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# Towards the clinical application of laser-induced breakdown spectroscopy for rapid pathogen diagnosis: the effect of mixed cultures and sample dilution on bacterial identification

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Laser-induced breakdown spectroscopy has been utilized to classify and identify bacterial specimens on the basis of their atomic composition. We have characterized the effect that the presence of a second bacterial species in the ablated specimen had on the identification of the majority species. Specimens with a reduced number of bacterial cells (approximately 2500) were identified with 100% accuracy when compared to undiluted specimens. In addition, a linear dependence of the total spectral power as a function of cell number was determined. Lastly, a high selectivity was obtained for a LIBS-based analysis of nine separate bacterial strains from four genera. © 2010 Optical Society of America

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## 1. Introduction

Laser-induced breakdown spectroscopy (LIBS) is a sensitive spectrochemical technique that has recently begun to be used for characterizing biological samples such as microorganisms (i.e., bacteria) and tissues [1–12]. The speed, portability, and robustness of the technique suggest that LIBS may be applicable as rapid point-of-care medical diagnostic technology. Specifically, it may offer a potentially much faster way to detect and identify harmful pathogens in clinical specimens at “time zero” (the time when a specimen of blood, urine, cerebral-spinal fluid (CSF), sputum, etc., is obtained from a patient). The identification of bacteria in such clinical samples is critical

in certain diseases that can kill within hours of symptoms appearing (i.e., bacterial meningitis), when the administration of antibiotics as early as possible is of the utmost importance [13,14]. As well, knowledge at time zero of the particular pathogen causing infection would help to reduce the overuse and abuse of broad spectrum antibiotics that contribute to the growing crisis in antibiotic resistance.

There are numerous other emerging and existing technologies that purport to provide a real-time medical diagnosis of pathogens, such as polymerase chain reaction [15,16], microarray assemblies [17,18], fluorescence *in situ* hybridization [19], and other fluorescence techniques [20] to name a few. These techniques all possess their own unique limitations. Chief among these are the need for *a priori* genetic (DNA/rRNA sequences) or serological (surface antigenic composition) knowledge needed to allow the

construction of sensitive consumables such as polymerase chain reaction primers, 16s rRNA probes, or fluorescently labeled antibodies [21,22]. Conversely, the advantages of a LIBS-based diagnostic technology include: no *a priori* genetic information or knowledge of conserved genome sequence is required, no amplification of genetic material (DNA/rRNA sequences) is required, no *a priori* knowledge of surface antigens is required, no consumables (unstable antibodies, fluorescent dyes, etc.) are required, speed (identification of an unknown pathogen can theoretically be obtained in under a second), and limited or no sample preparation is required. In addition, LIBS can possibly be applied for the detection and classification of biologically inactive specimens, such as “viable, but nonculturable” states of bacteria (as in the case of *V. cholerae*) and dormant nonreproducing spores (as in *B. anthracis*). These specimens are notoriously difficult to identify with other techniques.

The number of bacteria that may be present in a specimen to be tested via LIBS is dependent on the type of specimen (blood culture, contaminated water, tainted food product, etc.). Even in the case of clinical specimens, the number of bacteria present in an infected patient will vary from one organism to another, and the numbers present in specimens from asymptomatic patients will be different from symptomatic patients. For example, the infectious dose (the number of bacteria required to produce an infection) for intestinal diseases caused by *Shigella* or enterohemorrhagic *E. coli* is approximately 10 organisms [23]. In the case of *V. cholerae* (responsible for cholera) it is about one million organisms, while *Campylobacter* infections require several hundred organisms. Because of these low numbers, many other techniques require the culturing of bacteria or the amplification of genetic fragments (which can take several hours or several days) to identify the bacteria. It is therefore crucial to establish the lowest number of bacteria that can be identified with the LIBS technique. In this paper we will investigate the effect that reducing the number of bacterial cells has on the LIBS-based identification.

Bacteria may be present in mixed samples under some (but certainly not all) conditions. There are multiple clinical examples of sterile samples (i.e. blood, urine, CSF) where the bacteria causing an infection will be the only bacteria present [24]. In these situations, concerns about mixing with other bacteria are unfounded. Still, the presence of other biological material (i.e., cells in blood, proteins in urine) may have some effect on the LIBS-based diagnosis. In specimens obtained from stool, sputum, or contaminated food or water, or even specimens contaminated by environmental bacteria, the bacteria causing the infection may be present along with other minority bacteria. In this paper we will investigate the effect that the presence of a second bacterium has on the LIBS-based identification. Lastly, we will show that a discriminant function analysis of LIBS spectra obtained from multiple genera of clinically relevant

bacteria (such as *Escherichia*, *Streptococcus*, and *Staphylococcus*) yielded a discrimination between species that indicates an identification of unknown bacterial samples using a pre-compiled reference library of spectral fingerprints is feasible.

## 2. Experiment

### A. LIBS Setup

In our LIBS experiments, 1064 nm 10 ns laser pulses from an Nd:YAG laser (Spectra Physics, LAB-150-10) were used to ablate bacteria. Pulse energies were 10 mJ/pulse. The laser was focused by a 5× high-damage threshold microscope objective to a diameter of approximately 100 μm. An optical fiber with 600 μm core diameter (N.A. = 0.22, 1 m long) was used to collect the optical emission from the microplasma. This fiber was connected to an Echelle spectrometer (LLA Instruments GmbH, ESA 2000) equipped with a 1024 × 1024 (24 μm × 24 μm pixel area) ICCD array, with spectral coverage from 200 to 840 nm possessing a 0.005 nm resolution in the UV. LIBS spectra were collected in an argon environment at atmospheric pressure using a 315 cm<sup>3</sup> sampling chamber with a slow inlet flow (8 × 10<sup>-5</sup> m<sup>3</sup>/s) of argon to create a slight overpressure in the intentionally leaky chamber [25]. Five laser pulses were fired at every sampling location, and the spectra from five different locations were collected and averaged, resulting in a spectrum of 25 averaged laser pulses per bacterial spectrum. Although data from five sampling locations was used in this study, a signal-to-noise ratio sufficient for effective discrimination was usually achieved after only two locations, indicating that in the future the quantity of bacteria required could be reduced. LIBS spectra were acquired at a delay time of 2 μs after the ablation pulse with an integration gate width of 20 μs duration.

To obtain high signal-to-noise spectra, the bacteria samples (after preparation as described below) were mounted on the surface of a 1.4% nutrient-free bacto-agar substrate, as described in our previous work [26]. Ten microliters of a high-density bacterial suspension (pellet) were micropipetted to the surface of the bacto-agar, which was kept at room temperature. After absorption of the liquid in the bacterial pellet by the bacto-agar, the bacteria formed a thin pad approximately 0.5 cm<sup>2</sup> in area, which allowed multiple sampling locations (over 100) to be obtained per 10 μL pad (Fig. 1). Multiple pads could be mounted on a single agar substrate to test bacteria from different sources sequentially.

### B. Bacterial Sample Preparation

Multiple species of bacteria were prepared in two separate microbiology facilities in the course of this work. Gram-negative and Gram-positive bacterial species (*Escherichia*, *Streptococcus*, and *Staphylococcus*) were prepared in the following manner. Bacteria were cultured in a rich nutrient broth overnight at 37 °C. After that, bacteria were streaked on a trypti-

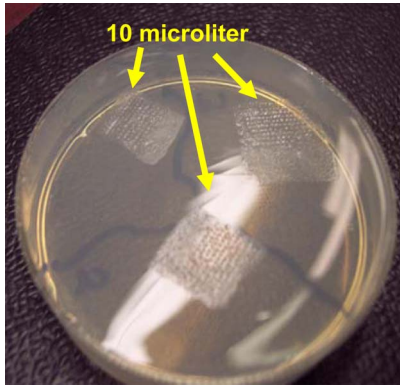


Fig. 1. (Color online) Photograph of three  $10\ \mu\text{L}$  bacterial “pads” mounted on a nutrient-free bacto-agar substrate. The pads are extremely thin, basically transparent, and very difficult to observe with the eye. These pads were made significantly thicker to aid in visualization. Visible in each pad is an array of dimpled craters where the LIBS laser has been rastered. Approximately 500–1500 bacteria were present in any single sampling location.

case soy agar plate by using a sterilized inoculating loop. The plate was then incubated for 24 h at  $37^\circ\text{C}$ . Two conditional mutant strains of *Mycobacterium smegmatis* bacteria were grown for 24 h on a 7H9/ADC agar plate containing 5 ng/ml tetracycline and 50 ng/ml hygromycin. In all cases, bacteria were harvested from the growth plates and suspended in 1.5 ml phosphate-buffered saline (PBS) or deionized water. Finally, bacterial pellets were produced by centrifuging the tubes for 3 minutes at 5000 rev/min at room temperature. The supernatant fluid was withdrawn and discarded. Spectra obtained from bacteria isolated from PBS and water were identical, indicating that any small volumes of residual buffer present after centrifugation were insignificant.

### C. Mixed Samples

Mixtures of known mixing fraction were prepared from suspensions *M. smegmatis* and *E. coli* C (Nino). The mixing of these two particular species would almost certainly never occur in a clinical setting, but the easily observed differences in the LIBS spectra of these two microbes (resulting from the physiological variation between the two, one being a Gram-neutral *Mycobacterium* and one a Gram-negative *Escherichia*) provided an optimal experiment in which to initiate bacterial mixing experiments compared to, for example, the use of a mixture of two highly similar *E. coli* strains. Morphologically however, the two microbes are fairly similar. Two separate suspensions (one of *M. smegmatis* and one of *E. coli*) were prepared prior to the mixing. A spectrophotometer was used to measure the optical density of the two bacterial suspensions to ensure equal concentrations prior to mixing. The turbidity or optical density of the suspension of bacteria cells was measured at a wavelength of 600 nm ( $\text{OD}_{600}$ ) with the bacteria in their mid-log phase of growth. The measured optical density was 1.83 for both. The fact that the cell sizes of the *M. smegmatis* and the *E. coli* C cells are very similar ( $1.5\text{--}4\ \mu\text{m}$  in length and

$0.3\text{--}0.5\ \mu\text{m}$  in width) [27] confirmed the initial numbers of bacteria were the same.

After establishing the initial bacterial concentration, six separate mixtures were prepared with a ratio *M. smegmatis* to *E. coli* C given by  $M_{1-x}:C_x$  with  $x = 0.0, 0.1, 0.2, 0.3, 0.5, 1.0$ . Multiple 1.5 mL tubes of these mixtures were prepared, thoroughly agitated via vortex mixing, then centrifuged for 3 min at 5000 rev/min. The supernatant was discarded to produce the bacterial pellet. Again,  $10\ \mu\text{L}$  of the dense pellet was mounted on the agar surface prior to LIBS testing.

### D. Sample Dilution

To study the effect of cell number on the LIBS-based identification of a bacterial target, three different bacterial concentrations of the wild-type (WT) strain of *M. smegmatis* were prepared. The first concentration was the standard undiluted concentration, which was  $4.7 \times 10^8$  bacteria/mL. This concentration was calculated in the standard microbiological way based on the bacterial growth curve. For the second concentration,  $10\ \mu\text{L}$  of the bacterial suspension was added to  $10\ \mu\text{L}$  PBS, while the third concentration was achieved by adding  $10\ \mu\text{L}$  of the bacterial suspension to  $20\ \mu\text{L}$  PBS. In order to ensure the homogeneity of the mixture, all samples were agitated with a vortex mixer.  $10\ \mu\text{L}$  from each concentration was then mounted on the agar surface.

### E. Data Analysis

LIBS spectra were collected and were analyzed with a discriminant function analysis (DFA) as described in our previous work [28]. The use of computerized chemometric techniques like DFA, principal component analysis, or principal least squares-discriminant analysis has greatly increased the selectivity of the LIBS technique and reduced the time required to make identifications of unknown targets based on their LIBS spectra [29–34]. DFA is a statistical technique used for classifying a set of observations into mutually exclusive groups on the basis of a set of independent variables (predictors). The DFA analysis was performed with commercial software (SPSS V17.0, SPSS Inc.). LIBS spectra were analyzed by measuring the intensity of 13 emission lines from 5 different elements: P, C, Mg, Ca, and Na. The 13 emission lines are shown in Table 1. The integrated area under each line was determined by a commercial software program (ESAWIN v3.20, LLA Instruments, GmbH). The intensity of each line was divided by the sum of all 13 line intensities (the total spectral power) to normalize the spectrum against shot-to-shot fluctuations. These relative line intensities constituted 13 independent variables that were used for the discrimination between the bacterial spectra.

## 3. Results and Discussion

### A. Mixing Experiment

LIBS spectra from pure samples of *M. smegmatis* WT, *Streptococcus viridans*, and *E. coli* C bacteria



**Table 1. Resolved Spectral Emission Lines from the LIBS Plasma Utilized as Independent Variables in the Discriminant Function Analysis of Bacteria<sup>a</sup>**

Wavelength (nm)	Line Identification
213.618	P I
214.914	P I
253.560	P I
255.326	P I
247.856	C I
279.553	Mg II
280.271	Mg II
285.213	Mg I
393.361	Ca II
396.837	Ca II
422.666	Ca II
588.995	Na I
589.593	Na I

<sup>a</sup>The source of the emission line from a neutral (I) or singly ionized (II) atom is indicated.

were collected. *S. viridians*, a Gram-positive organism, was included in the DFA of the *M. smegmatis*/*E. coli* mixtures to serve as a negative control. No mixing fraction should ever classify as the control. In order to investigate the differences between the bacteria, spectra from the agar substrates on which all the bacteria were ablated, which lacked many of the elements present in the bacteria, were included in the DFA. Figure 2 shows the first three discriminant function (DF) scores for these four sets of spectra. A “leave-one-out” (LOO) analysis of these data indicated that 100% of all samples were correctly classified which shows that LIBS spectra obtained from the pure bacterial samples were distinctly different from each other and from the agar substrate as well. In a LOO analysis, a single data point is omitted during the construction of the DFs. DF scores are calculated for the omitted (assumed to

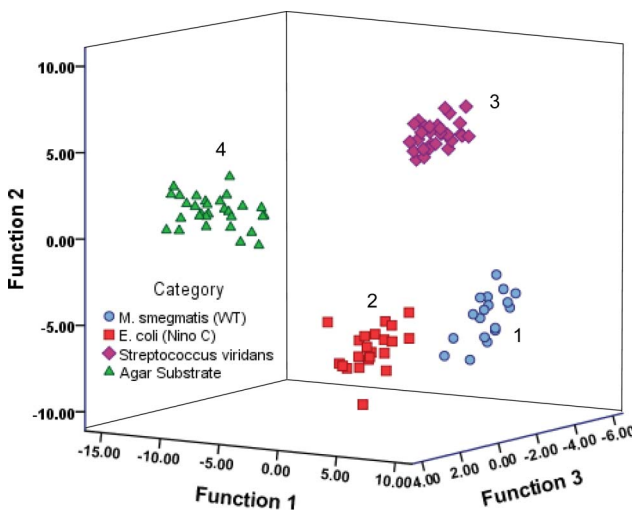


Fig. 2. (Color online) First three discriminant function scores from a DFA of the LIBS spectra from pure samples of three different bacteria: (1) *Mycobacterium smegmatis* (WT), (2) *E. coli* C, and (3) *Streptococcus viridians*, in addition to (4) the agar substrate on which they were ablated.

be unidentified) point using the new functions, and the unknown point is assigned a group classification on the basis of these scores. Therefore rule sets for classification are always created from “known” samples, but the specificity results are always obtained from “unknown” or unidentified samples and all data points are tested as unknown.

A useful output generated by the DFA analysis is the structure matrix table, which returns the statistical weights of the elements or the atomic transition lines that comprise the various discriminant function scores. The structure matrix from this analysis indicated that the two 213.618 nm and 214.914 nm phosphorus lines were primarily used in the discrimination between bacterial and agar spectra, while the 396.837 nm calcium line and 285.213 nm magnesium line were responsible for the discrimination between *E. coli* C and *M. smegmatis* (WT) bacterial spectra.

Figure 3 shows a plot of the first two DF scores from a DFA of the LIBS atomic emission spectra from pure samples of *M. smegmatis* (WT) and *E. coli* C, as well as the four mixtures with the mixing fractions described above. As established earlier, the spectrum from a sample of pure *M. smegmatis* (Group 1) was easily differentiable from a spectrum from a sample of pure *E. coli* (Group 6). Spectra from mixtures classified strongly with each other, not with spectra from pure samples, confirming the homogeneity of the mixtures. As the fraction of *E. coli* in the mixture became progressively higher, the DF1 score of the spectra from the mixtures (indicative of the primary discrimination between the two bacterial types) shifted closer to the DF1 score of pure *E. coli*.

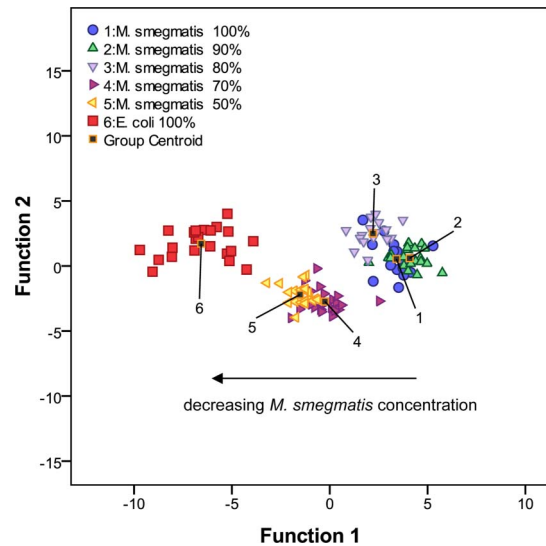


Fig. 3. (Color online) DFA plot showing the first two discriminant function scores for the spectra obtained from pure samples of two bacteria, (1) a wild type strain of *M. smegmatis* (WT) and (6) a strain of *E. coli* (C) and four mixtures of those two bacteria at various mixing fraction (2–5). As the fraction of *E. coli* in the mixture became progressively higher, the DF1 score of the mixture (indicative of the primary discrimination between the two bacterial types) shifted closer to the DF1 score of pure *E. coli*.

Moreover, the DF1 score of the centroid (which is the effective “center of mass” of the distribution of measurements) of the 50% mixture (Group 5) shifted approximately 50% of the way between Group 1 and Group 6. The spectra from the 90% and 80% mixtures were closely grouped with the 100% pure sample spectra. This means that spectra from *M. smegmatis* bacteria could be identified with a high confidence even in the presence of low concentrations of *E. coli*. The previous result is in good agreement with what may occur in some clinical samples in which microbial contamination can exist, but only at minority or trace concentrations. In this setting, clinical microbiologists need to isolate the mixed organisms from each other and grow them in pure culture in order to identify each organism. This process may take several days in order to determine the correct organism. In contrast, our results can be obtained almost instantaneously upon obtaining the mixed sample.

In order to determine the accuracy of the identification of the mixed samples, a DFA of the LIBS spectra from the two pure samples, the four mixed samples, and the pure *S. viridians* was performed. The DFA also performed a LOO classification on these spectra, which evaluated the selectivity of the experiment by calculating the misclassification percentage of each group. This is known as the resubstitution estimate, and the corresponding results are shown in Table 2. The spectra obtained from the control samples of *S. viridians* bacteria were completely distinct from any other samples, and no mixtures classified as the control. This test was repeated with additional species of bacteria, and the mixtures only ever classified with the species that comprised the mixture. The 90% and 80% mixtures classified 100% of the time with the majority species, indicating the strong likelihood that spectra from mixtures with only trace amounts or small minority fractions of contaminant bacteria will be easily identifiable as belonging to the majority species. These experiments will need to be reproduced with a greater number of specimens to statistically determine whether this identification accuracy is truly 100% or whether it is somewhat lower. The identification accuracy dropped quickly for mixing fractions below 80%, achieving the anticipated 50% level for 50:50 mixtures. Because the DFA must assign the spectrum to one of the two “pure” groups, it is not surprising that the classification accuracy tracked the mixing

fraction as the concentration of the majority species was decreased.

## B. Dilution Experiment

Figure 4 shows the first two DF scores for a DFA performed on spectra obtained from the three different concentrations of *M. smegmatis* (WT), a similar mutant called *M. smegmatis* (TE), and *S. viridians*. In this analysis, the group centroids of all concentrations of the *M. smegmatis* (WT) were closely grouped together. This indicates that the LIBS spectra for all concentrations of *M. smegmatis* (WT) were the same, regardless of the number of bacterial cells present. This was not unexpected, as the spectra were always normalized by the total spectral power, and therefore should be independent of the number of cells. The centroid location of Group 4, *M. smegmatis* (TE), was well-separated from that of Groups 1–3, *M. smegmatis* (WT), but possessed a similar DF1 score. This confirmed the fact that *M. smegmatis* (TE) is highly similar to *M. smegmatis* (WT) and possessed a highly similar LIBS spectrum, but both were completely distinct from the *S. viridians* (a Gram-positive bacterium) spectrum. 100% of all *M. smegmatis* (TE) spectra were correctly classified regardless of concentration. This is a significant result for a clinical diagnostic, as some clinical tests are dependent on the pathogen concentration or the absolute number of pathogens present. The LIBS-based chemometric identification is independent of these factors. Also, as we attempt to extend this diagnostic to clinical applications, a reference library of LIBS spectral fingerprints from important organisms will be constructed, most likely using well-characterized strains and samples with a high number of cells to provide excellent signal-to-noise. It is important to prove that the LIBS spectra obtained from clinical specimens, which will contain much lower numbers of bacteria, classify 100% of the time with the reference library spectra obtained from samples with much higher numbers.

Using the known initial titer of our liquid bacterial suspension, the volume of suspension mounted on the agar, the area of the mounted bacterial pad, and the area of our ablation craters we calculated the number of bacteria ablated in any given sampling location. This number was approximately 1500 cells for our normal “undiluted” samples. Because five sampling locations were averaged together to make one LIBS spectrum, our initial calculations showed

Table 2. Classification Results from the Discriminant Function Analysis of *M. smegmatis*/*E. coli* Mixed Samples

Category	# of Spectra	Classification Results		
		<i>M. smegmatis</i>	<i>E. coli</i>	<i>S. viridians</i>
100% <i>M. smegmatis</i> , 0% <i>E. coli</i>	21	100%	0%	0%
90% <i>M. smegmatis</i> , 10% <i>E. coli</i>	20	100%	0%	0%
80% <i>M. smegmatis</i> , 20% <i>E. coli</i>	16	100%	0%	0%
70% <i>M. smegmatis</i> , 40% <i>E. coli</i>	21	76%	24%	0%
50% <i>M. smegmatis</i> , 50% <i>E. coli</i>	19	47%	53%	0%
0% <i>M. smegmatis</i> , 100% <i>E. coli</i>	25	0%	100%	0%

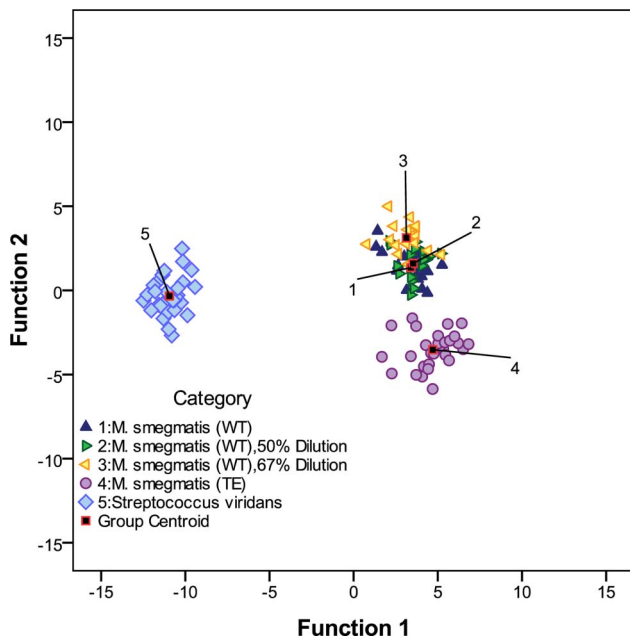


Fig. 4. (Color online) DFA plot showing the first two DF scores for three different concentrations of *M. smegmatis* (WT) (1–3), a highly similar mutant called *M. smegmatis* (TE) (4), and the Gram-positive *S. viridans* (5). The ability to identify and differentiate the *M. smegmatis* (WT) samples was independent of sample concentration.

that we were identifying approximately 7500 bacteria with every LIBS spectrum. All spectra from the two dilutions were 100% correctly identified, indicating that 3750 and 2500 bacteria were also identifiable. These estimates of the bacterial number have at least a 10% uncertainty.

Figure 5 shows a typical LIBS spectrum obtained from the lowest concentration tested in the dilution study. Approximately 2500 bacterial cells total were ablated to obtain this spectrum, which is dominated by emission from C, Mg, and Ca, and to a lesser ex-

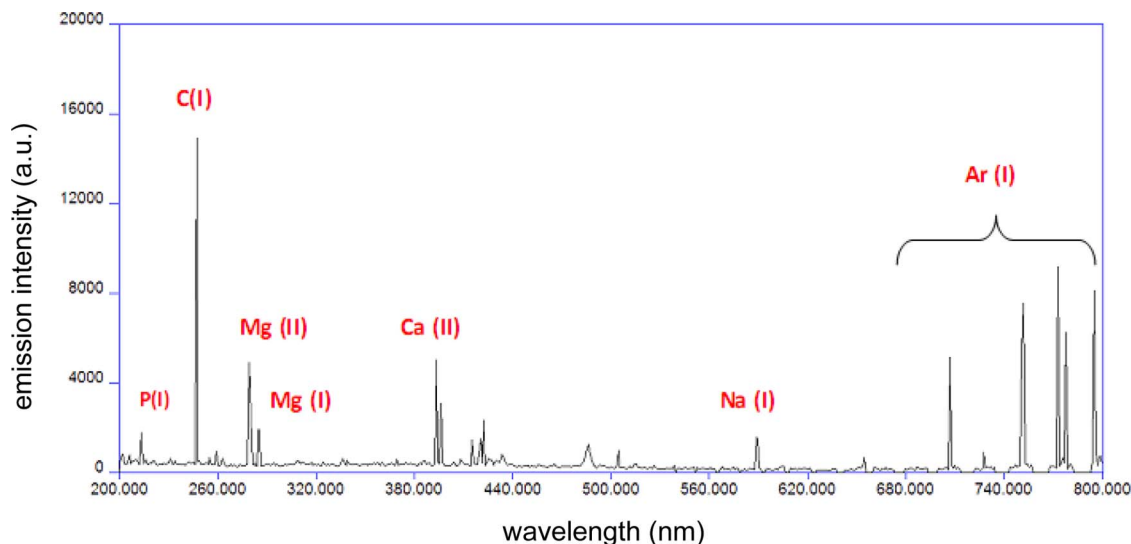


Fig. 5. (Color online) Typical LIBS spectrum from the lowest concentration of *M. smegmatis* (WT) tested in this study. The sample was ablated in argon, and the emission lines are identified. A total of approximately 2500 bacteria were ablated to create this spectrum.

tent emission from P and Na. The signal-to-noise of these emission lines was still completely adequate for identification purposes, as was shown in Fig. 4, and the background was small. The LIBS spectrum was acquired at the same experimental parameters as given before. These results are encouraging, as the required number of bacterial cells is lower than the infectious dose for many (not all) diseases as described earlier.

The limiting factor in the number of cells that can be identified was the emission intensities of the phosphorus lines at 253.560 and 255.326 nm which eventually decreased below the background intensity. However, our optical detection efficiency can be improved by constructing a new light collection optical system. As mentioned before we used only an optical fiber with a 600  $\mu\text{m}$  core mounted  $\sim 2$  cm away from the plasma to collect the emission. The percentage of total emission that is collected with this arrangement is less than 1%. The use of short focal length large diameter dual parabolic reflectors would increase the amount of collected light by a factor of 1000 based on calculations that assume a purge chamber similar to what we use now and commercially available parabolic reflectors. We also intend to explore the use of dual-pulse nanosecond LIBS, which could conservatively yield a factor of two increase in emission intensity, although this has not yet been demonstrated in bacterial systems. A second nanosecond Nd:YAG laser exists in our lab for this purpose. With these improvements we intend to lower the minimum number of bacterial cells to around ten.

The total spectral power measured from the various concentrations was linearly dependent on the number of bacterial cells ablated. This is shown in Fig. 6. The total spectral powers from all spectra from a given concentration were averaged and the standard deviation is shown as the uncertainty. A linear fit to these data ( $R^2 = 0.953$ ) shows the

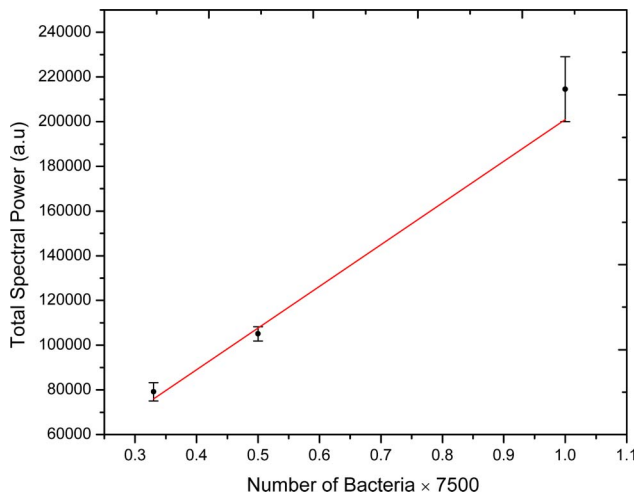


Fig. 6. (Color online) Total spectral power associated with each of the five elements observed in the LIBS spectrum of *M. smegmatis* (WT) ablated in argon as a function of bacterial cell number. A linear dependence was observed.

expected linear dependence of the LIBS signal intensity with bacterial cell number. Based on this result, it is possible we may be able to correlate the bacterial number with the measured total spectral power in future experiments. This could have relevance as a rapid check of bacterial resistance since many fast-growing bacterial species double their number every 15–20 min and since the LIBS total spectral power can easily resolve a doubling of the bacterial number. An experiment could be designed where a clinical bacterial sample is obtained and half is tested via LIBS and half is exposed to a rich nutrient medium

in the presence of an antibiotic. Twenty minutes later another LIBS spectrum could be obtained from the sample growing with the nutrient medium. Antibiotic-sensitive bacteria should show no increase or a decrease in LIBS total spectral power as the cells are unable to divide. Antibiotic-resistant bacteria on the other hand should multiply exponentially, and a corresponding increase in LIBS total spectral power from the first test should be observed. This process is traditionally done in a similar method, but with a “culture-and-count” confirmation of bacterial growth. This method can take from 24–72 h to determine the presence of antibiotic resistant strains.

### C. Bacterial Discrimination

Four strains of *E. coli* (enterohemorrhagic *E. coli* O157:H7, C (Nino), HF4714, and HfrK12), two conditional mutants of *M. smegmatis* (WT and TA), two *Staphylococcus* species (*aureus* and *saprophyticus*), and two *Streptococcus* species (*viridans* and *mutans*) were ablated as described above and the spectra were analyzed together using a DFA. The first two DF scores of this analysis are shown in Fig. 7. For a discrimination between  $N$  groups,  $N - 1$  discriminant functions were constructed. In this analysis, 79.0% of the variance between the groups was described by function 1, 12.2% by function 2, 3.6% by function 3, 2.5% by function 4, and the rest of the variance, 2.7%, was described by the remaining discriminant functions. Only the first two DF scores are plotted in Fig. 7, which contain most of the variance, yet a statistically significant amount of variation is contained in the rest of the functions,

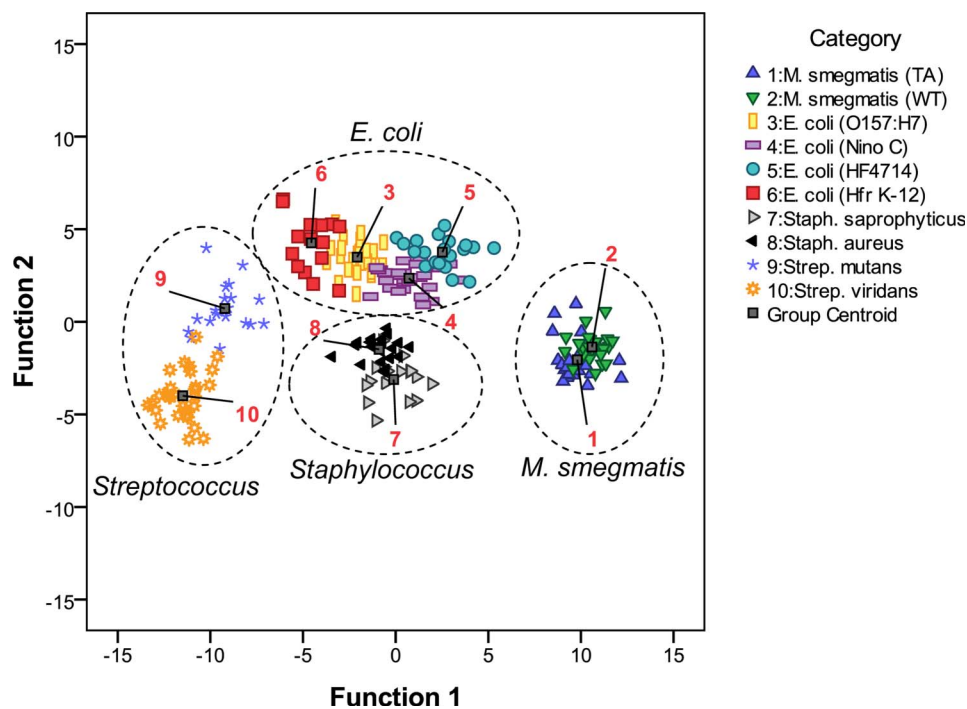


Fig. 7. (Color online) DFA plot showing the first two DF scores for LIBS spectra from two species of *Staphylococcus* (*aureus* and *saprophyticus*), two species of *Streptococcus* (*viridans* and *mutans*), two conditional mutants of *M. smegmatis* (WT and TE), and four strains of *E. coli* (enterohemorrhagic *E. coli* O157:H7, Nino C, HF4714, and HfrK12).



Table 3. Classification Results from the Discriminant Function Analysis of 10 Different Bacteria

Group	Predicted Group Membership (%)									
	1	2	3	4	5	6	7	8	9	10
1: <i>M. smegmatis</i> (TA)	82.4	17.6	0	0	0	0	0	0	0	0
2: <i>M. smegmatis</i> (WT)	28.0	72.0	0	0	0	0	0	0	0	0
3: <i>E. coli</i> O157:H7	0	0	96.0	4.0	0	0	0	0	0	0
4: <i>E. coli</i> (Nino C)	0	0	3.6	96.4	0	0	0	0	0	0
5: <i>E. coli</i> (HF4714)	0	0	0	0	100.0	0	0	0	0	0
6: <i>E. coli</i> (HfrK-12)	0	0	6.7	0	0	93.3	0	0	0	0
7: <i>Staph. saprophyticus</i>	0	0	0	0	0	0	94.1	5.9	0	0
8: <i>Staph. aureus</i>	0	0	0	0	0	0	0	100.0	0	0
9: <i>Strep. mutans</i>	0	0	0	0	0	0	0	0	95.0	5.0
10: <i>Strep. viridans</i>	0	0	0	0	0	0	0	0	0	100.0

which are not shown. In this analysis, 92.3% of all the original grouped cases were correctly classified in a LOO. All errors of identification occurred only between spectra belonging to the same genus or species, as can be seen in Table 3.

The results of this LIBS-based diagnostic applied to a variety of bacteria are indicative that the technique is not merely basing an identification/discrimination on random differences in the spectra. The fact that spectra are closely grouped by genus (*Staphylococcus* and *Streptococcus*) and are even more closely grouped by genus and species (*E. coli* and *M. smegmatis*) demonstrate that the technique is identifying the true microbiological diversity of these organisms. It is important to point out that because  $N - 1$  discriminant functions are always constructed when  $N$  groups are classified, as additional bacteria are added to the reference library of existing LIBS spectral fingerprints, the phase space of the DFA correspondingly increases. In this way, concerns about an “overcrowding” of discrimination space (and subsequent loss of selectivity) as additional bacteria are added to the reference library may be unfounded. Lastly, it is very important to note that as this diagnostic is extended toward clinical applications, patient case histories will play an extremely important role in the determination of which potential candidate bacteria are included in a reference library against which an unknown pathogen will be tested. In most circumstances, knowledge of the case history will preclude all but a few suspect pathogens. Therefore a DFA comparing an unknown pathogen against a reference library composed of all known pathogens will almost certainly not occur. This clinical fact reduces concerns about the ultimate selectivity of the technique based on the overcrowding of discrimination space.

#### 4. Conclusions

Nanosecond laser-induced breakdown spectroscopy followed by a discriminant function analysis clearly showed a discrimination between several bacterial species, with a close grouping based on specimen genus, species, and strain observed. The issues of sample dilution and sample mixing (important questions that must be addressed as the LIBS technology moves toward the goal of clinical diagnoses) have

been investigated. It was shown that in a mixture of two bacteria, accurate identification was possible down to a 80 : 20 mixing ratio, with a subsequent loss of selectivity observed at lower mixing fractions. At no time were spectra from mixed samples classified as anything other than one of the two bacteria comprising the mixture.

Bacterial specimens were diluted by a factor of two and three to determine the effect that reducing the number of bacterial cells in the LIBS plasma would have on the bacterial identification. All dilutions of a bacterial suspension classified 100% of the time with the most dense “control” concentration, even when compared to a closely related mutant of the same species. It was shown that for the lowest dilution, approximately 2500 bacteria were required for the accurate identification of the bacteria. This number can be reduced in the future (perhaps by a factor of 1000) with the construction of a better light collection system.

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