Biochemical detection and identification false alarm rate dependence on wavelength using laser induced native fluorescence

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Abstract

Most organic and many inorganic materials absorb strongly in specific wavelength ranges in the deep UV between about 220nm and 300nm. Excitation within these absorption bands results in native fluorescence emission. Each compound or composite material, such as a bacterial spore, has a unique excitation-emission fingerprint that can be used to provide information about the material. The sensitivity and specificity with which these materials can be detected and identified depends on the excitation wavelength and the number and location of observation wavelengths.

We will present data on our deep ultraviolet Targeted Ultraviolet Chemical Sensors that demonstrate the sensitivity and specificity of the sensors. In particular, we will demonstrate the ability to quantitatively differentiate a wide range of biochemical agent targets against a wide range of background materials. We will describe the relationship between spectral resolution and specificity in target identification, as well as simple, fast, algorithms to identify materials.

Hand-held, battery operated instruments using a deep UV laser and multi-band detection have been developed and deployed on missions to the Antarctic, the Arctic, and the deep ocean with the capability of detecting a single bacterial spore and to differentiate a wide range of organic and biological compounds.

Keywords: native fluorescence, biological and chemical, detection and classification

1. INTRODUCTION

There are many present-day techniques that can identify or classify biological or chemical materials in laboratories. In a laboratory setting, the size and power consumption of the instrument are not of concern. Nor is the need for sample handling, preparation, or the use of reagents. When dealing with in-situ, field environments, portability of the instrument, power requirements, environmental factors, and the use of consumable reagents affect the ability to migrate typical lab instruments and experiments to the field. Therefore to overcome the challenges of in-situ observations, we have focused on non-contact, non-destructive methods to detect biological and chemical materials that may be present on surfaces or in water. We employ laser excitation of endogenous fluorophors intrinsic to the target material being detected. Unlike Laser induced fluorescence (LIF) which uses a chemical/biological tag for the material of interest, Laser induced Native Fluorescence (LINF) is a reagentless method requiring no sample handling or preparation. In addition, this method avoids many of the problems of derivatization or samples with dye tags, and examines the sample in the natural, undisturbed, state.

Native Fluorescence is a well-known technique used in a variety of areas from differentiating malignant versus benign cancers, observing dissolved organic carbon (DOCs) in the ocean, detection of life in sandstones in Antarctica, to detection of weaponized Bacillus anthracis or other harmful materials on surfaces or in water. LINF provides an extremely sensitive method to provide preliminary information about the location and potential hazard of a trace contaminant. The sensors we are developing fall into the category of "trigger" since they are very sensitive and provide some level of information about the hazard. When detecting biological or chemical materials used in biochemical warfare, it is important that the technique used as a trigger provide as much information about the material such that false alarm rates are reduced, and secondary confirmatory systems are used only when necessary. The sensors we will describe here offer a higher level of specificity than has heretofore been possible with LINF sensor and therefore can reduce the demand on higher level confirmation sensors. The proposed technique

integrates well with confirmation sensors such as resonance Raman-based sensors. Our goal in this paper is to demonstrate the level both of sensitivity and specificity that is achievable with optimum choice of excitation and detection wavelengths to differentiate target from background interferants.

2. NATIVE FLUORESCENCE DETECTION AND CLASSIFICATION

When a molecule is irradiated at an excitation wavelength that lies within the absorption spectrum of a molecule, it will absorb the radiant energy and be activated from its ground state (S_0) to an excited singlet state (Sn), with the electron in the same spin as the ground state. The molecule can then relax back from the excited state to the ground state by giving up excitation energy either non-radiatively or radiatively, depending on the local environment. In a nonradiative transition, relaxation occurs by thermal generation. In a radiative transition, relaxation occurs via fluorescence at specific broad emission wavelengths.

Many molecules and most organic and biological molecules have fluorophors or electronic states corresponding to energy in the ultraviolet. Figure 1 shows an excitation-emission matrix (EEM) diagram of benzene over a range of excitation wavelengths from 200nm to 400nm and emission wavelengths from 200nm to 600nm. Shown are iso-intensity contours resulting from elastic scattering and fluorescence. This diagram illustrates several important features of the relationship between elastic and Raman scattering and fluorescence. The diagonal line in the diagram represents elastic scattering where the emission and excitation wavelengths are equal: $\lambda em = \lambda ex$. Fluorescence emission is always Stokes-shifted to longer wavelengths. The amount of Stokes shift varies from one compound to another and as a function of excitation wavelength. In 1984 S.Asher[i] showed that that no fluorescence is observable below 260nm for any sample, even with excitation at wavelengths as low as 220nm. This is also illustrated in Figure 1, where a fluorescence-free region is shown for excitation wavelengths below about 250nm and extending down to 200nm and below. Asher's explanation was that the smallest conjugated polyene or aromatic molecule to show significant fluorescence from its first excited state is benzene, which shows fluorescence only at wavelengths longer than 260nm. Larger aromatics show absorption bands below 260nm but excitation into these bands results in fast internal conversion of this energy into the lowest energy singlet or triplet excited state, and fluorescence or phosphorescence occurs at much longer wavelengths. As illustrated in Fig 1, shorter excitation wavelengths provide a wider range of fluorescence-free wavelengths in which to observe weak Raman scattering emissions without interference from fluorescence. At wavelengths below about 240nm, Raman scattering up to about 4000 cm^{-1} can be observed without interference from fluorescence. Figure 2 also clearly illustrates the fact that the fluorescence efficiency and Stokes shift is strongly dependent on excitation wavelength. At excitation wavelengths below about 220nm benzene shows little or no fluorescence at any wavelength. In this region, measurements can be made in full daylight conditions since the detection region is solar blind. S. Asher's 1984 paper opened up the field of ultraviolet resonance Raman spectroscopy, which is especially valuable for the elucidation of biological materials.

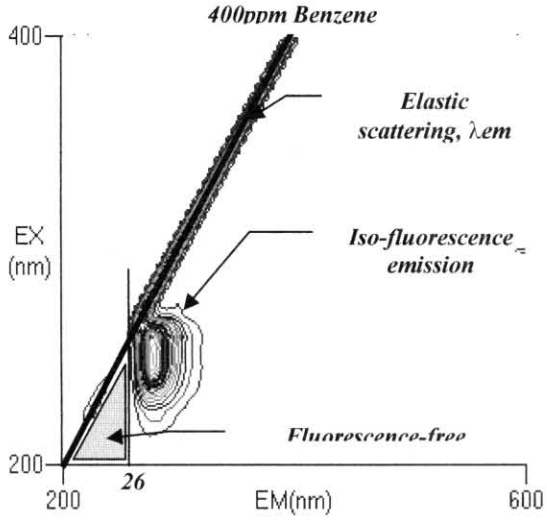


Figure 1. Excitation-Emission-Matrix diagram

When excitation wavelength is increased above about 240nm, fluorescence begins to interfere with Raman emissions occurring at larger Raman Stokes shifts. At excitation wavelengths above 260nm, fluorescence emissions will always overwhelm weak Raman emissions, even at small Raman Stokes shifts. A wide range of material fluoresces between about 260nm and 750nm, severely restricting the variety of samples for which Raman spectra can be obtained without fluorescence interference. For this reason, Raman spectroscopy is commonly performed in the near infrared or ultraviolet to avoid fluorescence interference.

3. *HIGH RESOLUTION DIFFERENTIABILITY OPTIMIZATION FOR AVAIALABLE SOURCES*

We have previously shown that separation of target molecules from background materials using high resolution native fluorescence spectra was optimal at an excitation wavelength of 235nm (SPIE ref). This was determined by using a multivariate technique, Principal Component Analysis, EEM spectra of 10 groups of targets and background materials. The target groups consisted 1 rings aromatic amino acids, bacterial spores, vegetative bacterial cells (Gram + and Gram -) with cellular components, 2 ring aromatics, indole-like organics, 3 ring polyaromatic hydrocarbons (PAHs), 4 ring PAHs, and >5 Ring PAHS. A "background" group, consisting pollen, dust, minerals, and household materials (sugar, flour, corn starch, etc), was shown not to interfere with the target groups. The optimal excitation region was found using the overall differentiability factor (ODf), an index that relates excitation wavelength with the ability to separate all groups from one another. This can be attributed to a unique double peak feature found in the emission spectrum of the spore group.

Presently, no commercial 235nm source is available. However, light emitting triodes (LETs) and Laser Triodes (LTs) that emit in and around 235nm are in development (PSI). For now, the available laser sources that can be used for portable in-situ measurements of surfaces and liquids are hollow cathode lasers at 224nm and 248nm as well as a 4rth harmonic Nd:Yag 266nm laser. It is therefore necessary to specify the targets and possible backgrounds to determine the optimal excitation wavelength(s) given the available sources.

LET image and Emission at 235

For the biochemical warfare application, the targets set includes bacterial spores, bacterial toxins, and vegetative cells (gram+ / gram -). These need to be separable from background materials or false positives that may be found in the environment. The background set includes pollen, dust, minerals, and household materials (sugar, flour, corn starch, etc), as well as diesel soot components such as >2 ring PAHs. In addition, the background set includes likely false positives such as free amino acids (tyrosine, phenylalanine, tryptophan) and small peptides containing aromatic ring amino acids. These are considered false positives since the target materials consist of these components and to ensure that false alarms are reduced, the available excitation sources need to separate between the constituents and targets.

PCA 235 and PCA 224 and PCA 248 and PCA 266

To test which source is most appropriate, the SDf (specific differentiability factor), described in (SPIE ref) is used. Similar to the ODf, the SDf, relates the separation in PCA space of a given target group from the nearest sample. We used the SDf in an iterative manner by selecting each target and determining the distance between the group and the nearest sample not within the group. Excitation with a 266nm or a 248nm source show that the separation between the bacterial spores and free tyrosine or small peptides containing tyrosine can only be determined with a confidence level of such ~70%. However, the SDf shows that a separation between bacterial spore group and the tyrosine subgroup was greater than 3 times the standard deviation of the spore group using an excitation wavelength of 224nm indicating that the spore group could be identified with 99.7% confidence. For the bacterial toxin analogues (bovine serum albumin and lysozyme), excitation at all three wavelengths provide >90% confidence in differentiating the toxins from any other sample tested in the database. For the final target group, the SDf shows that using a 224nm source identifies vegetative cells with 99.7% confidence where 248nm and 266nm provide a confidence level of 95%.

SDF vs wavelength (spores)

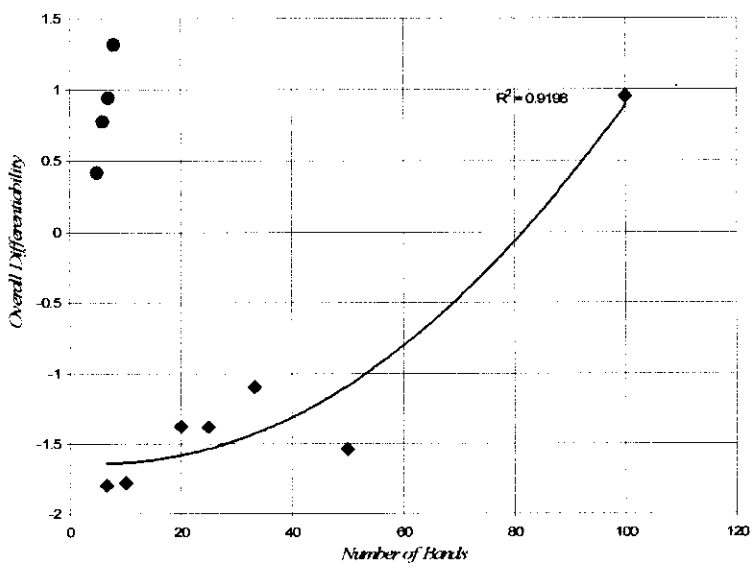
Using high resolution native fluorescence data shows that excitation with a 224nm laser differentiates between targets and backgrounds better than other available sources. The issue with using the 248 or 266nm sources for differentiability is in distinction between free amino acids such as tyrosine, and the bacterial spores. The fact that this distinction can be made using a 224nm source, the possibility of false alarm rates is dramatically decreased.

4. *LOW RESOLUTION DIFFERENTIABILITY OPTIMIZATION FOR AVAIALABLE SOURCES*

The Principal component analysis shows that the target groups and background groups that we have specified can be well separated using only 3 principal components. Qu et al (PCA filter ref) and others have used customized transmission filters that match the PCA loading vectors. For situations where only two targets need to be separated, the loading vectors are easier to model. However, with the targets and backgrounds we have, the PCA loading vectors are a present too complicated to develop into transmission filters. Therefore, we have used a set of 6 bandpass filters to match the 3 loading vectors established by the high resolution spectra In our analysis, each excitation wavelengths used, provided a slightly different set of vectors. Since the 224nm provided the greatest differentiability with the high resolution fluorescence spectra, we used the corresponding vectors to choose an appropriate filter set.

For in-situ applications, the consequence of using bandpass filters are that the sensor becomes more rugged since no monochrometer or spectrograph are necessary and it becomes more compact. Additionally, the sensitivity of the system is increased considerably since the band pass filters are relatively wide compared to a grating based system. Furthermore, the aperature, of a photomultiplier tube (PMT) coupled system is much greater than the entrance slit of a spectrometer. Thus the bandpass sensor has a considerable lower limit of detection than a grating based unit.

To understand the effect of using filters optimized using the PCA loading vectors, we compared it to spectra where the data was sequentially binned. This simulates how a fewer contiguous bands spanning the fluorescence region from 200-600nm would differentiate targets from the background groups and from one another.

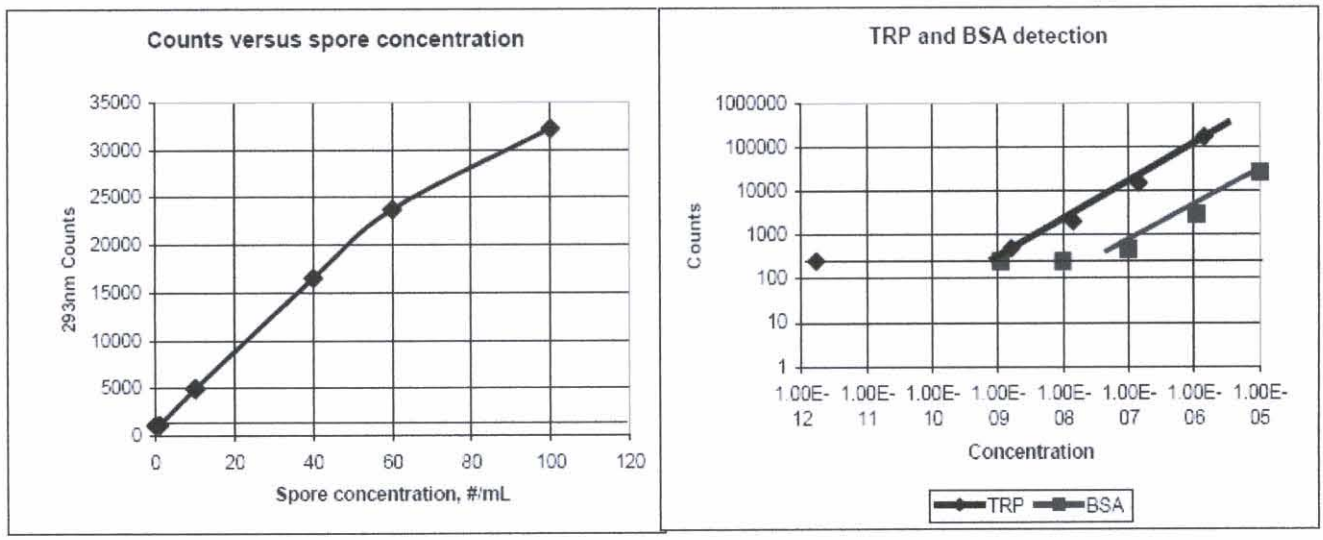


SDF vs #of Bands vs Contiguous bands

PCA regression loadings

PCA diagram of 224nm HR vs 224 LR

5. *LIMITS OF DETECTION*



LOD of spore, BSA, and TRP

6. *INSTRUMENT EXAMPLES*

Image of Antarctic image

Image of Biocounter

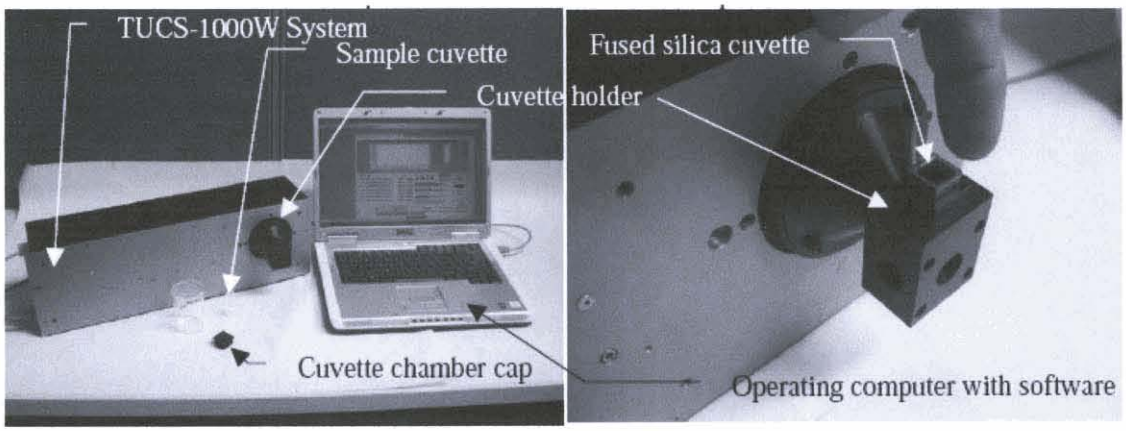


Image of TUCS -1000W

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[i] ***S.A.Asher, C.R. Johnson, Raman Spectroscopy of a Coal Liquid Shows That Fluorescence Interference Is Minimized with Ultraviolet Excitation, Science, 225, 311-313, 20 July 1984.***