Bacteria Identification | Hot Paper

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Identification of Bacteria in Water by a Fluorescent Array**

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Abstract: *We report a method for the rapid and efficient identification of bacteria making use of five probes having fluorescent characteristics (F-array) and subsequent statistical analysis. Eight kinds of bacteria, including normal and multidrug-resistant bacteria, are differentiated successfully. Our easy-to-perform and time-saving method consists of mixing bacteria and probes, recording fluorescent intensity data by automated flow cytometry, and statistical analysis. No washing steps are required in order to identify the different bacteria simultaneously.*

The identification of bacteria has become increasingly important because of the huge demand in biology, clinical diagnosis, and the food industry.[1] Rapid and efficient bacteria identification plays a vital role in identifying the origins of infectious diseases and food contamination, and providing guidance for antibiotic usage and environmental monitoring. Especially in clinical diagnosis, it is significant for directing the treatment of individual infected patients and monitoring the epidemiology of infectious disease. Bacteria identification can be also applied in discovering outbreaks of pathogens, monitoring trends in infection, and identifying the emergence of new threats.[2]

We present a multifluorescence array based on probes with fluorescent characteristics (F-array) for bacteria identification with the assistance of statistical methods, such as principal component analysis (PCA) and quadratic discriminant analysis (QDA). The molecules we chose have different charged groups and hydrophobic properties. Because of their different surface structures, the bacteria display various interactions with these probe molecules. The fluorescence of the F-array can be recorded by fluorescence microscopy or flow cytometry, and the data are applied to achieve an effective method for the identification of bacteria following mathematical analysis methods. In this report, eight kinds of bacteria, including two multidrug-resistant (MDR) bacteria, are used to test the efficiency of the array, and the analysis data demonstrate that the F-array is effective in differentiating species of bacteria, even between normal bacteria and multidrug-resistant bacteria.

Conventional methods of identifying bacteria include plating and culturing,[3] polymerase chain reaction (PCR),[4] gene microarray,[5] gene sequencing identification techniques,[6] fluorescence in situ hybridization (FISH),[7] and immunological techniques.[8] The plating and culturing method is usually time-consuming and requires more than 24 h. Gene-related techniques typically employ amplification, and the occurrence of false-positive results is unavoidable. FISH is designed to detect specific bacteria types and requires complex procedures. Immunological techniques require special antibodies, which are difficult to choose for unknown bacteria, and the cost of antibodies is usually high. With the development of new technologies, surface-enhanced Raman spectroscopy (SERS),[9] mass spectrometry,[10] and biochemical sensors (such as polymer sensors,[11] nanomaterial-based sensors,[12] and molecule probes[13]) are also applied in bacteria identification. However, SERS requires a reproducible rugged surface to increase the Raman signal, whose reproducibility and stability should be improved. Mass spectrometry requires pretreatment to obtain proteins or DNA and a comprehensive database to differentiate each kind of bacteria correctly. Some biochemical sensors also use fluorescence signals, often combined with mathematical analysis, to identify different bacteria.[14] Traditional fluorescent molecules have some disadvantages like photobleaching and strong background signals; conjugated polymer sensors also have the problem of strong background signals. The identification of bacteria remains a big challenge, even for advanced technologies such as automated biochemical instrumentation, MALDI-TOF, and strain-typing analysis systems, which are applied in hospitals and other authorized organizations; the accuracy rate is 90–95% and several hours are still needed to complete the identification (Table S1).[15] So a new and effective method for identification of bacteria is required.

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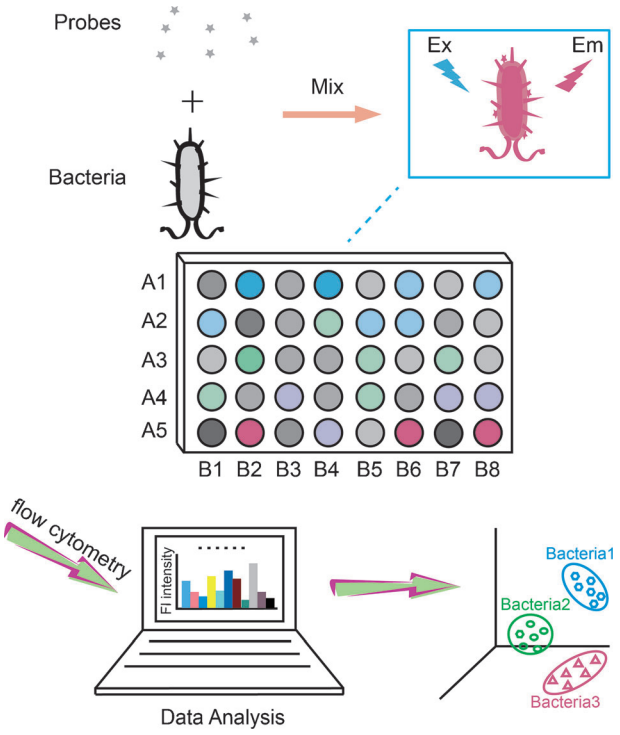
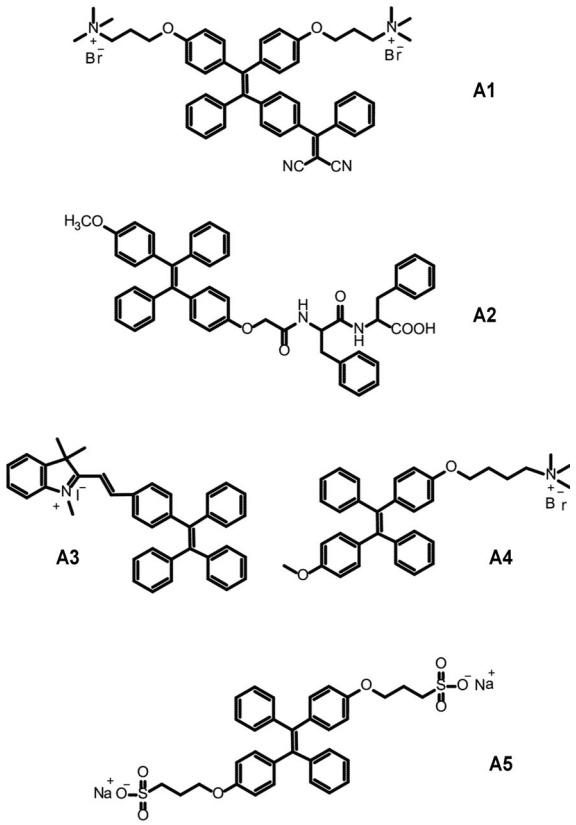
In this work, we used probes with particular characteristics to overcome the disadvantages of fluorescent molecules. The probes, derivatives of tetraphenylethylene, are unique luminophores that are nonluminescent when they are dispersed and dissolve well in solution, but show high fluorescence emission when aggregated.[16] For this reason there is essentially no background fluorescence and a high signal-to-noise ratio. These probe molecules display advantages like high quantum yield, good photostability, and satisfactory biocompatibility, which make them widely applicable in different areas.[17] No one has attempted to achieve bacteria identification using this kind of molecules.

The probes can be designed and synthesized with various electronic properties to achieve our proposal. We thought we could apply molecules with different electronic properties to identify different bacteria in some cases. Bacteria have different surface electronic properties, and based on this we made use of five probes with special fluorescent characteristics and different functional groups to identify diverse bacteria. The collective fluorescent signals of the labeled bacteria can be used to identify various bacteria, which is usually called sensor array. Some sensor arrays based on organic molecules are applied to identify anions, cations, and small molecules.[18] This kind of analytical strategy is conducive to high-throughput screening and can bue used to identify multiple analytes simultaneously. Our procedure based on a sensor array to identify bacteria includes mixing bacteria and probes, recording flow cytometry data, and conducting mathematical analysis; most of the work is carried out by the instrument and computer automatically and without any washing steps or extended incubation times (Scheme 1). Our introduced sensor array is easy to operate and fast, and satisfactory discrimination between different species of bacteria, and even between normal bacteria and multidrug-resistant bacteria, is achieved.



Scheme 1. The design of a sensor array based on five probes to achieve bacteria identification, in which **A1**–**A5** denote five different probes and **B1**–**B8** represent eight types of bacteria.

First, we detected the zeta potential of eight kinds of bacteria, finding that they are all different from each other (Table S2). So we chose probes with different electronic properties, expecting different levels of fluorescence when the probes were incubated with the bacteria (Scheme 2). Probe



Scheme 2. Structures of probes **A1**–**A5**.

A2 has one negatively charged group, **A3** and **A4** both have one positively charged group, and **A5** has two negatively charged groups.[19] For comparison, we also synthesized a new probe (**A1**) with two positively charged groups. The synthetic route and characterization data are given in the Supporting Information.

We incubated various bacteria with probes **A1**–**A5** and recorded their confocal microscopy images to confirm that different varieties of bacteria give rise to different levels of fluorescence emission. The images of eight kinds of bacteria stained with **A1**–**A5** were recorded as an array (Figure 1 shows images of four kinds of bacteria; a set of images for further four kinds of bacteria is shown in Figure S1). When one type of bacteria was incubated with different probes, different fluorescence responses resulted, and different bacteria incubated with the same sensors appeared to have different fluorescence responses in fluorescence intensity and

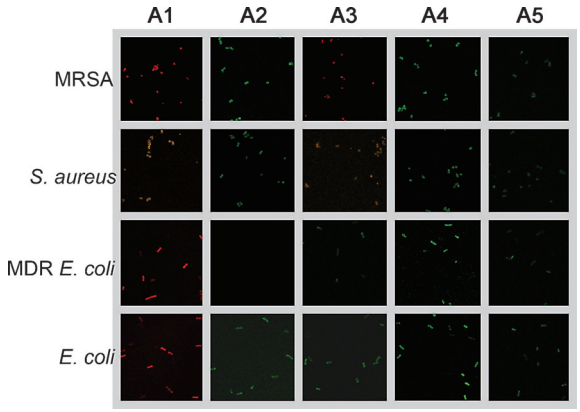


Figure 1. Confocal microscopy fluorescent images of four kinds of bacteria in the presence of **A1**–**A5** in our F-array. The different color representation (red, orange, green) is consistent with the maximum value of fluorescence recorded in flow cytometry.

emission wavelength. This phenomenon is suitable for fabricating a sensor array. Next we used flow cytometry, a mature easy-to-perform technique,[20] to record the quantitative fluorescence of each type of bacteria incubated with probes **A1**–**A5**.[20] The fluorescence emission results (Figure 2 and Figure S2) indicated that different bacteria with different sensors induced different types of fluorescence. We utilize this data to carry out mathematical analysis to identify different bacteria.

In order to understand the interaction between the probes and bacteria, we explored the binding site of the probes by taking **A1** and *E. coli* as an example. We used lysozyme to digest the cell walls of the bacteria. Without the cell wall, *E. coli* became smaller and some of the cells are round-shaped. When treated *E. coli* was incubated with probe **A1**, the fluorescence still appeared (Figure S3a). We speculate that the protoplasm rather than the cell wall plays an important role in the interaction of **A1** and *E. coli*. Furthermore, *E. coli* was treated with 1% trypsin to remove the surface proteins from the bacteria. After the incubation of **A1** and *E. coli*, the fluorescence of bacteria appeared (Figure S3b). So we deduce that the fluorescence of bacteria comes from the interaction of the probe and protoplasm. A reasonable explanation is that the probes enter the bacteria, aggregate in the protoplasm, and produce the fluorescence.[21] Additionally, the charge of the probe is an important factor to induce the molecules entering bacteria.[22]

Each type of bacteria was incubated with probes **A1**–**A5** and each incubation was repeated six times. After recording the fluorescence data from flow cytometry, we calculated the logarithms of the average fluorescence data to eliminate the scale differences and processed the standardization to make the data more comparable. We conducted principal component analysis (PCA) using the fluorescent intensity data (only processing the standardization after the logarithmic treatment) of eight bacteria with five probes. PCA is a statistical procedure that converts a group of variables into some linearly uncorrelated variables using orthogonal transformation. The "top" components corresponding to the larger eigenvalues account for most of the variability in the data.

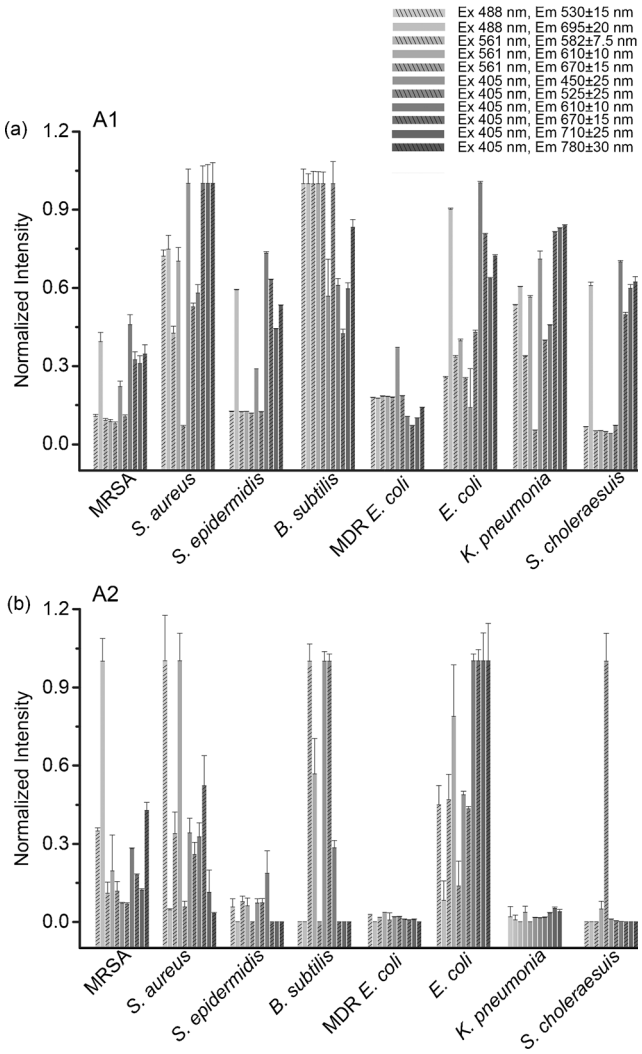


Figure 2. The normalized fluorescence intensity of eight kinds of bacteria stained with probes **A1** (a) and **A2** (b). We calculated the normalized intensity by using fluorescence value in each channel divided by the highest fluorescence value of the same channel for one probe. Each value is an average of six repeated measurements. Each type of bacteria incubated with one probe has eleven fluorescence values from 11 channels using flow cytometry. The excitation wavelengths are 488 nm, 561 nm, and 405 nm; the eleven channels correspond to the emission wavelengths listed in the figure.

Each kind of bacteria was incubated with five probes, and after each incubation we recorded fluorescence emission in 11 different channels to obtain 11 data points. Therefore, one kind of bacteria has 55 fluorescence data points. After calculation, eight principal components (PCs) of the data for each kind of bacteria out of 55 components describe 96.0% rate of contribution, and the rates of contribution for the top three PCs are 38.7%, 21.1%, and 11.3% . Figure 3 shows the results of 48 subjects (eight kinds of bacteria, each has six replicates) using the top three PCs.

To classify the bacteria and assess the discriminating power of the F-array, quadratic discriminant analysis (QDA) is used, where the Mahalanobis distances of the new case to the respective centroids of eight groups are calculated. The minimal Mahalanobis distance indicates it belongs to that

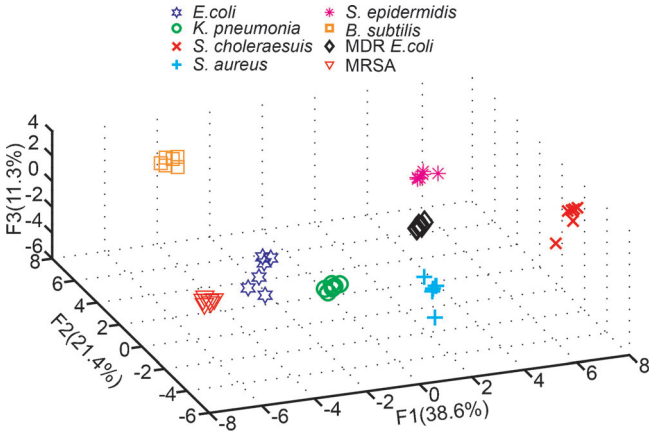


Figure 3. Transformed score of the fluorescence response pattern for eight kinds of bacteria incubated with five probes determined by PCA. Eight kinds of bacteria, each have six replicates. F1, F2, and F3 are the top three rates of contribution.

group. In contrast to the traditional linear discriminant analysis (LDA), QDA use the quadratic curve to replace the linear curve as the boundary to discriminate the subjects in different groups. The results based on QDA show that there is 100% accuracy in identifying the eight bacteria using the fluorescence data from **A1**–**A5**, which means our F-array is highly suitable for bacteria identification.

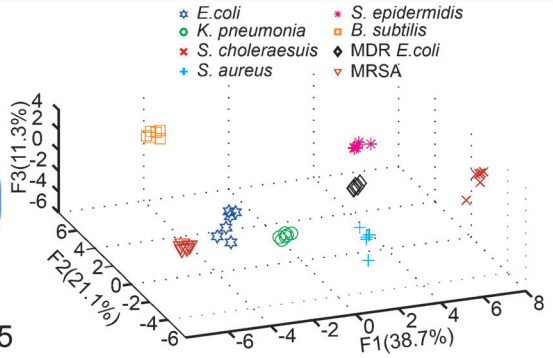
To see whether the F-array can discriminate the bacteria correctly, the cross-validation procedure is employed. Each kind of bacteria has six replicates, which refer to as six subjects. In each case, five subjects in each bacteria group are used to determine the parameter of the discrimination model (the mean and variance of each feature for the quadratic discrimination) and the remaining subject is used to evaluate the accuracy of the prediction (Figure S4). As expected, the F-array has high accuracy with a prediction probability of 93.75%.

(a)

PCA	PC1	PC2	PC3
Eigenvalue	999.34	546.51	292.48
proportion	38.7%	21.1%	11.3%
A1	22.04%	20.87%	32.51%
A2	20.95%	13.59%	12.95%
A3	20.58%	33.09%	5.63%
A4	19.00%	22.66%	26.71%
A5	17.43%	9.79%	22.20%

A1,A2 A3,A4,A5

Excluded A2,A5

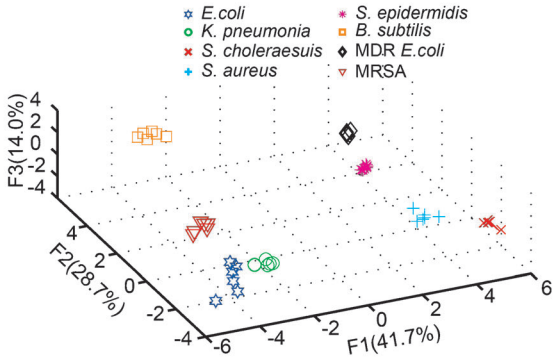


(b)

PCA	PC1	PC2	PC3
Eigenvalue	647.79	445.33	216.82
proportion	41.77%	28.71%	13.98%
A1	39.34%	31.62%	23.32%
A2	-	-	-
A3	29.06%	39.42%	30.38%
A4	31.60%	28.96%	46.30%
A5	-	-	-

A1,A3,A4

Excluded A4



(c)

PCA	PC1	PC2	PC3
Eigenvalue	465.18	308.03	138.59
proportion	44.99%	29.79%	13.40%
A1	51.90%	54.41%	25.12%
A2	-	-	-
A3	48.10%	45.59%	74.88%
A4	-	-	-
A5	-	-	-

A1,A3

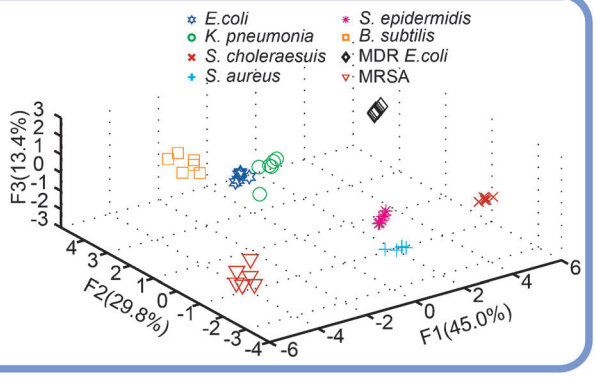


Figure 4. Schematic representation of the investigation of the most important contributors in F-array: a) PCA for all five probes (**A1**–**A5**) shows that the main contributors to the bacteria identification are **A1**, **A3**, and **A4**. b) **A2** and **A5** were excluded from the data set and the remaining data were analyzed again with PCA, showing that the main contributors were **A1** and **A3**. c) A2 was excluded and the remaining data were analyzed again with PCA.

Subsequently, we tried to decrease the number of probes to simplify the F-array. In the F-array, five probes were introduced to identify different bacteria. Among the five probes, three have positive charge and two have negative charge. The surface of the bacteria we used is predominatly negatively charged. We hypothesize the positively charged probes have interact with the bacteria more strongly than the negatively charged probes and may contribute more in differentiating bacteria. It would be ideal if a lower number of probes could achieve asimilar level of classification accuracy, which may simplify the F-array. For this reason we utilized the PCA method to process the screening of the five probes. The probes contributing most in distinguishing different kinds of bacteria will remain in the F-array, whereas the less contributing probes are excluded (Figure 4).

To screen the five probes, the standard strategy for judging the discriminatory capacity is determining the contribution of the principal components (PC1, PC2, PC3) for every probe. The results of PCA using five probes showed that **A1**, **A3**, and **A4** have the highest contributing PCs (Figure 4a). This finding is consistent with our assumption, because these probes are positively charged and would interact more strongly with bacteria than the negatively charged probes. The higher affinity would have more impact on the variance within the response data that is found in other arrays.[17a] We then apply QDA to judge the accuracy of bacteria identification. It demonstrates that the three probes can still provide 100% correct identification. Next, we apply PCA to identify bacteria only using **A1**, **A3**, and **A4** (Figure 4b). Since the two highest contributors are **A1** and **A3**, we can exclude **A4**. Using **A1** and **A3** alone to distinguish the same eight kinds of bacteria through PCA (Figure 4c), 100% accuracy is still maintained. These results indicate that the combined utilization of two or three positively charged probes achieves the ideal effect. It gives us some inspiration for the design of F-array for bacteria identification.

To further test the ability to predict unknown bacteria using the F-array, we selected 12 unknown bacterial samples randomly from the eight bacterial species grown in different batches. We tested the twelve unknown bacteria using the procedures developed above and the fluorescence data were collected through flow cytometry. We first used the original data to build a discriminant model where the mean and covariance matrix of eight PCs (96.0% rate of contribution) were calculated. We determined the Mahalanobis distance of a detected sample to the respective centroids of the eight bacteria types such that we. have eight Mahalanobis distance values for one kind of unknown bacteria. The smallest Mahalanobis distance value indicates that the sample belongs to that kind of bacteria group. 11 out of 12 bacterial subjects were correctly determined and the detection accuracy is 91.7% (Table S3). The identification of MDR *E. coli* and methicillin-resistant *S. aureus* from samples of *E. coli* and *S. aureus* may have potential applications in clinical diagnostics.

We made use of the high signal-to-noise ratio of fluorescent probes to identify bacteria; normal bacteria and antibiotics-resistant bacteria could be differentiated, which is very important for clinical diagnosis. The rapid and accurate identification of bacteria can provide important and timely information for the treatment of infectious patients. Moreover, our procedure for bacteria identification is simple: after the incubation of probes and bacteria, there is no need to get rid of background fluorescence through washing steps, which is helpful for the future applications of this method.

Currently we are investigating the mode of action to understand the probe–bacteria system in more detail for better system design. The application of F-array in clinical samples for bacteria identification is our goal in the following work. Our system has great potential. Based on the results of bacteria identification, other cells, proteins, and particles may also be differentiated by similar methods.

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