

Supplementary Information

A ratiometric fluorescent sensor array as a versatile tool for bacterial pathogen identification and analysis

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1. Synthesis of the dyes

To 1 g of a N,N-disubstituted aminobenzaldehyde, 1 equiv. of 2-hydroxyacetophenone (or 2-hydroxynaphthophenone to obtain BfDMAF) was added in a 50 mL round-bottom flask, and the compounds were dissolved in 10 mL of DMF. Subsequently, 3 equiv. of sodium methylate were added, and the mixture was stirred at room temperature for 3 hours. After that, the mixture was transferred into a bigger flask and diluted with 50 mL of ethanol. An excess of 30 equiv. of sodium methoxide was then added, followed by an excess of 20 excess of 20 equiv. of hydrogen peroxide (30% v/v), that was added dropwise while stirring. Formation of the gel of the intermediate epoxide product was observed, which then dissolved upon further addition of H₂O₂. The mixture was subsequently refluxed for 2-3 minutes and left to cool down. After that, the reaction mixture was diluted with 50 mL of ultrapure water, and neutralized to pH 6-7. The product was filtered (extracted with ... in case of DOAF) and purified as indicated below.

DMAF: the crude product was recrystallized from methanol. Yield 0.71 g (35.5 %). ¹H NMR (500 MHz, DMSO-d₆): 3.01 (s, 6H), 6.84 (d, 2H), 7.42 (t, 1H), 7.73 (m, 2H), 8.07 (d, 1H), 8.12 (d, 2H), 9.16 (s, 1H).

BfDMAF: the crude product was recrystallized from methanol. Yield 0.92 g (41.5 %). ¹H NMR (500 MHz, DMSO-d₆): 3.02 (s, 6H), 6.87 (d, 2H), 7.66 (t, 1H), 7.77-7.85 (m, 2H), 8.08 (d, 1H), 8.16 (d, 2H), 8.27 (d, 1H), 9.23 (s, 1H), 10.01 (d, 1H).

DOAF: the crude product was purified by column chromatography (silica gel, chloroform). Yield 0.31 g (22.2 %). ¹H NMR (500 MHz, DMSO-d₆): 0.84 (t, 6H), 1.24-1.27 (m, 20H), 1.47 (m, 4H), 3.20 (t, 4H), 6.50 (t, 1H), 6.59 (d, 2H), 6.71 (d, 1H), 7.07 (t, 2H), 7.69 (d, 1H), 8.07 (d, 1H), 9.60 (s, 1H).

DPAF: the crude product was recrystallized from methanol. Yield 0.51 g (34.7 %). ¹H NMR (500 MHz, DMSO-d₆): 7.04 (d, 2H), 7.14 (m, 6H), 7.37 (m, 4H), 7.45 (t, 1H), 7.70 (d, 1H), 7.78 (t, 1H), 8.09 (t, 3H), 9.31 (s, broad, 1H).

Table S1. Hydrodynamic radii and polydispersity indices of the dye-lodaded nanoparticles based on data from the dynamic light scattering measurements

Dye	Radius, nm	PDI
DMAF	440 ± 160	0.670 ± 0.120
BfDMAF	855 ± 50	0.465 ± 0.030
DOAF	292 ± 7	0.065 ± 0.050
DPAF	290 ± 16	0.385 ± 0.040

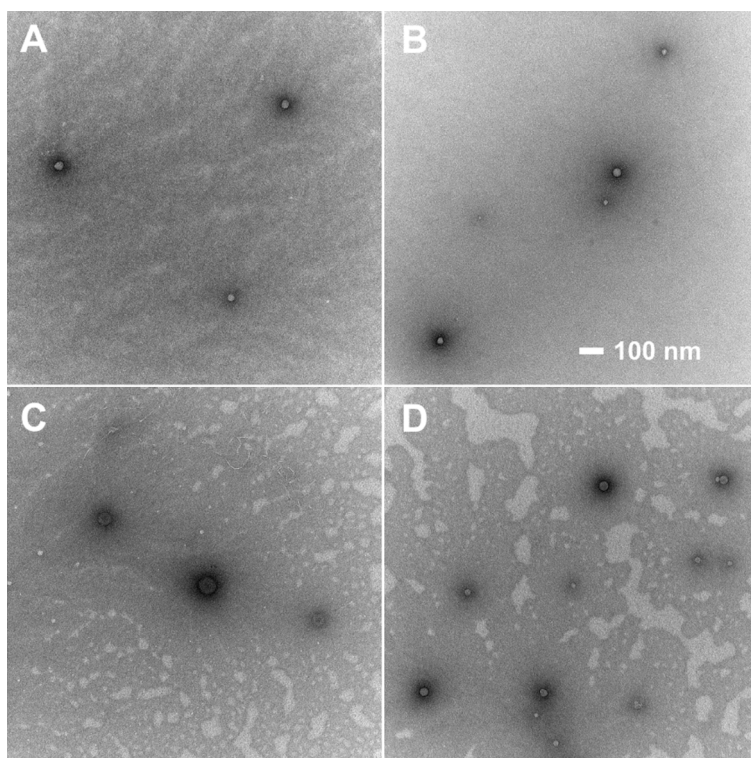


Figure S1. TEM micrographs of 0.5 mg/mL solutions of freshly prepared HA nanoparticles loaded with DMAF (A), BfDMAF (B), DOAF (C), and DPAF (D).

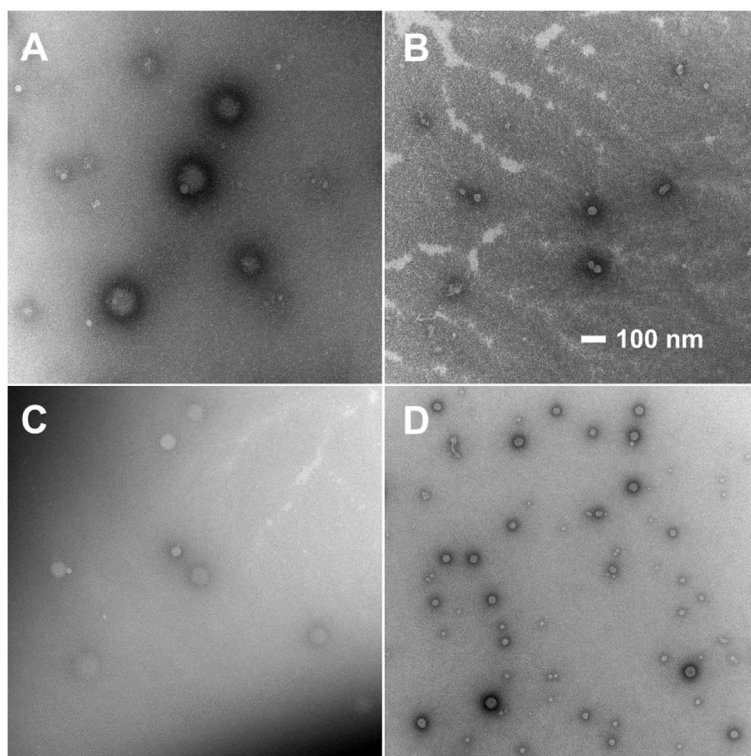


Figure S2. TEM micrographs of 0.5 mg/mL solutions of HA nanoparticles loaded with DMAF (A), BfDMAF (B), DOAF (C), and DPAF (D). Solutions were prepared 8 months prior to imaging.

2. Fluorescent response upon interaction with bacteria

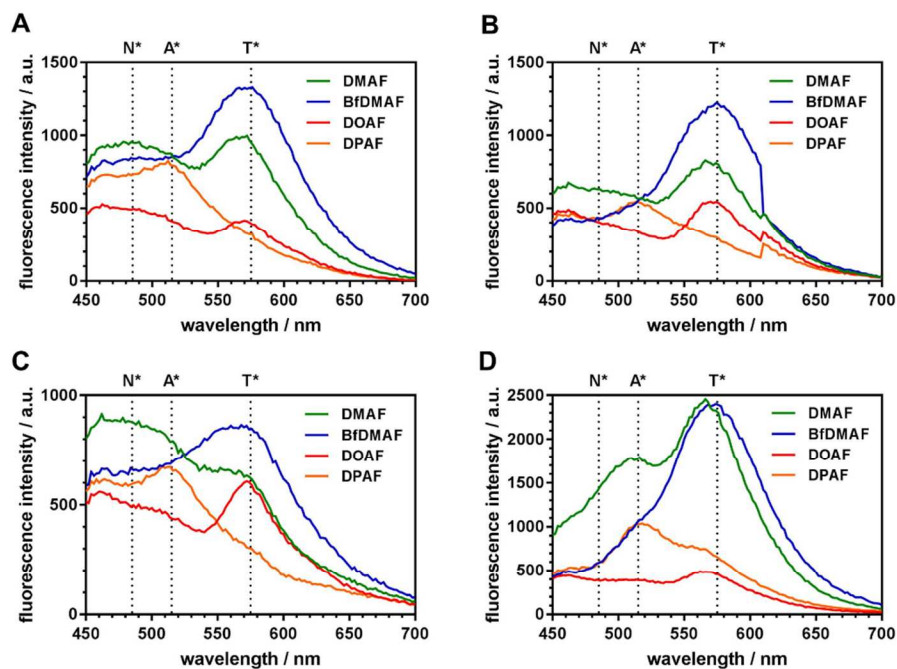


Figure S3. Fluorescence spectra of the ratiometric dyes upon interaction with Gram-positive bacteria: *Staphylococcus aureus* (A), *Staphylococcus epidermidis* (B), *Bacillus subtilis* (C), and *Enterococcus faecalis* (D).

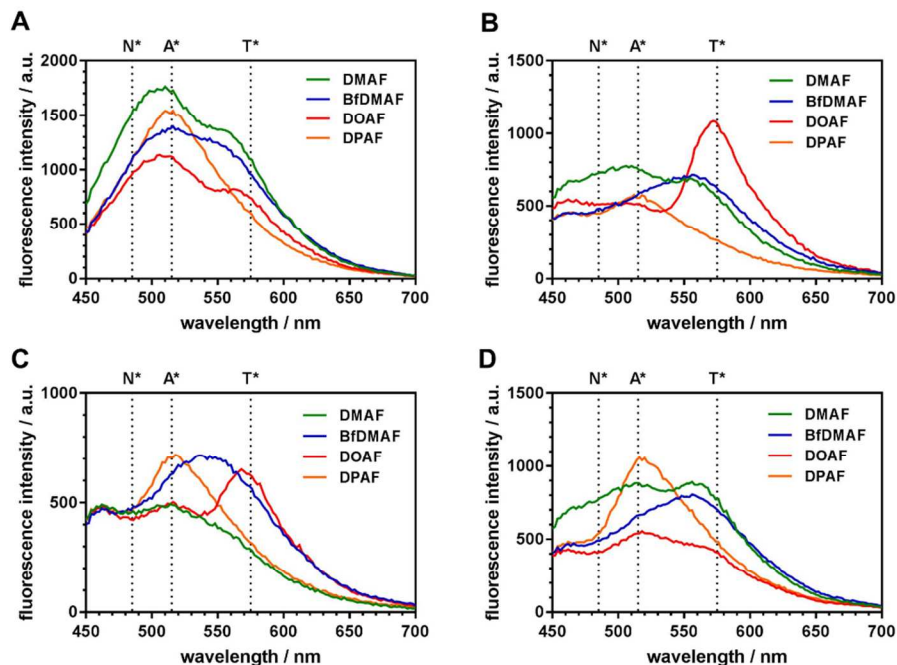


Figure S4. Fluorescence spectra of the ratiometric dyes upon interaction with Gram-negative bacteria: *Escherichia coli* (A), *Acinetobacter baumannii* (B), *Klebsiella pneumoniae* (C), and *Citrobacter freundii* (D).

3. Multichannel sensing

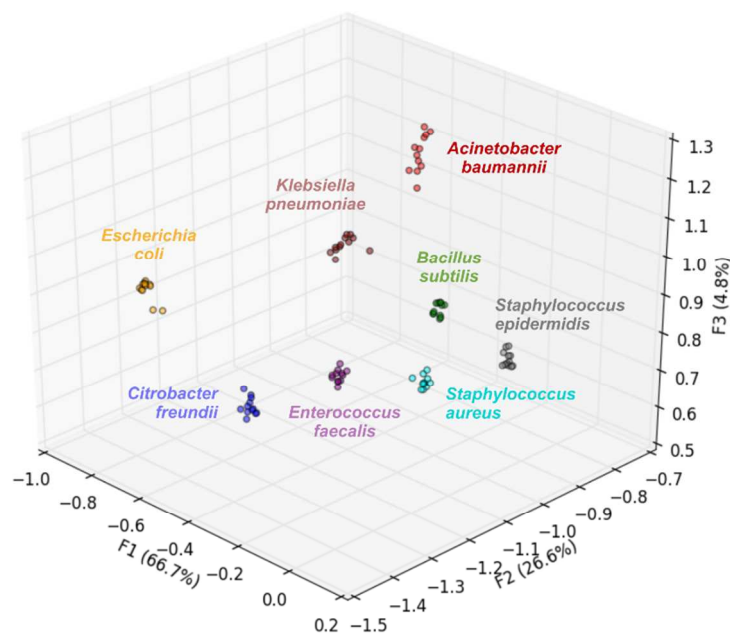


Figure S5. Canonical plot of the double-channel ratiometric response from the four sensor components in the subspace of the first three discriminants.

4. Analysis of unknown samples

Table S2. Results of validation of the sensor array using randomized strain-proportional training (75%) and test (25%) subsets of data

Unknown species	Correct attribution to the corresponding class								Total
	1	2	3	4	5	6	7	8	
<i>Staphylococcus aureus</i>	3/3	3/3	3/3	3/3	3/3	3/3	3/3	3/3	24/24 100%
<i>Escherichia coli</i>	3/3	3/3	3/3	3/3	3/3	3/3	3/3	3/3	24/24 100%
<i>Staphylococcus epidermidis</i>	3/3	3/3	3/3	3/3	3/3	3/3	3/3	3/3	24/24 100%
<i>Acinetobacter baumannii</i>	3/3	3/3	3/3	3/3	3/3	3/3	3/3	3/3	24/24 100%
<i>Klebsiella pneumoniae</i>	3/3	3/3	3/3	3/3	3/3	3/3	3/3	3/3	24/24 100%
<i>Bacillus subtilis</i>	3/3	3/3	3/3	3/3	3/3	3/3	3/3	3/3	24/24 100%

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<i>Citrobacter freundii</i>	3/3	3/3	3/3	3/3	3/3	3/3	3/3	3/3	24/24 100%
<i>Enterococcus faecalis</i>	3/3	3/3	3/3	3/3	3/3	3/3	3/3	3/3	24/24 100%
TOTAL	24/24	24/24	24/24	24/24	24/24	24/24	24/24	24/24	192/192 100%

5. Predictive potential of the sensor array

Table S3. Determination of the Gram status for the unknown samples. Data for pairs of bacterial strains, one Gram(+) and one Gram(-) in each pair (shaded), were excluded from the main dataset and used as the test subset; data for the remaining 6 bacterial species were used as the training subset

Unknown species	Correct determination of the Gram status	
	samples	%
<i>Staphylococcus aureus</i>	12/12	100
<i>Escherichia coli</i>	12/12	100
<i>Staphylococcus epidermidis</i>	12/12	100
<i>Acinetobacter baumannii</i>	12/12	100
<i>Klebsiella pneumoniae</i>	12/12	100
<i>Bacillus subtilis</i>	12/12	100
<i>Citrobacter freundii</i>	12/12	100
<i>Enterococcus faecalis</i>	12/12	100
TOTAL	96/96	100

Table S4. Classification of the unknown samples outside of the training dataset in the “one against the rest” discrimination analysis. Data for the “test species” were excluded from the main dataset and used as a test subset; data for the remaining 7 bacterial species were used as a training subset – the target species against the other 6 species

Target species	Test species	Correct attribution to the “Other bacteria” class	
		samples	%
<i>Staphylococcus aureus</i>	<i>Escherichia coli</i>	7/12	58
	<i>Staphylococcus epidermidis</i>	10/12	83
	<i>Acinetobacter baumannii</i>	12/12	100
	<i>Klebsiella pneumoniae</i>	12/12	100
	<i>Bacillus subtilis</i>	4/12	33
	<i>Citrobacter freundii</i>	11/12	92
	<i>Enterococcus faecalis</i>	12/12	100

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<i>Escherichia coli</i>	<i>Staphylococcus aureus</i>	12/12	100
	<i>Staphylococcus epidermidis</i>	12/12	100
	<i>Acinetobacter baumannii</i>	12/12	100
	<i>Klebsiella pneumoniae</i>	12/12	100
	<i>Bacillus subtilis</i>	12/12	100
	<i>Citrobacter freundii</i>	12/12	100
	<i>Enterococcus faecalis</i>	2/12	16
<i>Staphylococcus epidermidis</i>	<i>Staphylococcus aureus</i>	12/12	100
	<i>Escherichia coli</i>	12/12	100
	<i>Acinetobacter baumannii</i>	7/12	58
	<i>Klebsiella pneumoniae</i>	12/12	100
	<i>Bacillus subtilis</i>	12/12	100
	<i>Citrobacter freundii</i>	12/12	100
	<i>Enterococcus faecalis</i>	12/12	100
<i>Acinetobacter baumannii</i>	<i>Staphylococcus aureus</i>	12/12	100
	<i>Escherichia coli</i>	11/12	92
	<i>Staphylococcus epidermidis</i>	3/12	25
	<i>Klebsiella pneumoniae</i>	10/12	83
	<i>Bacillus subtilis</i>	12/12	100
	<i>Citrobacter freundii</i>	12/12	100
	<i>Enterococcus faecalis</i>	12/12	100
<i>Klebsiella pneumoniae</i>	<i>Staphylococcus aureus</i>	12/12	100
	<i>Escherichia coli</i>	11/12	92
	<i>Staphylococcus epidermidis</i>	12/12	100
	<i>Acinetobacter baumannii</i>	12/12	100
	<i>Bacillus subtilis</i>	3/12	25
	<i>Citrobacter freundii</i>	10/12	83
	<i>Enterococcus faecalis</i>	12/12	100
<i>Bacillus subtilis</i>	<i>Staphylococcus aureus</i>	2/12	16
	<i>Escherichia coli</i>	12/12	100
	<i>Staphylococcus epidermidis</i>	12/12	100
	<i>Acinetobacter baumannii</i>	12/12	100
	<i>Klebsiella pneumoniae</i>	12/12	100
	<i>Citrobacter freundii</i>	12/12	100
	<i>Enterococcus faecalis</i>	5/12	42
<i>Citrobacter freundii</i>	<i>Staphylococcus aureus</i>	12/12	100
	<i>Escherichia coli</i>	11/12	92
	<i>Staphylococcus epidermidis</i>	12/12	100
	<i>Acinetobacter baumannii</i>	12/12	100
	<i>Klebsiella pneumoniae</i>	12/12	100
	<i>Bacillus subtilis</i>	12/12	100
	<i>Enterococcus faecalis</i>	9/12	75
<i>Enterococcus faecalis</i>	<i>Staphylococcus aureus</i>	12/12	100
	<i>Escherichia coli</i>	11/12	92
	<i>Staphylococcus epidermidis</i>	12/12	100
	<i>Acinetobacter baumannii</i>	12/12	100
	<i>Klebsiella pneumoniae</i>	12/12	100
	<i>Bacillus subtilis</i>	12/12	100
	<i>Citrobacter freundii</i>	12/12	100
TOTAL		595/672	89