Distinguishability of biological material by use of ultraviolet multispectral fluorescence

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Recent interest in the detection and analysis of biological samples by spectroscopic methods has led to questions concerning the degree of distinguishability and biological variability of the UV fluorescent spectra from such complex samples. We show that the degree of distinguishability of such spectra is readily determined numerically. As a practical example of this technique, we show its application to the analysis of UV fluorescence spectra taken of *E. coli, S. aureus*, and *S. typhimurium*. The use of this analysis to determine the degree of biological variability and also to verify that measurements are being made in a linear regime in which analytic methods such as multivariate analysis are valid is discussed.
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1. Introduction

Recently there has been a great deal of interest in the detection and analysis of biological samples, such as proteins and microorganisms, by various spectroscopic methods[1–9] (e.g., UV or IR fluorescence, absorption, or both and enhanced Raman scatter). If practicable, there are wide-ranging scientific and commercial applications for such methods, from the determination of the biological variability of a particular micro-organism during the cell cycle to the detection of biological contaminants. The difficulty with these methods is that, unlike a chemical such as benzene for which the spectrum is unique with detailed structure appearing in both absorption and fluorescence, the spectrum from a protein or an entire microorganism tends to be broad and somewhat featureless. The UV fluorescence from such samples is dominated by the fluorescence signal from the heterocyclic amino acids in proteins,[10] which are shifted in wavelength because of differences in the local environments of the individual residues. Thus the overall spectrum from an entire microorganism can be composed of signals from hundreds of unique amino acid residues in various proteins. One is then left with various questions concerning the utility of the information in such spectra: To what degree are the spectra distinguishable? What amount of variability can one expect in the spectra? What sample concentration ensures that the measurement is being made in a linear regime (i.e., where the spectrum scales linearly with concentration)? And so forth. Nonlinear cases are under study and in general can be dealt with by correction of algorithms or nonlinear methods such as neural nets.

At first glance, the above issues might seem difficult to address; however, in this paper we show that with a relatively simple analysis one can obtain a quantitative measure of the degree of distinguishability among spectra of different species or of the same species taken at different times or under differing conditions. The method applied to a single microorganism yields a measure of variability of the spectrum that is due to biological variability or variability of the experimental technique. We first describe our ongoing experiments in the multispectral UV fluorescence measurement of biological material and then describe the analysis that leads to the determination of distinguishability of such spectra.

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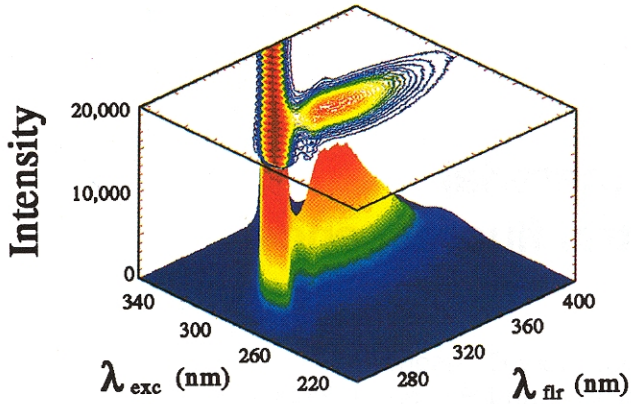

Fig. 1. Multispectral signal of UV fluorescence from *E. coli*.

2. Multispectral Ultraviolet Fluorescence

We measured the UV fluorescence spectra from various microbes. The instrument used to generate the data presented in this paper was designed by Sandia National Laboratories and built by Allied Signal. Similar instruments are available from various commercial manufacturers. For exciting fluorescence the instrument contains as a light source a high-pressure xenon lamp with peak illumination occurring near $\lambda = 300$ nm. A monochromator is then used to pick a particular excitation wavelength from the broadband xenon light source in a wavelength range from 200 to 350 nm in 5-nm increments for the examples shown (the resolution was typically 7 nm). The fluorescence from the sample is then passed through a spectrometer and sampled with an intensified diode array consisting of 1024 channels that sample the signal at wavelengths between 248 and 402 nm with a resolution of approximately 7 nm, thus building a two-dimensional (λ_{exc}, λ_{flr}) spectrum (hence multispectral).

Figure 1 shows an example of a spectrum of *E. coli* taken with this instrument. The two main features of the spectrum are the first-order elastically scattered light (discussed below) and a broad peak typical of fluorescence from the organisms discussed in this paper. The relative intensity in Fig. 1 is given in counts (the detector for this instrument is a 14-bit analog-to-digital converter), and the amplitude is similar for all three organisms used in our examples. The spectra were not corrected for variation of the instrument sensitivity with the detection wavelength nor for the wavelength variation of excitation. These variations do not affect the analysis because the spectra were measured with the same instrument under similar conditions and the spectral signatures did not change over the duration of the study.

The three bacteria considered in this paper—*E. coli* (ATCC 25922), *S. typhimurium* (ATCC 14028), and *S. aureus* (ATCC 25922)—were obtained from the American Type Culture Collection (Rockville, Md.). Samples were held in the culture collection at the U.S. Meat Animal Research Center at −20 °C in 75% glycerol suspensions. Organisms were grown out in tryptic soy broth with 0.5% wt./vol. yeast extract (TSBYE) (Difco, Detroit, Mich.) at 37 °C for 16 h. They were then grown on TSBYE agar slants and held at 5 °C to use as working cultures during the course of these experiments.

Bacteria were grown in TSBYE at 35 °C for 16 h and then washed three times in sterile phosphate-buffered saline by centrifugation at 3000g for 20 min at 5 °C to pellet, decanted, and resuspended to the original volume. The resulting cell populations were assayed at approximately 10^9 colony-forming units (i.e., bacteria) per milliliter. Subsequent dilutions and mixtures were made aseptically in sterile phosphate-buffered saline immediately prior to collecting spectra. Organism suspensions were prepared fresh daily and discarded by autoclaving at the end of the work day.

Figure 2 shows the fluorescence spectra taken with the above-described instrument for *E. coli, S. aureus,*

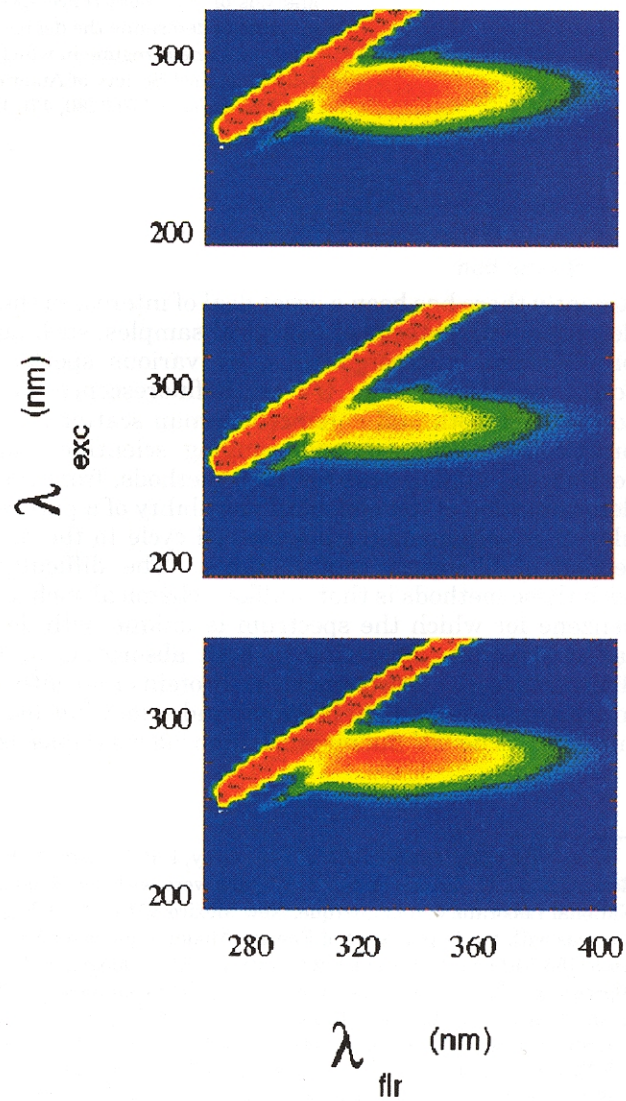

Fig. 2. Examples of fluorescence spectra taken with the U.S. Department of Agriculture fluorometer: top, *E. coli*; middle, *S. aureus*; bottom, *S. typhimurium*.

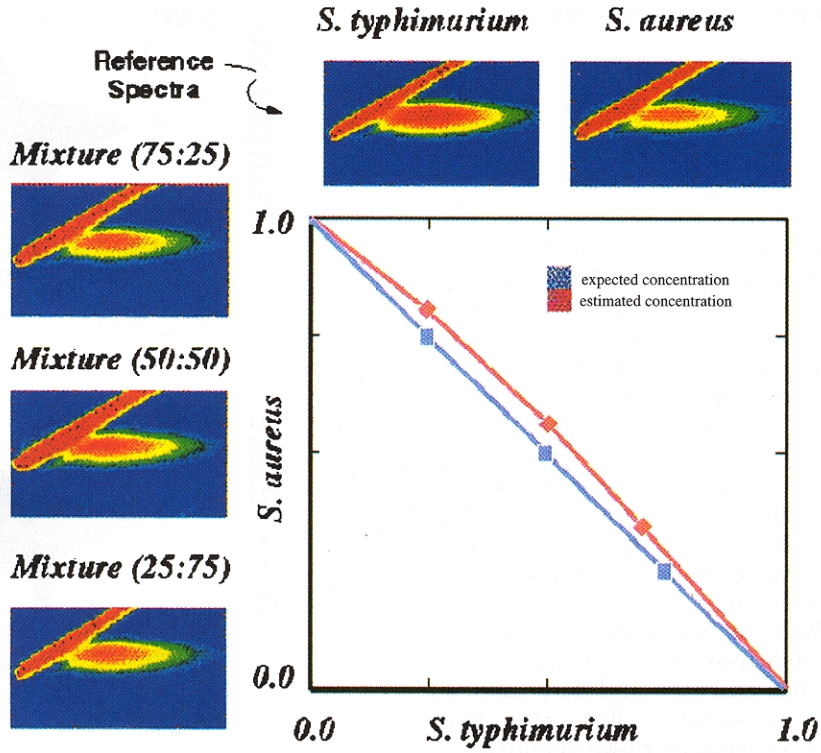

Fig. 3. MVA analysis of mixtures of *S. aureus* and *S. typhimurium*. Shown are the reference spectra and the spectra of three mixtures (25% *S. aureus*, 75% *S. typhimurium*; 50% *S. aureus*, 50% *S. typhimurium*; and 75% *S. aureus*, 25% *S. typhimurium*).

and *S. typhimurium*. Note that the strongest part of the light measured is the elastic scatter from the lamp (i.e., $\lambda_{\text{scatter}} = \lambda_{\text{excitation}}$) and is seen as the diagonal stripe (lower left to upper right) in Fig. 2. Also seen in the spectra are the water Raman lines, which are much weaker and appear as a diagonal stripe just to the higher-fluorescence-wavelength side of the elastic scatter. For the data presented in this paper the signal does not saturate the detector, and the scattered light carries information on the scattering and the absorption of the sample. Unfortunately, the usual case is that the signal from the elastic scatter saturates, so information from this part of the spectrum is not useful for analysis.

Although it would appear to be difficult to discriminate among these spectra, the application of multivariate analysis[11–13] (MVA) shows that there is sufficient difference to decompose mixtures. Figure 3 shows the results from a version of MVA[14]; the method used was singular-value decomposition[11] applied to measurements of a mixture of *S. aureus* and *S. typhimurium*. The figure shows the relative concentrations (known and MVA estimated) for mixtures in terms of stock concentrations known to be sufficiently dilute to yield a linear spectral response to dilution (i.e., concentrations not optically dense). As can be seen for the case shown, there is no difficulty in determining the relative concentrations of the two components from the combined spectra to within the pipetting uncertainty. We performed measurements for various combinations of microbes with varying degrees of success. We had difficulty discriminating between *E. coli* and *S. typhimurium* in these studies, the reason for which we demonstrate in Section 3.

3. Distinguishability of Spectra

We wished to obtain a quantitative measure of the difference between two spectra, which we denote as $S_\alpha = 1$ or $\alpha = 2$. The dimensionality of S_α is arbitrary, but the validity of the analysis below depends on the ability to define the inner product $S_1 S_2$ (in our example the spectra are two dimensional, and the inner product is a conventional scalar product). Furthermore, the spectra can be from the same sample at different times in the case of determination of temporal variability [i.e., spectra taken at times t_1 and t_2, $S_1 = S(t = t_1)$ and $S_2 = S(t = t_2)$]. We now write S_1 in terms of S_2 as

$$S_1 = CS_2 + \delta S_{1,2}, \tag{1}$$

where C is a constant to be determined and $\delta S_{1,2}$ is the part of S_1 not expressible in terms of S_2 or orthogonal to S_2. To determine $\delta S_{1,2}$, we minimize it in the least-squares sense with respect to C, that is,

$$\delta S_{1,2}{}^2 = (S_1 - CS_2)^2 \equiv \chi^2. \tag{2}$$

Then the expression

$$\frac{\partial \chi^2}{\partial C} = 0 \tag{3}$$

yields the expression for C that minimizes $\delta S_{1,2}$:

$$C_{\max} = \frac{S_1 S_2}{S_2^{\,2}}. \tag{4}$$

Then $\delta S_{1,2}$ is simply

$$\delta S_{1,2} = S_1 - C_{\max} S_2. \tag{5}$$

Two things to observe are that (1) $\delta S_{1,2}$ is limited in magnitude by S_1 because if S_1 and S_2 have no overlap, $C \equiv 0$ and thus $\delta S_{1,2} = S_1$; and (2) $\delta S_{1,2} \neq \delta S_{2,1}$. Also note that, unlike the spectra, $\delta S_{1,2}$ is not positive definite. We have shown the comparison of two spectra; however, the method is readily extended to multiple spectra and is nothing more than the Grahm–Schmidt orthogonalization.[15] One could use this approach to decompose the spectrum from a mixture into its components, and the values of C would then give the relative concentrations (a better approach would be to use singular-value decomposition because of the lack of bias[11]).

Following the analytic method outlined above, we can compare the spectra from our studies of multispectral UV fluorescence pairwise and determine to what extent each is unique. For our two-dimensional UV fluorescence spectra we denote $S_\alpha \rightarrow S_{i,j}^{\,\alpha}$, where α again denotes the species, i is the excitation wavelength, and j is the fluorescence wavelength. With this notation we can write $S_{i,j}^{\,1}$ in terms of $S_{i,j}^{\,2}$ as in Eq. (1):

$$S_{i,j}^{\,1} = C S_{i,j}^{\,2} + \delta S_{i,j}^{\,1,2}. \tag{6}$$

Then our minimization of $\delta S_{i,j}^{\,1,2}$ gives us

$$C_{\max} = \frac{\sum_{i,j} S_{i,j}^{\,1} S_{i,j}^{\,2}}{\sum_{i,j} (S_{i,j}^{\,2})^2}. \tag{7}$$

We can then compute $\delta S_{i,j}^{\,1,2}$.

Figure 4 shows $\delta S_{i,j}^{\,1,2}$ computed numerically for the three spectra shown in Fig. 2. Shown are the cases computed with (top) *E. coli* as S^1 and *S. aureus* as S^2, (middle) *S. typhimurium* as as S^1 and *S. aureus* as S^2, and (bottom) *S. typhimurium* as S^1 with *E. coli* as S^2. We calculated the differences shown in Fig. 4 by masking out the first-order elastically scattered light. We did this to accentuate the differences that are dominated by the fluorescence part of the spectrum alone. The signal strengths in the top and middle cases are generally 10% to 20% of the overall signal strength in the fluorescence spectrum from any of the samples. The bottom case shows that the signal available from fluorescence for distinguishing between *S. typhimurium* and *E. coli* is significantly lower than that for the other two cases (by a factor of 2 to 4). Thus, to distinguish these two components from each other in a mixture, it is necessary to have a higher signal-to-noise ratio, or alternatively some additional information needs to be added to the data (for example, a change in polarization of the excitation and the emission light). The fact that *S. typhimurium* and *E. coli* are difficult to discern is consistent with results from the analyses of spectra of mixtures of the two.

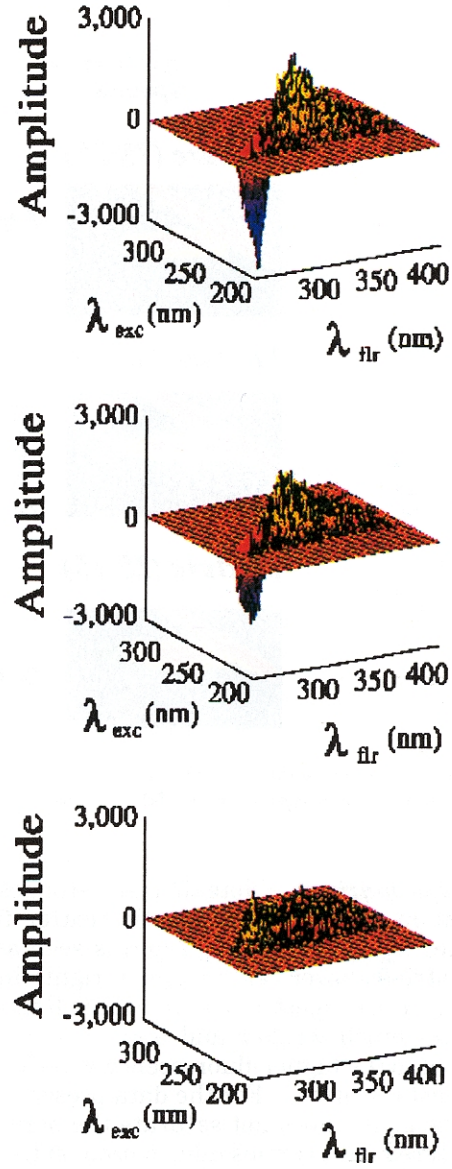

Fig. 4. Orthogonal component for (top) *E. coli* versus *S. aureus*, (middle) *S. typhimurium* versus *S. aureus*, and (bottom) *S. typhimurium* versus *E. coli*. Differences were calculated with part of the signal resulting from fluorescence alone. Note that the signal is significantly greater for the top and the middle spectra compared with the bottom spectrum.

Figure 4 demonstrates where in the spectra the information lies that is pertinent to discrimination between any two of our particular choices of species. Figure 4 was generated by use of the raw data from spectra taken with approximately equal amplitude signals. If one were to normalize S_1 and S_2 to unit power, the amount of power in S_1 that is available to discriminate S_1 from S_2 is just $1 - C_{\max}$. This gives a true measure of the signal-to-noise ratio in terms of the power that is available for the analysis of mixtures.

4. Discussion

An important issue for microbial detection and identification is validation of database spectra and evaluation of system performance in the event of minor genetic variation in bacterial isolates from different sources or those differences that occur over the course of time owing to natural variation and selection. These changes and differences or the appearance of totally new strains of bacteria will manifest themselves in phenotypic variation. Current noncultural detection methods often rely on gene probes or antibody-based methods that must be updated periodically to detect differences in the detection-assay target molecules. Spectroscopic detection and characterization of bacteria would have the advantage that such phenotypic changes (if detectable) would be immediately apparent. This potential benefit would be especially useful for outbreaks of disease caused by agents for which no prior probes have been developed. Immunoassay and gene-probe development usually require several months to years to complete.

As we have shown with our example of the UV multispectral fluorescence spectra of biological samples, the determination of the degree to which spectral signatures are distinguishable is readily obtained by simple analytic techniques. The determination of the orthogonal component $\delta S_{1,2}$ has various uses. If one wishes to use MVA methods to analyze mixtures such as those shown in Fig. 2, there is an implicit assumption that the data were taken in a linear regime (i.e., that the spectral shape does not change with amplitude or sample concentration). The method we have outlined can be used to verify this by calculation of the residual $\delta S_{1,2}$ for two samples taken at different dilutions. In the linear regime the residual is of the order of the instrument noise. Similarly, with an appropriate choice of standard sample (e.g., the buffer in which the sample is suspended), the part of the signal that is due to the instrument or is purely associated with the protocol for making the measurement can be isolated. In the case of biological variability in which changes in the spectrum from a sample are due to a difference in the mixture of proteins that are the source of fluorescence at differing stages of the cell cycle, calculating the residual from spectra taken of the same species at different times in the cell cycle should allow one to discern the degree of variability.

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