>600</sub> reached 0.6. The medium was diluted into fresh LB medium containing RB-TPP (100 μM) and DAPI (1 μM) to OD<sub>600</sub> ~0.3. After 30 min incubation, the cells were centrifuged, washed with LB medium for three times and then resuspended in sodium phosphate buffer (100 mM) of pH 7.0 or pH 4.0. The cells were visualized by confocal fluorescence microscopic analysis.

### Phagocytosis of RB-TPP-loaded bacteria by Raw 264.7 cells

Bacterial phagocytosis by Raw 264.7 macrophages was performed in accordance with a previously reported method<sup>[3]</sup>. RB-TPP loaded *E. coli*, prepared as described above, was washed with LB medium for three times, and then resuspended in DMEM. Raw 264.7 macrophages were washed with PBS and then added to DMEM containing RB-TPP loaded *E. coli* at the amount of infection (MOI) of 10. The macrophage-bacteria system was incubated at 37 °C for 1 h. After 1 h incubation, macrophage cells were washed with cold PBS, resuspended in DMEM, and then analyzed by confocal fluorescence microscopy.

### pH dependent fluorescence of ROX-lactam-L-Lys labelled bacteria

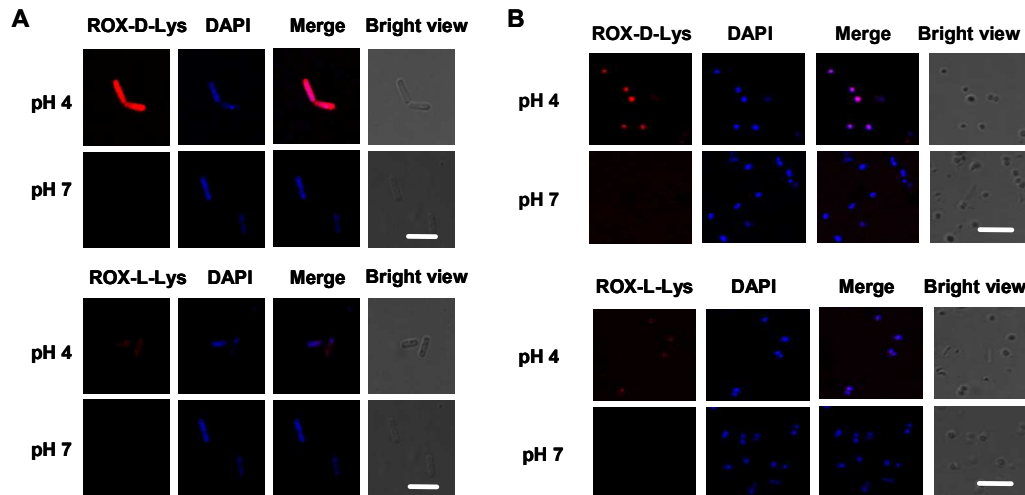
*E. coli* and *S. aureus* were respectively grown at 37 °C in LB medium until the OD<sub>600</sub> was 0.6. The medium was diluted to OD<sub>600</sub> ~0.3 with fresh medium containing ROX-lactam-L-Lys (100 μM) and DAPI (1 μM). After 30 min incubation, the cells were harvested by centrifugation, washed with LB medium, and then resuspended in sodium phosphate buffer (100 mM) of pH 7.0 or pH 4.0. In control experiments, the bacteria were labelled with ROX-lactam-d-Lys (100 μM), and then suspended in sodium phosphate buffer (100 mM) of pH 7 or pH 4. These cells were analyzed by confocal fluorescence microscopy.



**Figure S3.** Fluorescence emission of of ROX-lactam-d-Lys-labelled *E. coli*. in buffer of pH 4.0 or 7.0 (A). pH mediated fluorescence activation of ROX-lactam-d-Lys-labelled *E. coli* (B). *E. coli* were cultured in LB medium supplemented with DPAI (1 μM) and ROX-lactm-d-Lys (100 μM) for 30 min. The cells were harvested, washed with PBS, resuspended in phosphate buffer (100 mM, pH 7.0 or pH 4.0) and then analyzed by confocal fluorescence microscopy. DAPI signal was shown in blue and ROX signal was shown in red. Bars, 5 μm.

### pH dependent fluorescence of ROX-lactam-d-Lys labelled bacteria

*E. coli* were grown at 37 °C in LB medium until the OD<sub>600</sub> was 0.6. The medium was diluted to OD<sub>600</sub> ~0.3 with fresh medium containing ROX-lactam-d-Lys (100 μM) and DAPI (1 μM). After 30 min incubation, the cells were harvested by centrifugation, washed with LB medium, and then resuspended in sodium phosphate buffer (100 mM) of pH 7.0 or pH 4.0. The cells were analyzed by confocal fluorescence microscopy.



**Figure S4** Fluorescence emission of ROX-lactam-L-Lys-labelled *E. coli* (A) and *S. aureus* (B) in sodium phosphate buffer of pH 4.0 or pH 7.0 using ROX-lactam-L-Lys-labelled bacteria as the controls. Bacteria were cultured in LB medium supplemented with DPAI (1 μM) and ROX-lactm-L-Lys (100 μM) or ROX-lactm-D-Lys (100 μM) for 30 min. The cells were harvested, washed with PBS, resuspended in sodium phosphate buffer (100 mM, pH 7.0 or pH 4.0) and then visualized by fluorescence microscopy. DAPI signal was shown in blue and ROX signal was shown in red. Bars, 5 μm.

#### ***In vitro* characterization of ROX-lactam-d-Lys and FITC-d-Lys loaded *E. coli* and *S. aureus***

*E. coli* and *S. aureus* were respectively grown at 37 °C in LB medium until OD<sub>600</sub> reached 0.6. The medium was diluted to OD<sub>600</sub> ~0.3 with fresh medium containing ROX-lactam-d-Lys (100 μM) and FITC-d-Lys (100 μM). After 30 min incubation, the cells were centrifuged, washed with LB medium, and then resuspended in sodium phosphate buffer (100 mM, pH 4~8). The cells were subjected to confocal fluorescence microscopy analysis.

#### **Phagocytosis of ROX-lactam-d-Lys and FITC-d-Lys loaded *E. coli* and *S. aureus* by Raw 264.7 macrophages**

*E. coli* and *S. aureus* pre-labelled with ROX-lactam-d-Lys and FITC-d-Lys were washed with PBS for three times and resuspended in DMEM media. Macrophages were washed f with PBS and then incubated with dye-labelled *E. coli* or *S. aureus* at a amount of infection (MOI) of 10 in DMEM at 37 °C. At different incubation time (0-2 h), a portion of macrophage cells was pipetted, washed with cold PBS, and then incubaed with lysotracker (1 μM) in DMEM for 30 min. The resultant macrophages were washed with PBS for three times, resuspended in DMEM, and then probed by confocal fluorescence microscopy.

#### **Effets of BFA on imaging of bacterial phagocytosis by Raw 264.7 cells**

Raw 264.7 cells were repectively incubated in DMEM for 30 min with *E. coli* or *S. aureus* that have been labelled with ROX-lactam-d-Lys and FITC-d-Lys. After 1 h incubation, Raw 264.7 cells were washed with PBS for three times and then in incubated in DMEM spiked with or without BFA (50 nM) for 8 h at 37 °C. The resultant macrophages were stained with lysotracker blue (1 μM) for 30 min, washed with PBS, resuspended in DMEM and then imaged by confocal fluorescence microscopy.

**References:**

- 1) E. Kuru, H. V. Hughes, P. J. Brown, E. Hall, S. Tekkam, F. Cava, M. A. de Pedro, Y. V. Brun, M. S. VanNieuwenhze, *Angew Chem Int Ed Engl* 2012, *51*, 12519-12523.
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- 3) L. Kong, B. X. Ge, *Cell research* 2008, *18*, 745-755.