Electronic Supplementary Information (ESI)

A Biocompatible Dual-AIEgen System without Spectral Overlap for Quantitation of Microbial Viability and Monitoring of Biofilm Formation

Wei He[‡ac] Zheng Zheng[‡a], Haotian Bai*[a], Linghong Xiong[b], Lei Wang[b], Yinghui Li[b], Ryan T. K. Kwok[ac], Jacky W. Y. Lam[ac], Qinghua Hu[b], Jinquan Cheng*[b], Ben Zhong Tang*[acd]

a Department of Chemistry, Department of Chemical and Biological Engineering, Hong Kong Branch of Chinese National Engineering Research Center for Tissue Restoration and Reconstruction, Institute of Advanced Study and Division of Life Science The Hong Kong University of Science and Technology, Clear Water Bay, Kowloon, Hong Kong, China

b Shenzhen Center for Disease Control and Prevention, Shenzhen 518055, China

c HKUST-Shenzhen Research Institute, No. 9 Yuexing 1st RD, South Area, Hi-tech Park Nanshan, Shenzhen 518057, China

d Center for Aggregation-Induced Emission, SCUT-HKUST Joint Research Laboratory and State Key Laboratory of Luminescent Materials and Devices, South China University of Technology, Guangzhou 510640, China

*e-mail: tangbenz@ust.hk; cjinquan@szcdc.net, baihaotian@ust.hk

‡These authors contributed equally to the work.

Materials and Instruments

All chemicals and reagents were commercially available and used as received without further purification. 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyltetrazolium bromide (MTT) were purchased from Sigma-Aldrich and used as received. For cell culture, Dulbecco's Modified Eagle's Medium (DMEM), fetal bovine serum (FBS), penicillin, streptomycin solution, CellMask Green were purchased from Invitrogen. ^{1}H and ^{13}C NMR spectra were measured on a Bruker ARX 400 NMR spectrometer using CDCl3 and DMSO-d6 as solvents and tetramethylsilane (TMS; δ = 0 ppm) was chosen as internal reference. High-resolution mass spectra (HR-MS) were obtained on a Finnigan MAT TSQ 7000 Mass Spectrometer System operated in a MALDI-TOF mode. Absorption spectra were measured on a Milton Roy Spectronic 3000 Array spectrophotometer. Steady-state photoluminescence (PL) spectra were measured on a Perkin-Elmer spectrofluorometer LS 55. Absolute fluorescence quantum yield was measured by a calibrated integrating sphere (Labsphere). Laser confocal scanning microscope images were collected on Zeiss laser scanning confocal microscope (LSM 800) and analyzed using ZEN 2009 software (Carl Zeiss). The statistical analysis of cells was measured by flow cytometry (BD FACS Aria IIIu). Fluorescence intensity that was used for quantative analysis was obtained on a microplate reader (Varioskan LUX multimode microplate reader) using black 96-well plates (Thermo Scientific, light-tight, flat bottom, nonsterile).

Synthesis

Synthesis of DCQA. 7-(diphenylamino)-9-ethyl-9H-carbazole-2-carbaldehyde (0.50 g, 1.28 mmol) and 1-(3-trimethylammoniopropyl)-4-methylquinolinium dibromide (0.52 g, 1.28 mmol) were dissolved in dry ethanol (15 mL). 2 drops of piperidine was added and the solution was refluxed for overnight under nitrogen. After cooling to room temperature, the solvent was removed by evaporation under reduced pressure. The residue was purified by neutral aluminum oxide column chromatography eluting with dichloromethane/methanol mixture solvent giving DCQA as dark brown crystalline solid (0.62 g, yield: 62%). ^{1}H NMR (400 MHz, DMSO-d_6, ppm): δ9.45 (d, J = 6.5 Hz, 1H), 9.17 (d, J = 8.7 Hz, 1H), 8.64-8.59 (m, 2H), 8.51-8.41 (m, 2H), 8.30-8.26 (m, 2H), 8.17 (d, J = 8.1 Hz, 1H), 8.11-8.06 (m, 2H), 7.82 (d, J = 8.3 Hz, 1H), 7.33-7.29 (m, 4H), 7.18 (s, 1H), 7.08-7.03 (m, 6H), 6.87 (d, J = 8.4 Hz, 1H), 5.03 (t, J = 7.3 Hz, 2H),

4.41-4.35 (m, 2H), 3.60-3.56 (m, 2H), 3.08 (s, 9H), 2.45-2.41 (m, 2H), 1.27 (t, J = 7.1 Hz, 3H). ^{13}C NMR (100 MHz, DMSO-d_6, ppm): δ 153.27, 147.46, 147.32, 146.70, 145.13, 141.80, 140.12, 137.80, 135.16, 132.09, 129.36, 128.96, 126.48, 124.61, 123.64, 122.84, 121.89, 120.97, 120.00, 118.13, 117.56, 116.65, 115.76, 109.12, 103.98, 61.93, 53.02, 52.34, 36.86, 23.09, 13.56. MS: m/z calculated for 308.425, found 308.674.

Strain information

S. aureus (ATCC 6538), *B. subtilis* (ATCC 6633), *M. luteus* (ATCC 9341), *E. coli* (8099), *K. pneumoniae* (ATCC 700603), *S. marcescens* (CMCC 41002), *C. albicans* (ATCC 10231), *S. cerevisiae* (ATCC 9763).

Culture medium information

LB broth was chosen for *S. aureus*, *B. subtilis*, *M. luteus*, *E. coli*, *K. pneumoniae*, and *S. marcescens*. YPD broth was applied for the culture of *C. albicans* and *S. cerevisiae*.

Bacterial staining

A single colony of bacteria on solid culture medium was transferred to 5 mL of liquid culture medium and grown at 37 °C for 10 h. After reaching logarithmic phase, 500 μL of bacteria was transferred to a 1.5 mL microcentrifuge tube. Bacteria were harvested by centrifuging at 11700 g for 3 min. After removal of supernatant, bacteria were either killed by 200 μL 75 % alcohol (dead bacteria) or dealt with equal amount of PBS (live bacteria), following by washing with PBS. Then, 1 mL dye solution in saline at appropriate concentration was added into the microcentrifuge tube. After dispersing with vortex, the bacteria were incubated in a shaking incubator at 37 °C for designed time. Finally imaged by ZEISS LSM800.

Fungal staining

A single colony of fungi on solid culture medium was transferred to 5 mL of liquid culture medium and grown at 25 °C for 10 h. After reaching logarithmic phase, 500 μL of fungi was transferred to a 1.5 mL microcentrifuge tube. Fungi were harvested by centrifuging at 11700 g for 3 min. After removal of supernatant, fungi were either killed by 200 μL 75 % alcohol (dead fungi) or dealt with equal amount of PBS (live fungi), following by washing with PBS. Then, 1 mL dye solution in saline at appropriate

concentration was added into the microcentrifuge tube. After dispersing with vortex, the fungi were incubated in a shaking incubator at 25 °C for designed time. Finally imaged by ZEISS LSM800.

Biofilm imaging

An overnight culture of microbes was subcultured at an OD600 of 0.5 into relevant medium (*S. aureus*: TSB supplemented with glucose. *E. coli*: LB supplemented with casein hydrolysate. *C. albicans*: YPD broth). Then, 2 mL of bacteria/fungi suspension was added to a confocal dish. The dishes were static cultured at different temperature (37 °C for *S. aureus* and *E. coli*, 25 °C for *C. albicans*). After 72 h, the medium was removed and the plates were washed with sterile 1× PBS. Then, 1 mL of the probes was added into the dishes incubated for designed time. Finally imaged by ZEISS LSM800.

Quantitation of *E. coli* with different viabilities by plate reader

A single colony of *E. coli* on solid culture medium was transferred to 5 mL of liquid culture medium and grown at 37 °C for 10 h. After reaching logarithmic phase, 500 μL of culture suspension was transferred to a 1.5 mL microcentrifuge tube. Bacteria were harvested by centrifuging at 11700 g for 3 min. After removal of supernatant, bacteria were either killed by 1.5 mL 75 % alcohol (dead bacteria) or dealt with equal amount of PBS (living bacteria). Then live and dead bacteria were mixed at different ratios (L/D ratio), i.e., 0, 10 %, 30 %, 50 %, 70 %, 90 % and 100 %. Next, bacteria were harvested by centrifuging at 11700 g for 3 min. Then, 200 μL dye solution in buffer (pH = 10) at appropriate concentration was added into the microcentrifuge tube. After dispersing with vortex, 100 μL suspension were transferred into 96-well plate and incubated at 37 °C for designed time. Finally, the fluorescence peaks were recorded by microplate reader (Varioskan LUX multimode microplate reader) using black 96-well plates (Thermo Scientific, lighttight, flat bottom, non-sterile). The integrated intensities of the blue (460 ± 5 nm) and red (730 ± 5 nm) emission of suspensions excited at 310 ± 10 nm and 519 ± 10 nm were acquired, and the red/blue fluorescence ratios (Ratio R/B) were calculated for each proportion of live/dead *E. coli*. Each point represents the mean of four measurements.

Quantitation of *E. coli* with different viabilities by flow cytometry.

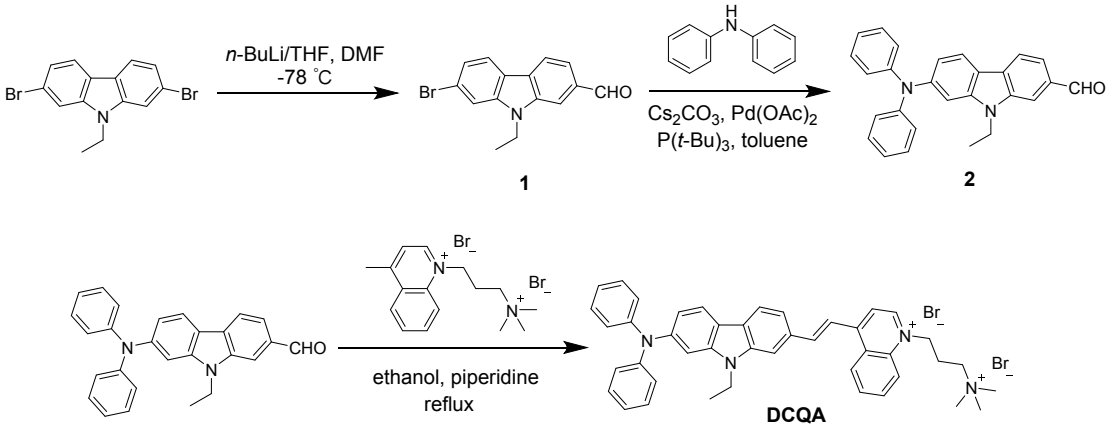
A single colony of *E. coli* on solid culture medium was transferred to 5 mL of liquid culture medium and grown at 37 °C for 10 h. After reaching logarithmic phase, 500 μL of bacteria suspension was transferred to a 1.5 mL microcentrifuge tube. Bacteria were harvested by centrifuging at 11700 g for 3 min. After removal of supernatant, bacteria were either killed by 1.5 mL 75 % alcohol (dead bacteria) or dealt with equal amount of PBS (living bacteria). Then live and dead bacteria were mixed at different ratios (L/D ratio), i.e., 0, 10 %, 30 %, 50 %, 70 %, 90 % and 100 %. Next, bacteria/fungi were harvested by centrifuging at 11700 g for 3 min. Then, 2 mL dye solution in buffer (pH = 10) at appropriate concentration was added into the microcentrifuge tube. After dispersing with vortex, the suspensions were incubated at 37 °C for designed time. Finally, the bacteria were measured by flow cytometry (BD FACS Aria IIIu). The integrated intensities of the blue (450 ± 40 nm) and red (780 ± 60 nm) emission of suspensions excited at 405 nm and 561 nm were acquired, and the red/blue fluorescence ratios (Ratio R/B) were calculated for each proportion of living/dead *E. coli*. Each point represents the mean of 5000 counts.

Quantitation of *E. coli* with different viabilities by confocal laser scanning microscope (CLSM).

A single colony of *E. coli* on solid culture medium was transferred to 5 mL of liquid culture medium and grown at 37 °C for 10 h. After reaching logarithmic phase, 500 μL of bacteria was transferred to a 1.5 mL microcentrifuge tube. Bacteria were harvested by centrifuging at 11700 g for 3 min. After removal of supernatant, bacteria were either killed by 1.5 mL 75 % alcohol (dead bacteria) or dealt with equal amount of PBS (living bacteria). Then live and dead bacteria were mixed at different ratios (L/D ratio), i.e., 0, 10 %, 30 %, 50 %, 70 %, 90 % and 100 %. Next, bacteria were harvested by centrifuging at 11700 g for 3 min. Then, 100 μL dye solution in buffer (pH=10) at appropriate concentration was added into the microcentrifuge tube. After dispersing with vortex, the suspensions were incubated at 37 °C for designed time. Finally, the confocal fluorescent images were obtained with Zeiss LSM 800 confocal laser scanning microscope. Different given percent live *E. coli* were stained with DCQA and TPE-2BA. Viability of *E. coli* suspension were calculated by CLSM images of each given percentage of live *E. coli*.

Experimental procedures for minimum inhibition concentration (MIC) test

A single colony of *S. aureus*, *E. coli* or *C. albicans* was grown in liquid medium (Mueller-Hinton broth for *S. aureus* and *E. coli*, RPMI-1640 for *C. albicans*). After reaching logarithmic phase, 1×10^6 cells mL^{-1} were adjusted for MIC test. DCQA and TPE-2BA were diluted in gradient through a sterile liquid medium. After the probes had been diluted, a volume of the standardized inoculum equal to the volume of the diluted probes was added to each dilution vessel, bringing the microbial concentration to approximately 5×10^5 cells mL^{-1}. The 96-well plates were incubated in incubator at specific temperature (37 °C for *S. aureus* and *E. coli*, 25 °C for *C. albicans*) for 24 h. After incubation, the series of dilution vessels is observed for microbial growth, usually indicated by turbidity. The last tube in the dilution series that does not demonstrate was recorded as the MIC values of the probes.



Scheme S1. Synthesis route to DCQA.

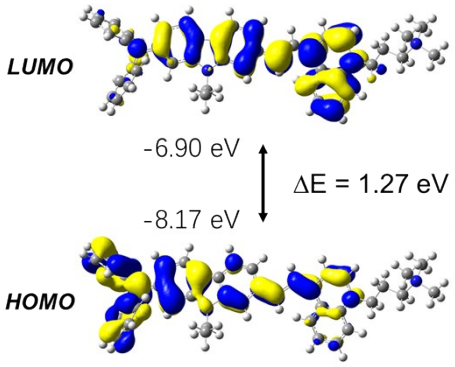


Figure S1. Molecular orbital amplitude plots of the HOMO and LUMO energy levels of DCQA.

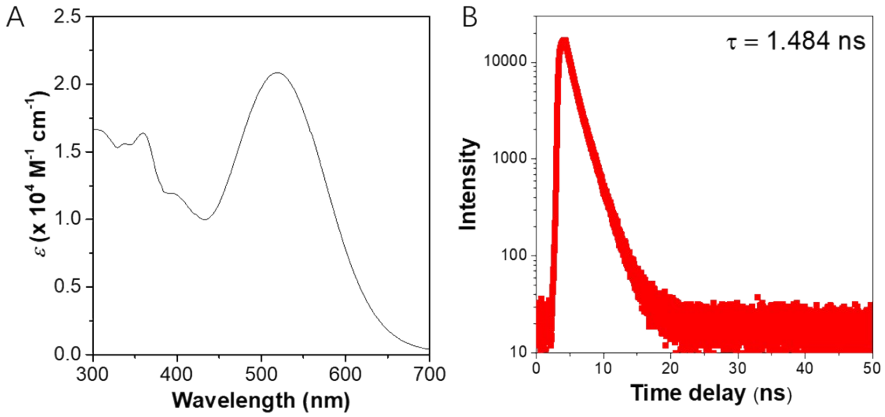


Figure S2. (A) Absorption spectra of DCQA in DMSO. (B) Fluorescence decay curves of DCQA in the solid state.

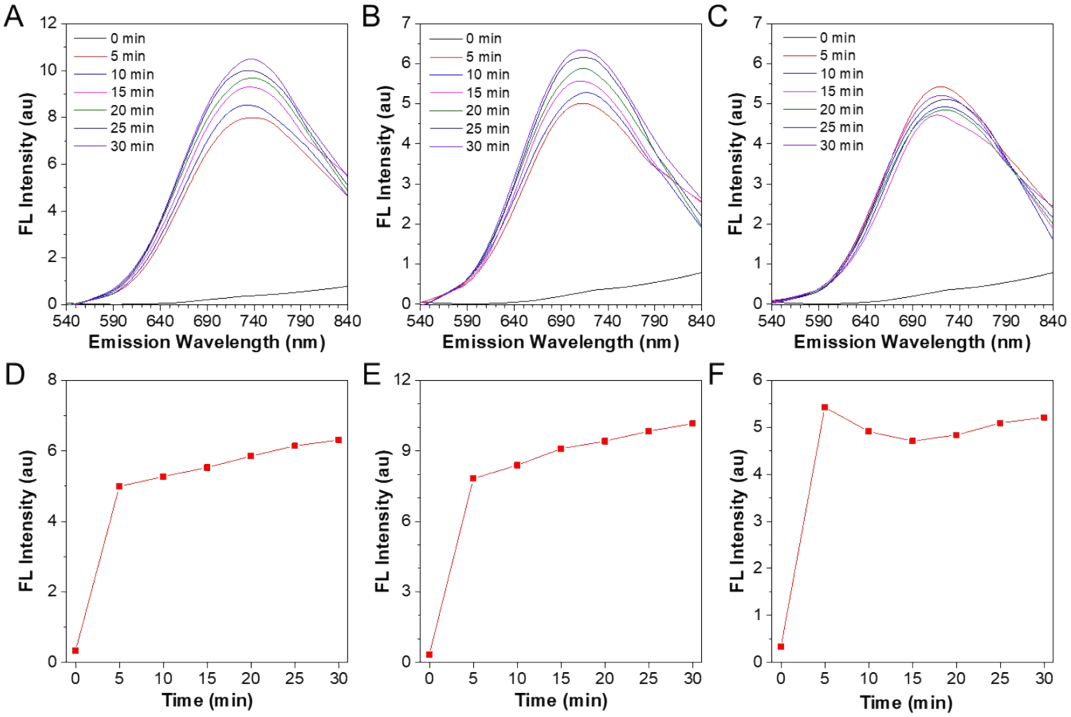


Figure S3. FL spectra of DCQA stained microbes at different time points. (A) *S. aureus*. (B) *E. coli*. (C) *C. albicans*. Excitation wavelength: 519 nm. Emission range: 540 – 840 nm. Concentration of DCQA is 10 μM. (D-E) FL intensity at different time. Wavelength peak = 720 nm.

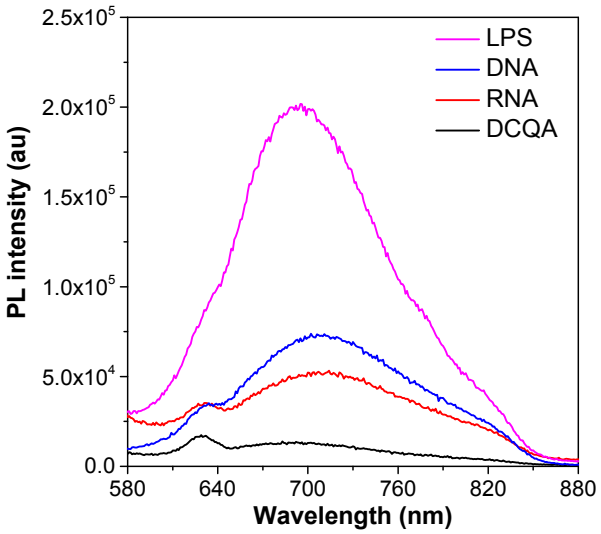


Figure S4. PL intensities of different bacterial component interacted with DCQA. Excitation wavelength: 519 nm. Emission range: 530 – 900 nm. Concentration of DCQA is 10 μM. 1× PBS was used as the medium.

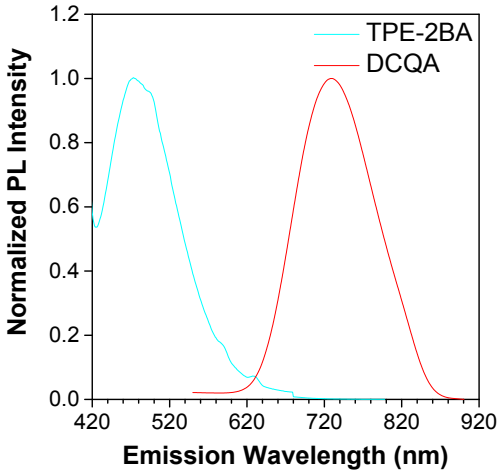


Figure S5. Photoluminescence (PL) spectra of TPE-2BA and DCQA. TPE-2BA: 10 μM in DMSO/water mixture. λ_{ex} = 405 nm. DCQA: 10 μM in DMSO/toluene mixture. λ_{ex} = 488 nm.

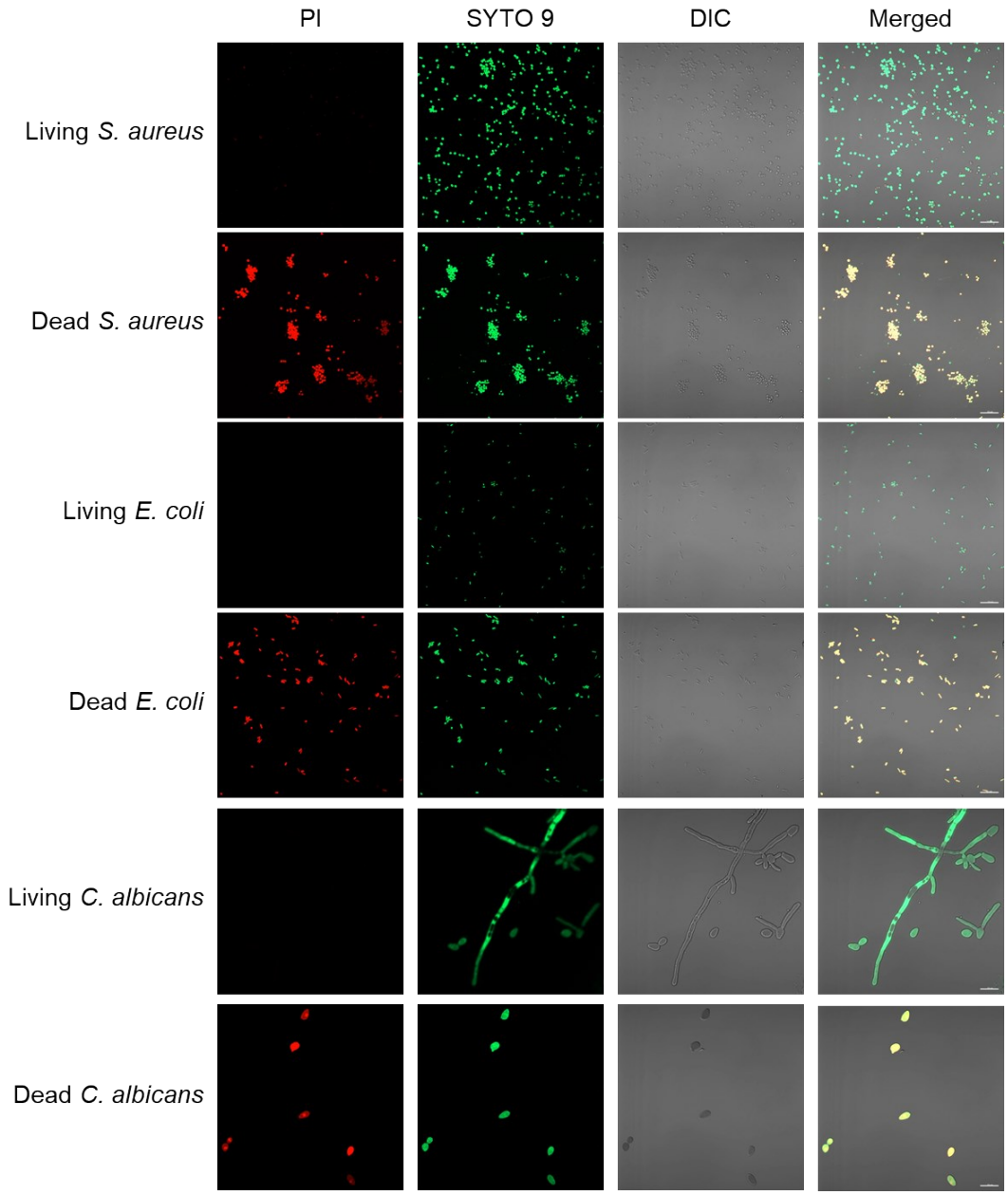


Figure S6. CSLM images of *S. aureus*, *E. coli* and *C. albicans* in both dead (treated with 75% ethonal and then washed with 1× PBS) and live (washed with 1× PBS only) status, which stained with Baclight Live/Dead Kit. For PI channel: λ_{ex} = 488 nm, λ_{em} = 570 – 700 nm. For SOTY 9 channel: λ_{ex} = 488 nm, λ_{em} = 500 – 560 nm.

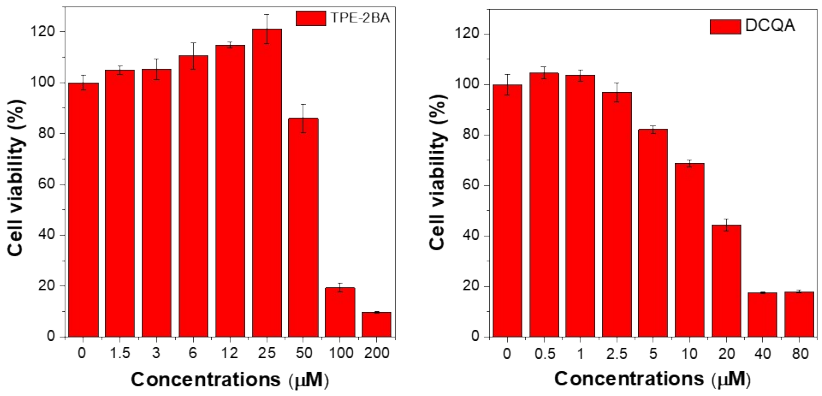


Figure S7. Cytotoxicity of AIEgens. Left: COS-7 cells incubated with different concentration of TPE-2BA. Right: COS-7 cells incubated with different concentration of DCQA.

Table S1. MIC results of DCQA and TPE-2BA toward three indicator strains

Microorganisms	MIC (μM)	
	DaCQA	TPE-2BA
S. aureus	> 80	> 400
E. coli	> 80	> 400
C. albicans	20	> 400