Electronic Supplementary Material (ESI) for Chemical Science.

This journal is © The Royal Society of Chemistry 2019

RGB-Emitting Molecular Cocktail for the Detection of Bacterial Fingerprints

Sheng Hong,‡[a] Di-Wei Zheng,‡[a] Qiu-Ling Zhang,[a] Wei-Wei Deng,[b] Wen-Fang Song,[a] Si-Xue Cheng,[a] Zhi-Jun Sun[b] and Xian-Zheng Zhang*[a]

[a] Key Laboratory of Biomedical Polymers of Ministry of Education & Department of Chemistry, Wuhan University, Wuhan 430072, P. R. China

[b] Department of Oral Maxillofacial Head Neck Oncology, School and Hospital of Stomatology, Wuhan University, Wuhan 430072, P. R. China.

* Corresponding author. E-mail: xz-zhang@whu.edu.cn (X.Z. Zhang).

‡ These authors contributed equally to this work.

Experimental Section

Materials. Boc-D-2,3-diaminopropionic acid was purchased from GL Biochem Ltd. Dimethylformamide (DMF), D-galactosamine hydrochloride, FITC and rhodamine B isothiocyanate (RB) were obtained from Aladdin Industrial Corporation. N-Succinimidyl 7-hydroxycoumarin-3-carboxylate was purchased from Heowns Biochem Technologies LLC. D-glucosamine hydrochloride was purchased from Thermofisher Scientific. Cy7-NHS was purchased from AmyJet Scientific Inc.

Characterization. Confocal laser scanning microscope (CLSM) images were obtained on a TCS SP8 STED (Leica) confocal laser scanning microscope. The fluorescence imaging was performed with an In Vivo Imaging system (IVIS, Perkin Elmer). Fluorescence spectra were recorded with a fluorescence spectrophotometer (Perkin Elmer). The point-of-care analysis of fluorescent samples was performed with "RealHex" mobile application on an IOS-operating smartphone (iPhone 8, Apple) or "Pixolor" mobile application on an Android-operating smartphone (Xiaomi Mi 8SE, Mi).

Bacterial strains. *Bacillus subtilis*, *Bacillus thuringiensis*, *Escherichia coli* (strain MG1655), *Clostridium butyricum*, *Clostridium difficile*, *Peptostreptococcus anaerobius*, *Staphylococcus aureus*, *Bacteriodes fragilis* and *Enterococcus faecalis* strains were obtained from the China Center for Type Culture Collection (CCTCC) and China General Microbiological Culture Collection Center (CGMCC). Brain Heart Infusion (BHI) broth and Luria-Bertani (LB) broth (Qingdao Hope Biotechnology, China) were used for the bacteria culture.

Synthesis of fluorescent monomers. *Coumarin-alanine (B-Ala)*. N-succinimidyl 7-hydroxycoumarin-3-carboxylate (91.0 mg, 0.3 mmol, 1.0 equiv) dissolved in anhydrous DMF (20 mL) was added into a round-bottom flask. Boc-D-2,3-diaminopropionic acid

(73.5 mg, 0.36 mmol, 1.2 equiv) was added and the mixture was reacted at RT overnight (17 h) under an atmosphere of nitrogen in the dark. The product was obtained by removing the solvent in a vacuum followed by treatment with trifluoroacetic acid/dichloromethane (1:1, 10 mL). After 30 min string, the solvent was evaporated in a vacuum. The product was characterized by ^{1}H NMR and mass spectrometry. Cm-Ala δ = 2.42 (s, 1H), 3.72- 3.79 (m, 1H), 3.84-3.90 (m, 1H), 4.05 (t, 1H), 6.84 (d, 1H), 6.88 (dd, 1H), 7.85 (d, 1H), 8.31 (s, 3H), 8.83 (s, 1H), 8.91 (t, 1H), 11.25 (s, 1H). ESI m/z calcd for $C_{13}H_{12}N_2O_6$ ([M-H]$^-$): 291.1, Found 291.2.

Fluorescein-glucose (G-Glu). D-glucosamine hydrochloride (77.6 mg, 0.36 mmol, 1.2 equiv) and Et_3N (91.1 mg, 0.9 mmol, 3.0 equiv) were mixed in DMF (10 mL). After 1 h stirring, the fluorescein (116.8 mg, 0.3 mmol, 1.0 equiv) was added. The reaction mixture was stirred for 16 h at RT in the dark. Then the product was obtained by filtering out the solid followed by concentration the filtrate in vacuo. The product was characterized by ^{1}H NMR and mass spectrometry. FITC-Glu δ = 3.0-3.1 (dd, 2H), 3.54-3.6 (m, 1H), 3.66-3.76 (m, 1H), 3.96-4.01 (m, 1H), 5.65 (d, 1H), 6.58-6.62 (d, 2H), 7.17-7.24 (m, 2H), 7.54-7.57 (dd, 1H), 7.75 (d, 1H). ESI m/z calcd for $C_{27}H_{24}N_2O_{10}S$ ([M-H]$^-$): 567.1, Found 567.0.

Rhodamine-galactose (R-Gal). D-galactosamine hydrochloride (77.6 mg, 0.36 mmol, 1.2 equiv) and Et_3N (91.1 mg, 0.9 mmol, 3.0 equiv) were dissolved in DMF (10 mL). After 1 h stirring, the rhodamine B isothiocyanate (160.8 mg, 0.3 mmol, 1.0 equiv) was added. The mixture was stirred for 16 h at RT in the dark. Then the mixture was filtered and the filtrate was dried in vacuo to get the product. The product was characterized by ^{1}H NMR and mass spectrometry. Rh-Gal δ = 1.13 (t, 12H), 3.03-3.09 (dd, 2H), 3.56-3.61 (m, 1H), 3.83-3.86 (m, 1H), 5.59 (s, 1H), 6.38 (d, 2H), 6.64-6.68 (m, 2H), 7.07-

7.11 (m, 2H), 7.59-7.61 (dd, 1H), 7.69 (s, 1H), 7.80 (s, 1H). ESI m/z calcd for $C_{35}H_{43}N_4O_8S^+$ ($[M]^+$): 679.3, Found 679.0.

Bacteremia modeling. The 6-8-week Balb/c mice were used for bacteremia modeling. The mice were intravenously injected with 100 μL different concentrations of S. aureus (1×10^6 CFU mL^{-1}, 1×10^7 CFU mL^{-1}, 1×10^8 CFU $mL^{-1}$and 1×10^9 CFU mL^{-1} in PBS). 24 h after the injection, blood samples were collected from the heart of mice and further analyzed by Microcolor system.

Skin infection modeling. The 6-8-week Balb/c mice were used for skin infection modeling. The mice were anaesthetized by isoflurane and a 7 mm length wounds were made on the right sides of the backbone followed by injection of 100 μL *S. aureus* or *E. coil* (1.0×10^7 CFU mL^{-1}) into the wounds. 3 Days after the inoculation, 5 μL of pus was collected and further analyzed by Microcolor system.

Bacterial strain labeling. The bacteria were grown overnight until the OD600 reached ~ 0.4 in the media. The medium containing three fluorescent monomers (0.5 mM) was prepared. DMSO (1%) was added to the medium to solubilize the Cm-Ala. Then the bacteria (100 μL) was added into the medium containing three fluorescent monomers (0.5 mM) and incubated for 12 h at 37 ^oC. After that, the bacteria were washed with PBS followed by centrifugation (1500 g, 3 min) for 3 times and then suspended in PBS buffer for later assay. 1% of DMSO did not affect bacteria labeling or growth.

Antibiotics test. Piperacillin (128 μg mL^{-1}) and cloxacillin (8 μg mL^{-1}) were used to kill *E. coli* (ATCC 25922) and *S. aureus*, respectively. 10^4 CFU of *E. coli* and *S. aureus* were added into the medium containing three fluorescent monomers (0.5 mM) or antibiotics and incubated for 24 h at 37 ^oC. For antimicrobial therapy in vivo, the piperacillin (1.28 mg mL^{-1}) and cloxacillin (80 μg mL^{-1}) were injected (100 μL) to the

mice infected with *E. coli* and *S. aureus*, respectively. The mice without antibiotic treatment were considered as control.

In vivo sample collection and processing. The samples collected from bacteremia model were performed by drawing blood from the heart of mice. And the bacteria samples were then separated from the blood by centrifugation (150 g) followed by directly addition into the Luria-Bertani broth (LB) medium contained with three fluorescent monomers (0.5 mM for each monomer) and incubated in the dark for 6 h at 37 ^{o}C.

The samples collected from skin infection model were performed by pipetting sanious (10 μL) from the wounds followed by directly addition into the LB medium contained with three fluorescent monomers (0.5 mM for each monomer) and incubated in the dark for 12 h at 37 ^{o}C.

The saliva samples obtained from healthy volunteers and patients suffering from OSCC were added into the Salivette followed by centrifugation at 150 g for 3min. All saliva samples were collected before the morning meal. No antibiotics were used 2 weeks before the sampling. Then, the supernate (100 μL) after centrifugation was added into the medium contained with three fluorescent monomers (0.5 mM for each monomer) and incubated in the dark for 12 h at 37 ^{o}C.

Readout of RGB values. The bacteria solution (1 mL) was add into a glass cell. Then, the solution was put in the portable spectrometer with three tunable band-pass filters. Under UV light, the RGB values were readout by the commercialized “RealHex” mobile application (app) based on an IOS-operating smartphone or “Pixolor” mobile application on an Android-operating smartphone. Images read from three channels were merged into RGB pictures.

Mice. All animal studies were approved by the Institutional Animal Care and Use Committee (IACUC) of the Animal Experiment Center of Wuhan University (Wuhan, China). All mouse experimental procedures were performed in accordance with the Regulations for the Administration of Affairs Concerning Experimental Animals approved by the State Council of People's Republic of China.

Patient samples. Saliva samples were obtained from patients in the School and Hospital of Stomatology Wuhan University. The School and Hospital of Stomatology of Wuhan University Medical Ethics Committee approved this study, and informed consent was obtained from the patients before they underwent surgery. The clinical stages of their HNSCC were classified according to the guidelines of the International Union Against Cancer (UICC 2002).

Statistical analysis. Unless indicated otherwise, 'average values' was defined as mean, and the error bars in each Figure represent S.D. of at least three independent experiments measured. Photographs of the three channels of RGB obtained from the portable spectrophotometer are superimposed into true color photographs. For statistical analyses, two-tailed Student's t-tests were performed by using Microsoft Excel 2013. Significance among more than two groups was calculated using ANOVA Turkey's test by using SPSS 22.0. The logistic regression analysis was performed with SPSS 22.0. For in vivo experiments, operations were blinded to treated groups. Animals were randomly divided into different groups.

Table S1. Summary of information and detection result of patients and health volunteers.

Individual Status	Staging	R	G	B	Rank (after normalization)
OSCC	II	162	95	113	-82.37
OSCC	IVa	150	110	217	-65.61
OSCC	IVa	141	163	254	-44.47
OSCC	II	94	78	147	-7.59
OSCC	III	150	36	31	-80.42
OSCC	II	107	87	152	-158.26
OSCC	III	106	64	150	-131.75
OSCC	II	137	77	96	-132.21
OSCC	I	84	56	107	-141.95
OSCC	II	127	54	99	-128.69
OSCC	II	131	73	178	-124.76
OSCC	II	97	50	105	-118.85
OSCC	II	119	40	76	-123.39
OSCC	III	118	48	58	-131.94
OSCC	III	121	60	140	-130.43
OSCC	II	115	58	115	-126.84
OSCC	IVa	108	77	168	-126.83
OSCC	III	109	59	137	-136.45
OSCC	II	56	44	93	-139.85
OSCC	IVa	137	79	186	-147.74
OSCC	IVa	123	73	113	-149.77
OSCC	III	135	78	165	-121.52
OSCC	III	96	76	120	-119.64
OSCC	II	95	71	152	-133.08
OSCC	IVb	52	17	44	-152.30
OSCC	II	40	36	40	-109.67
OSCC	III	40	46	36	-119.21
OSCC	III	35	30	27	-146.66
OSCC	II	54	60	116	4.97

Control	N/A	72	52	171	12.24
Control	N/A	85	46	186	-3.95
Control	N/A	36	27	116	48.51
Control	N/A	31	56	79	60.34
Control	N/A	29	20	65	55.21
Control	N/A	51	85	173	43.16
Control	N/A	66	73	133	23.51
Control	N/A	36	60	116	55.33
Control	N/A	31	54	81	59.92
Control	N/A	29	40	80	59.32
Precancerosis	N/A	29	30	65	57.29
Precancerosis	N/A	29	32	126	57.57
Precancerosis	N/A	27	29	65	59.38
Precancerosis	N/A	52	46	87	34.13
Benign tumor	N/A	4	48	23	89.93
Precancerosis	N/A	78	48	75	4.70
Precancerosis	N/A	31	32	83	55.36
Precancerosis	N/A	56	25	70	25.25
Precancerosis	N/A	44	25	62	39.05
Precancerosis	N/A	54	32	58	29.03

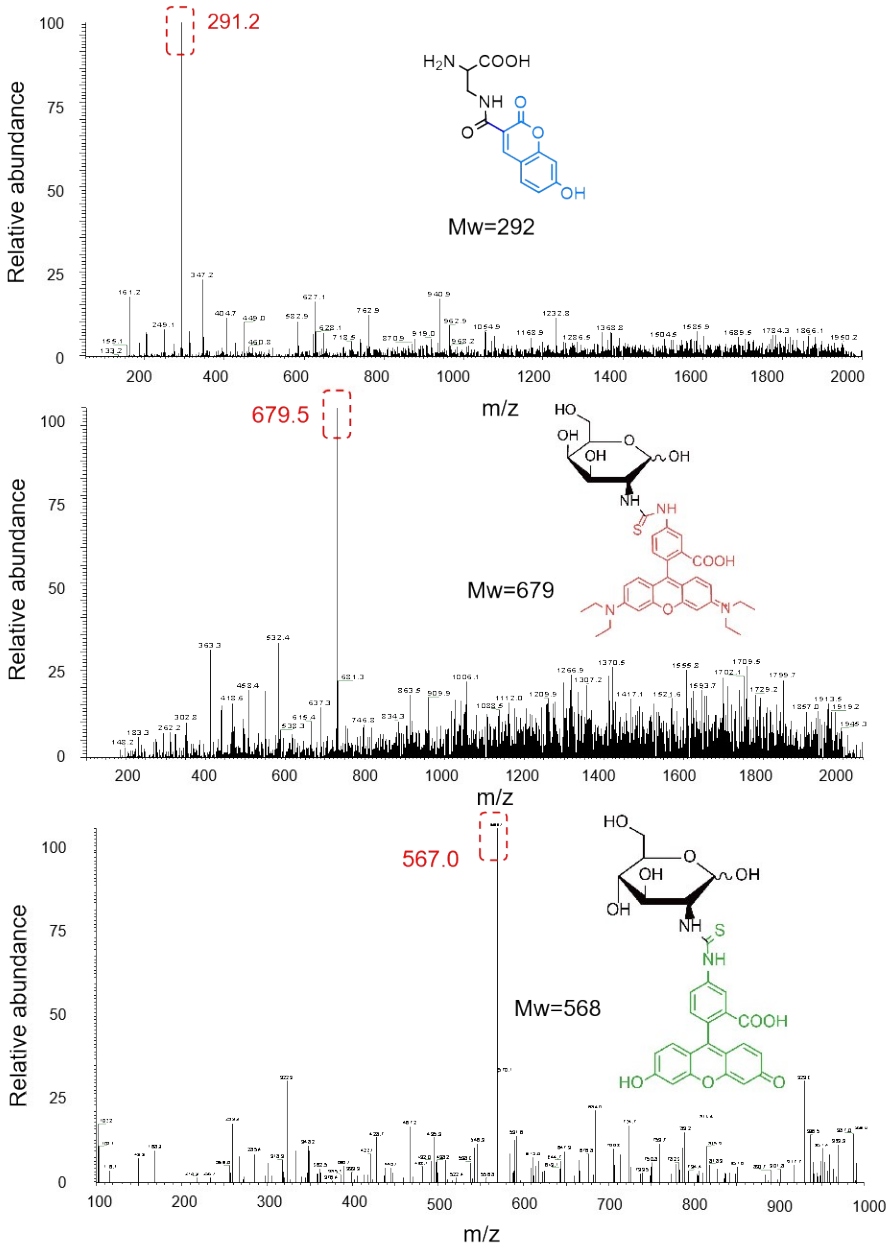

Fig. S1 ESI-MS analysis of coumarin-alanine, fluorescein-glucose and rhodamine-galactose. Coumarin-alanine: calculated: 291 $[M-H]^-$, found: 291; rhodamine-galactose: calculated: 679 $[M]^+$, found: 679; fluorescein-glucose: calculated: 567 $[M-H]^-$, found: 567.

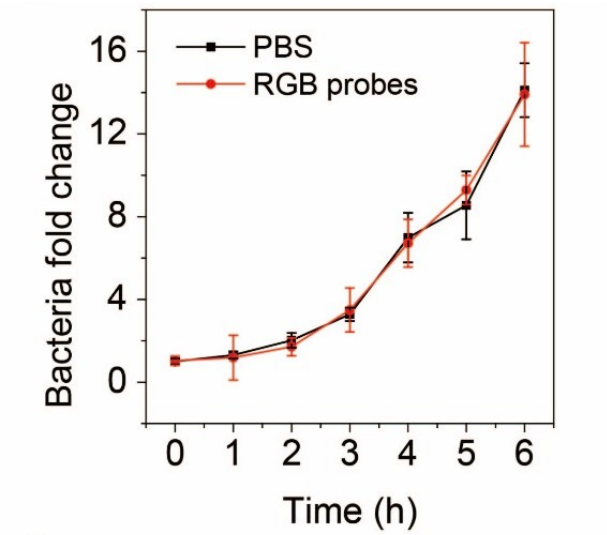

Fig. S2 The influence of RGB probes towards the growth of *E. coli*. The entire labeling procedure did not display any unfavorable effect on bacterial growth. As a control, the same volume of PBS was added to the culture medium.

Fig. S3 Three view drawing of the portable spectrometer for automating the colorimetric analysis with a smartphone. The portable spectrometer equipped with three tunable band-pass filters (blue, green and red optical filters).

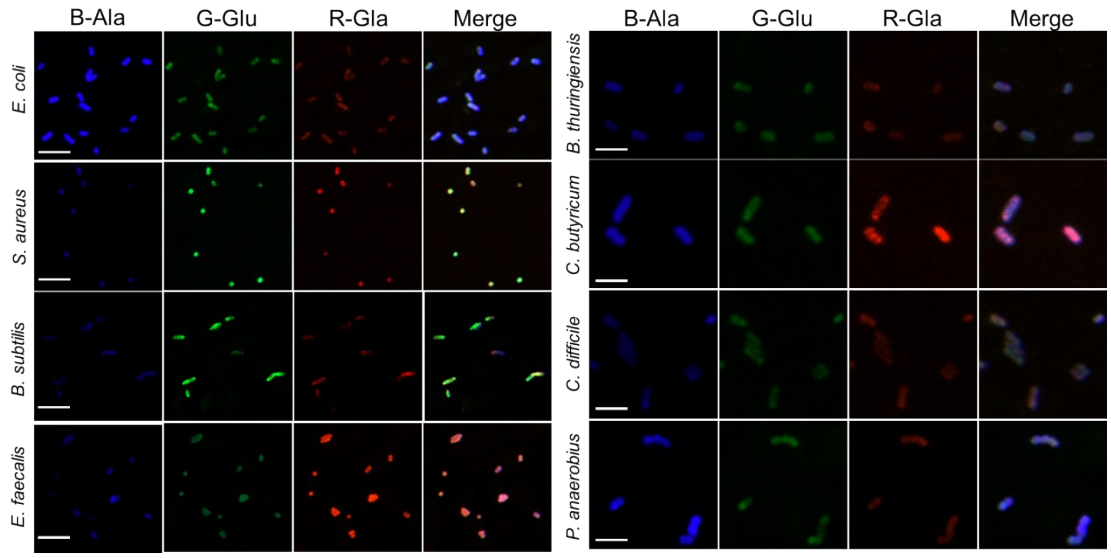

Fig. S4 Fluorescence images of *B. thuringiensis*, *B. subtilis*, *E. coli*, *C. difficile*, *C. butyricum*, *P. anaerobius*, *S. aureus* and *E. faecalis* (scale bar: 5 μm).

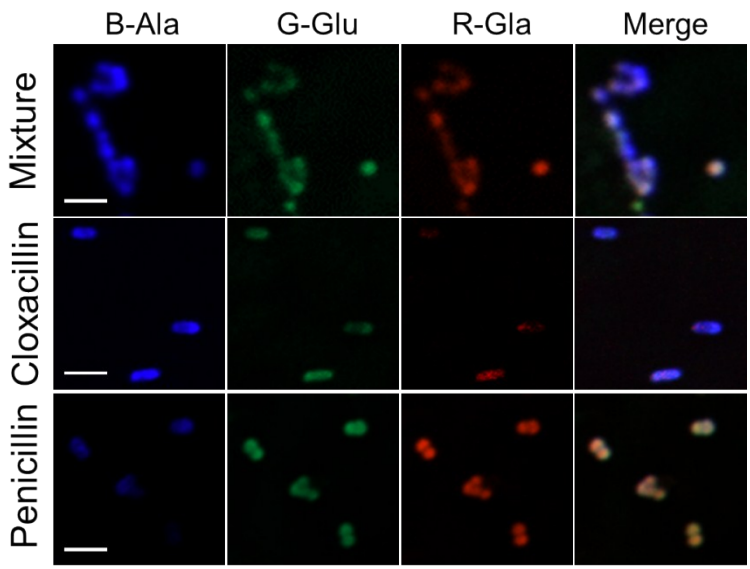

Fig. S5 Fluorescence images of the mixture of *E. coli* and *S. aureus* and the mixture after treatment with Piperacillin and cloxacillin. Piperacillin and cloxacillin were used to kill *E. coli* and *S. aureus*, respectively (scale bar: 8 μm).

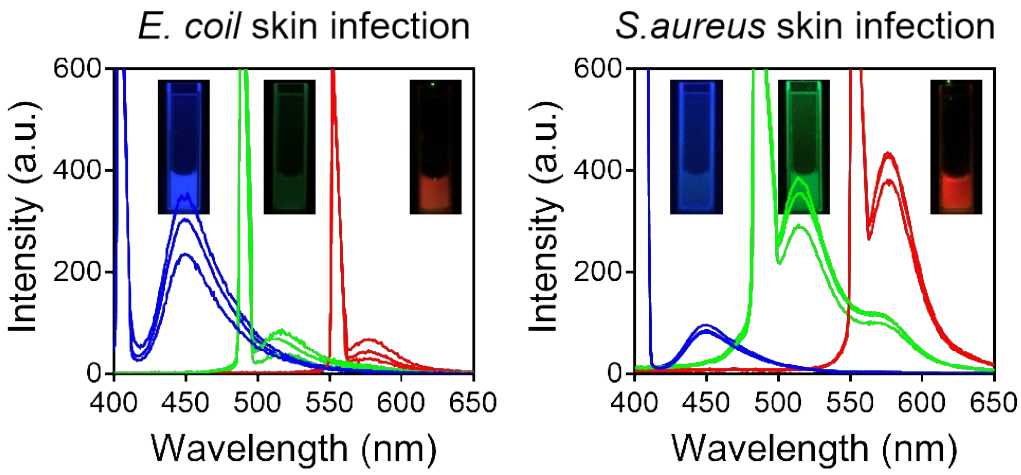

Fig. S6 Microcolor system for identifying mice underwent bacterial skin infection. Pus samples were collected from *E. coli* or *S. aureus* infected wound and further detected by Microcolor system and fluorescence spectrophotometer. All results were repeated in three independent experiments.

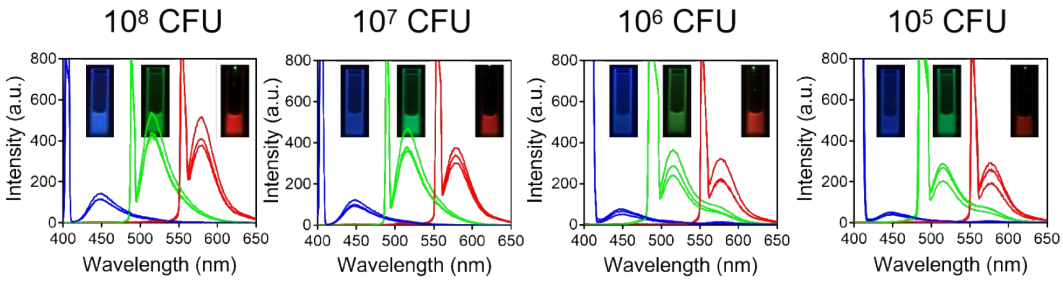

Fig. S7 Fluorescence fingerprint for identifying mice underwent bacteremia. Blood samples were obtained from *S. aureus* bacteremia bearing mice and further detected by Microcolor system and fluorescence spectrophotometer. All results were repeated in three independent experiments.

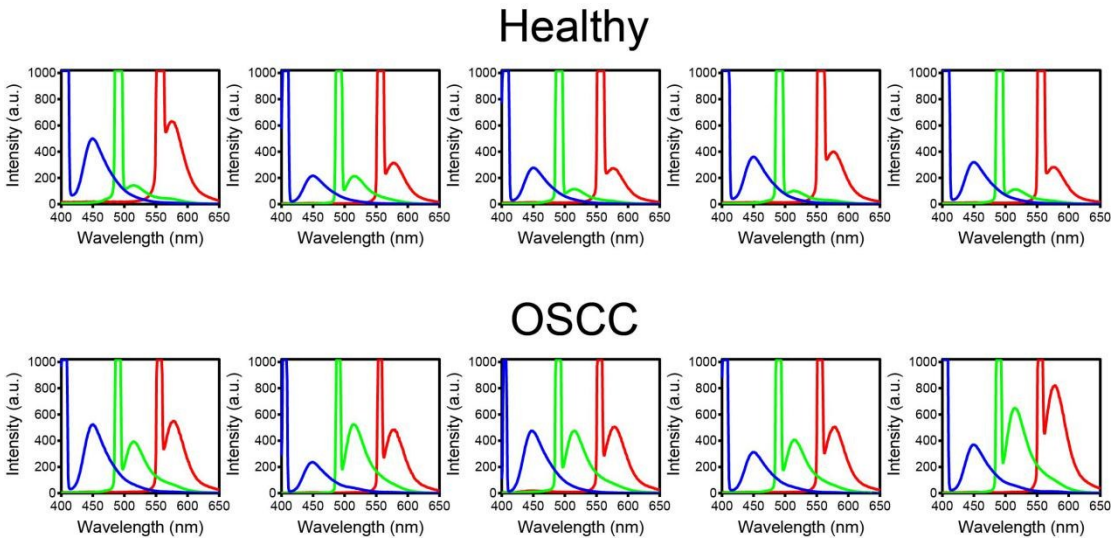

Fig. S8 Variation of fluorescence spectra between OSCC patients and healthy people.

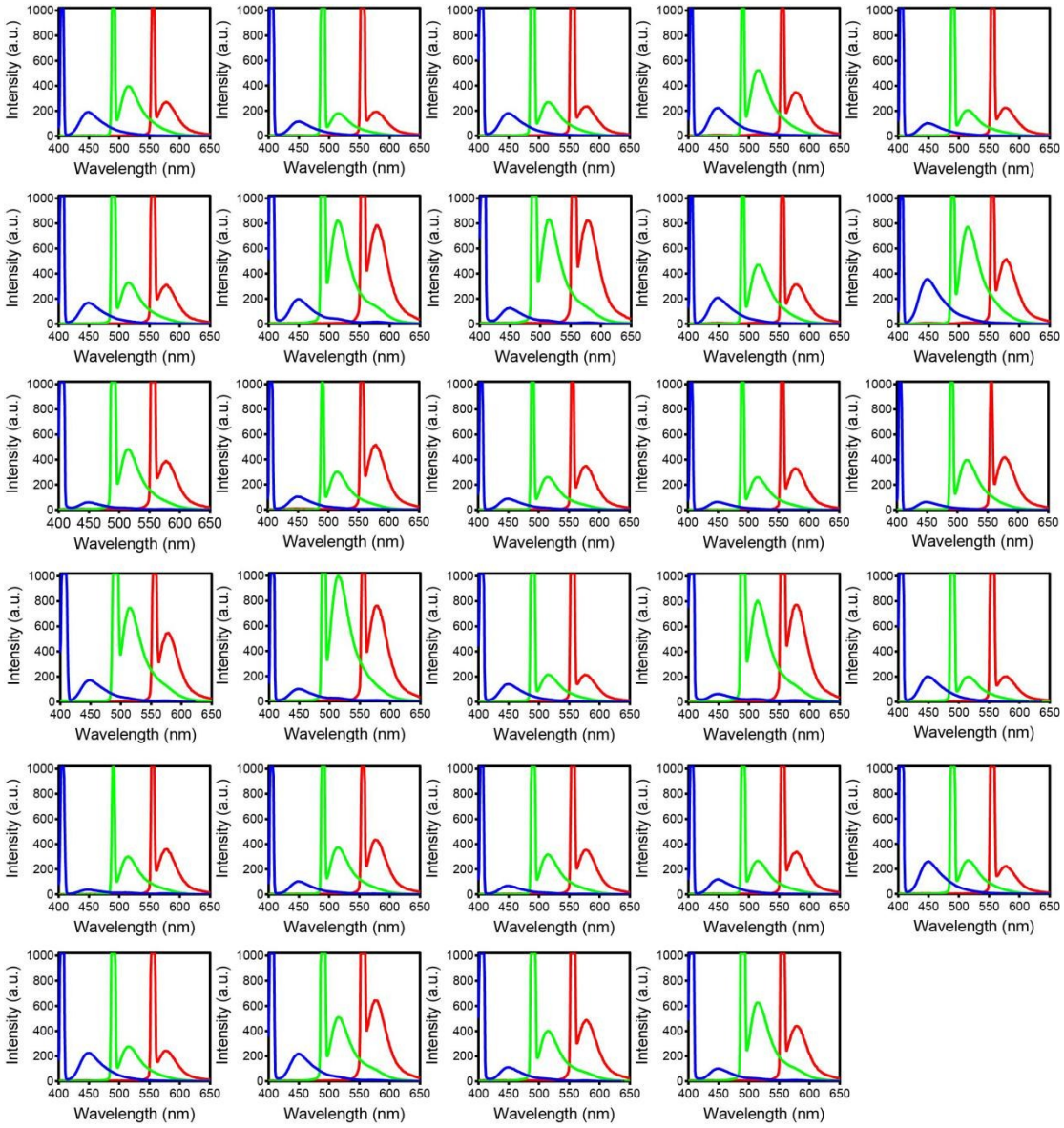

Fig. S9 Fluorescence spectra of saliva samples obtained from OSCC patients. Saliva samples from 29 patients were used for the fluorescence spectrophotometer detection.

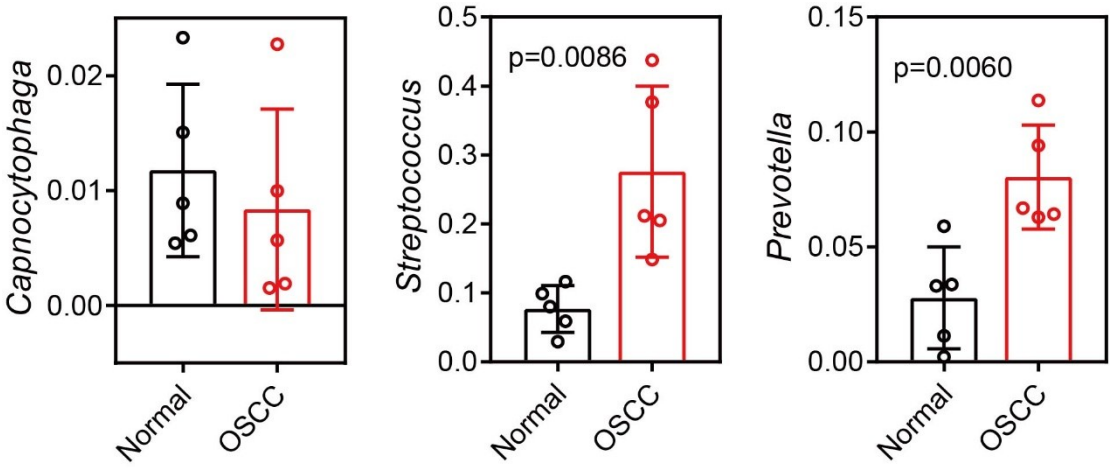

Fig. S10 Oral microbiome assay of OSCC patients and healthy people.

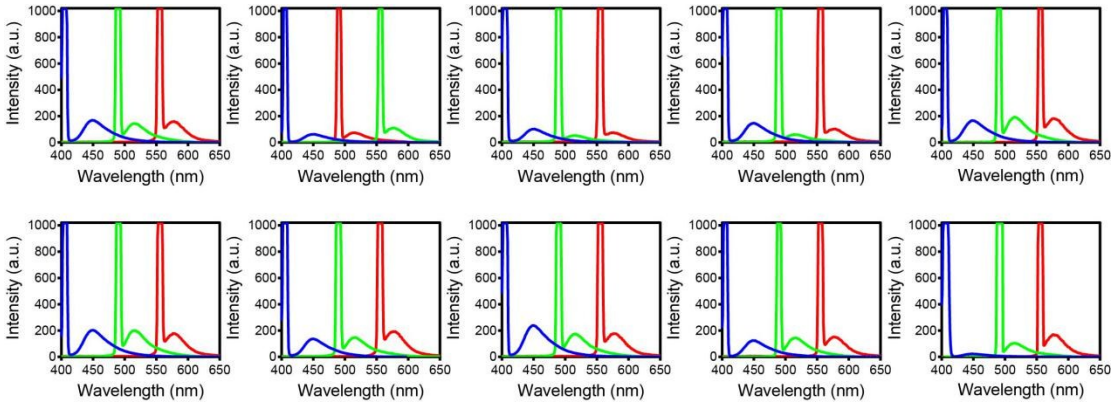

Fig. S11 Fluorescence spectra of saliva samples obtained from patients with precancerous lesions. Saliva samples from 10 patients with precancerous lesions were used for the fluorescence spectrophotometer detection.