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1. REPORT DATE: /1/92
2. REPORT TYPE AND DATES COVERED: Final 10/1/88-12/31/91

The Rapid Detection of Single Bacterial Cells by Deep UV Micro Raman Spectroscopy

3. FUNDING NUMBERS: DAA103-88-K-0093

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APR 30 1992
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8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES):
U. S. Army Research Office
P. O. Box 12211
Research Triangle Park, NC 27709-2211

10. SPONSORING/MONITORING AGENCY REPORT NUMBER: ARO 25476-12-45

11. SUPPLEMENTARY NOTES: The view, opinions and/or findings contained in this report are those of the author(s) and should not be construed as an official Department of the Army position, policy, or decision, unless so designated by other documentation.

12a. DISTRIBUTION/AVAILABILITY STATEMENT: Approved for public release; distribution unlimited.

12b. DISTRIBUTION CODE

13. ABSTRACT (Maximum 200 words)
A specially-designed micro-Raman spectrograph capable of selectively exciting single bacterial cells has been constructed. Detection limits have been shown to be one bacterial cell. With modest redesign, but with no change in basic technology, sensitivity is sufficient to allow identification of single bacterial cells in a matter of seconds.
Live bacteria were immobilized on glass slides by means of 0.1 M polylysine solution. The wet sample was placed on a microscope stage adjusted to maintain a temperature of 0 C. The sample was illuminated by the CW 257 nm output of a Spectra Physics Model 395B argon ion laser cavity extender. Ten percent of the beam (less than 3 mw) was directed by a beam splitter down the microscope optical axis and focussed onto a 5 micron spot on the sample. A Cassagrain objective focussed the beam and collected the back-scattered resonance Raman light. The Raman-scattered light was analyzed using a Spex Triplemate equipped with a blue-sensitive EG&G OMAII optical multichannel analyzer which was able to obtain a spectrum in 16 microseconds. Best sensitivity required scans of 30-60 seconds.

14. SUBJECT TERMS: Detection, Identification, Bacteria UV Micro Raman Spectra, Instrumentation

15. NUMBER OF PAGES: 19

16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT: UNCLASSIFIED

18. SECURITY CLASSIFICATION OF THIS PAGE: UNCLASSIFIED

19. SECURITY CLASSIFICATION OF ABSTRACT: UNCLASSIFIED

20. LIMITATION OF ABSTRACT: UL

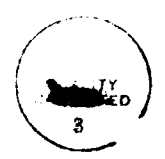
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A. The Problem Studied

Rapid and reliable methods for the detection and identification of microorganisms are very important. Fortunately, many effective means for bacterial identification have been developed. On the other hand only a few of these are rapid, accurate, sensitive and cost effective. In most instances, if general examinations of bacteria must be performed, presently-used techniques are very slow and may not be helpful unless extremely tedious procedures are followed. Traditional methods which are based upon visual microscopic examination, biochemical reactions and physiological functions of bacteria are inherently slow, time-consuming and tedious. Even today, important decisions related to the presence of pathogens have to be made before the results of microbiological tests are available. This situation is changing rapidly, however.

Advances in molecular biology, matched by progress in electronics, lasers and computer technology have allowed the introduction of totally-new methods. Although many new approaches are being applied with varying degrees of success, all are due to rapid advances in our detailed knowledge of the structures and properties of the molecular components of microorganisms. In the past decade bacterial cell components(1,2) have been especially carefully studied and documented. One consequence has been the development of sensitive detection methods for bacteria based upon physical and chemical properties. This is possible because of the much-improved sensitivity and specificity of newly-developed physical and chemical methods which can be applied to detect molecular components. Rapid identification as well as detection has become possible since molecular information can be used effectively to establish relationships at all levels of the bacterial taxonomic hierarchy.

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An array of physico-chemical methods have been developed for the purpose of rapid bacterial detection. Techniques include mass spectroscopy and its various combinations with chromatography and pyrolysis methods(3-5), protein electrophoresis(6), total luminescence(7-10), flow cytometry(11), impedance(12), circular intensity differential light scattering(13-16), NMR(17) and FTIR(18,19).

While DNA itself is most unique as a molecular basis for the classification of bacteria, many components have proved to be of more practical use in bacterial classification methods. For example, nucleic acid sequences of 16S r-RNA(1,20-22), the composition of lipids(23,24), peptidoglycans(25), cytochromes(23) and isoprenoid quinones(26) all have been used. Several monographs have been devoted to these topics(5,7,27-30). Our interest has centered on the use of UV resonance Raman spectroscopy to identify bacteria by sensitive, selective detection of taxonomic markers.

In the work which has been funded by this DOD equipment grant, emphasis has been upon extension of exciting laser frequencies to the deep UV, i.e., to the region below 218 nm, and, especially, to the investigation of the sensitivity limits of the UV resonance Raman method. As a means to determine sensitivities in studies of bacteria, a unique, yet relatively low-cost, UV micro-Raman microscope with reflecting optics has been constructed and used to study precisely-defined, very small numbers of bacteria.

B. Summary of Important Results

The sensitivity limits of the UV resonance Raman experiment for bacterial detection and identification have been investigated. For that purpose we have built a specially-designed micro-Raman spectrograph capable of exciting single

bacterial cells and small groups of cells selectively.

Figure 1 schematically represents the new UV Raman micro system. UV laser light is produced by frequency doubling the 514.5 nm output of a Spectra Physics Model 2000 Argon ion laser equipped with a Model 395B cavity extender. A relatively small fraction of the laser beam (typically less than 3 mw) was directed by a beam splitter down the microscope optical axis and focussed onto a 5 micron spot on the sample by means of the optical system shown in Figure 2.

The optical system collected the Raman scattered light originating from the sample and focussed it on the slits of a Spex Triplemate spectrograph equipped with gratings optimized at 250 nm. The dispersed spectra were detected with a blue-sensitive EG&G OMA II optical multichannel analyzer which was able to obtain a complete spectrum in 16 microseconds. Usually, for the best sensitivity, scan times of up to one minute were used.

The optical system shown in Figure 2 required the use of a 90/10 quartz beam splitter which passed only 10% of the laser beam to the Ealing Optics 36X Cassagrain objective. The Cassagrain objective focussed the beam on the sample and collected the Raman scattered light. This Raman scattered light spectrum was then 90% transmitted through the beam splitter and focussed by means of a quartz lens on the slits of the spectrograph. By inserting a mirror into the optical axis an image of the sample was directed to a video camera which directed the image of the sample onto a video monitor. The spot size of the beam at the sample was determined visually for the purpose of counting the organisms by noting the bright blue fluorescence produced by the bacteria when illuminated by the UV light. The spot size has been confirmed by burning a spot onto a calibrated plate.

Live bacteria were immobilized on glass slides by means of 0.1 M polylysine

solution which was allowed to dry on the slide surface before bacteria were introduced. Recently-cultured bacteria suspended in buffer were applied directly to treated plates and pressed gently with a cover slip to enhance adhesion. The wet slide was placed on a cold microscope stage adjusted to maintain the temperature near 0 Centigrade. Bacteria maintained in this fashion could be illuminated for up to one minute without showing changes in spectra. Spectra were identical to those obtained from flow samples.

Typical spectra of Flavobacterium capsulatum taken from a monolayer of attached cells, which partially-filled the laser beam spot, are shown in Figure 3. Such spectra are the result of illuminating 30 cells for 45 seconds. Figure 4 shows spectra of 50 Bacillus subtilis bacteria taken under similar conditions. By choosing a section of the plate having a relatively low bacterial density it was possible to excite bacteria in precisely-known numbers.

Spectra of single cells to date have been of marginal quality even though nucleic acid Raman peaks can be detected reproducibly. This suggests that under the conditions described more than one cell needs to be excited if quality spectra are to be produced. However, considering the 3% throughput of the spectrograph used, conservatively, in practice it will be possible to increase the signal level by at least a factor of 10 without changing the dispersive optical system or the laser power. Simple replacement of the filter stage of the spectrograph with a solution filter or atomic line filter to reject the Rayleigh line will increase the sensitivity ten fold.

Consequently, it is possible with present technology to detect single bacterial cells using UV resonance Raman spectroscopy. Furthermore, with additional improvements in detector sensitivity it appears certain that such spectra will be of high enough quality to allow the use of all resonance Raman

information currently extracted from bulk samples. Hence, it should be possible to use such spectra for very rapid (in a matter of seconds) bacterial identification.

C. A Review of Results to Date

Two major reviews of our microbiological identification studies have appeared in the last six months. The first(30), entitled "UV Resonance Raman Spectroscopic Detection and Identification of Bacteria and Other Microorganisms" was written as a book chapter intended for reading by biologists. A second review(31) intended for spectroscopists appeared very recently in Applied Spectroscopy Reviews. Because both of these lengthy articles describe nearly all of our significant work in detail through early 1991 I will not present this research in detail, but will merely outline the results.

Bacteria(31-35), bacterial endospores(34,36), viruses(37) and cyanobacteria(38) yield high-quality UV-excited resonance Raman spectra. Sensitivities are high since the fluorescence background is extremely low throughout the 190-251 nm region. Most of the spectra appear to be due to various UV-absorbing protein and nucleic acid components, but it has been noted that calcium dipicolinate in spores, and quinones in cyanobacteria and pollens are very strongly excited at characteristic UV wavelengths. Spectra range from the very simple to those of moderate complexity. Due to the selectivity of resonance Raman excitation they are much less complex than conventional Raman spectra of mixtures. Bacteria, endospores, cyanobacteria and pollens give rise to very-different, characteristic, UV-excited resonance Raman spectra. Spectra of all spora vary strongly as a function of excitation wavelength.

Illumination in the 242-257 nm range which selectively excites quinones,

dipicolinates, and nucleic acids produces useful spectra for all the organisms studied. Excitation wavelengths in the 218-231 nm range on the other hand appear more useful in the study of bacteria alone. Bacterial spectra excited at 231 and 222 nm are not dependent upon cultural conditions(31) and 222 nm-excited spectra, especially, show significant spectral differences from species to species and selective excitation of DNA. Consequently, the 222 nm-excited spectra appear to be best suited for bacterial fingerprinting. It is very easy to discriminate Gram-type on the basis of these spectra and, generally, spectra excited at 222 nm show more detail than 231 nm-excited spectra. It is believed that wavelengths in the 231-222 nm range may be best for the study of bacterial protein structure. In contrast, bacterial spectra excited near 242 nm predominately reflect nucleic acid composition and structure. While spectra excited at 242 nm are strongly culturally dependent because of their sensitivity to varying amounts of ribosomal RNA, they allow calculation of DNA GC/AT molar base pair ratios and may be ideal for assessing changes in the relative amounts of protein and RNA.

Upon first consideration it may appear that 242 nm and 251 nm-excited spectra are not promising for use in bacterial detection and identification, and that only 222 nm-excited spectra will be of use in analysis. This may appear to be true because of the very large dependence of 242 nm-excited spectra on the amount of r-RNA present. Clearly, the 242 nm-excited spectra reflect both DNA and RNA contributions. The RNA amounts per cell vary enormously depending upon the growth medium used and especially upon the rate of growth.

Known DNA(G+C/A+T) base-pair ratios relate simply to the intensity ratios of whole-cell Raman peaks due to cytosine near 1530 cm^{-1} and combined A+G scattering near 1485 cm^{-1} . The height of the 1485 cm^{-1} peak can be taken as closely proportional to the total amount of nucleic acid on a molar

basis(DNA+RNA) in the cell. In a more straight-forward fashion the height of the 1530 cm^{-1} peak can be taken as proportional to the moles cytosine in the nucleic acids. It follows that the mole percent G+C in the bacteria should be proportional to the intensity of the 1530 cm^{-1} peak divided by the intensity of the 1485 cm^{-1} peak, i.e., (I_{1530}/I_{1485}) . It has been observed(31) that the plots of peak intensity ratios vs. molar percent G+C are linear and essentially culturally independent.

The cultural independence of the (I_{1530}/I_{1485}) peak ratios indicates that the I_{1530}/I_{1485} ratios may be very useful in bacterial identification even though the 242 nm-excited spectra are not as promising as "fingerprints". Even though the RNA and protein content vary substantially, apparently the ratio of 1530 cm^{-1} and 1485 cm^{-1} peak intensities remains a measure of DNA molar percent(G+C). While base pair ratios will hardly allow unambiguous identification of bacteria, such knowledge certainly can limit a list of candidate organisms in a useful manner.

For selected organisms having extreme values in their base-pair ratios, I_{1530}/I_{1485} intensity ratios have been plotted versus DNA (G+C/A+T) values. Cultures ranged from early log to late stationary phase. While there is some scatter in the data, the linear relationship between the mole percent G+C and the Raman peak intensity ratios has been maintained even when subjected to the extremes of growth conditions.

It is clear that with either 200 or 218 nm excitation the spectra are culturally dependent. Consequently, it may not be simple to use these excitation wavelengths for the purposes of fingerprinting. At present 222, 231, 242, 251 and 257 nm all seem more attractive as exciting wavelengths.

Excitation at 200 nm has shown that resultant bacterial Raman spectra are

due to combinations of amino acid and nucleic acid contributions. E. coli have been studied using both log and stationary phase cultures. A peak near 860 cm^{-1} is probably due to tyrosine. Another strong peak near 1000 cm^{-1} has been assigned to tyrosine and phenylalanine. Between 1200 and 1300 cm^{-1} there is a broad band due to tyr at 1210 and 1263 cm^{-1} , and cytosine at 1294 cm^{-1} . A weak feature at 1413 cm^{-1} is not understood. The total absence of A+G contributions at 1485 cm^{-1} also is surprising, since corresponding nucleoside peak cross-sections are very large. The largest peak is centered near 1580 cm^{-1} and is due to G+A. A smaller peak at 1624 cm^{-1} is assigned to tyr. A substantial peak at 1677 cm^{-1} has been assigned to guanine.

It appears that UV-excited resonance Raman spectroscopy will be a valuable tool both in biophysical studies and in bioanalytical applications. The high sensitivity of the technique promises that it will be the basis for methods of rapid and specific microbiological analysis. Spectra also will provide important information about excited states of molecular components, and potentially, information about interactions between macromolecules in vivo. UV micro Raman instrumentation of modest cost can be developed which is capable of obtaining quality spectra from single bacterial cells. It seems likely that Raman-based methods will be established which will allow the very rapid detection and identification of bacteria and spores from aerosols, as well as the rapid assessment of the extent and manner to which bacteria change when exposed to antibiotics.

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D. Participating Scientific Personnel

1. W.H. Nelson, PI, URI, Chemistry Department
2. J.F. Sperry, Co-investigator, URI, Microbiology Department
3. R. Manoharan*, Postdoctoral, URI, Chemistry Department
4. S. Chadha, Graduate Student, URI, Chemistry Department
5. E. Ghiamati**, Graduate Student, URI, Chemistry Department

* Presently employed at MIT, Harrison Spectroscopy Lab., Cambridge, Mass.

** Earned a Ph.D. during the grant period.

E. Major Equipment Purchased

The budget of \$76,064.00 submitted to USARO September 13, 1988 was subjected to a major revision March 31, 1990 because the price of the Leitz UV microscope we had planned to purchase had increased far beyond original estimates. Still, as planned, a major portion of the money, \$36,000.00, was spent very early on the Spectra Physics Model 395B Ion Laser Cavity Frequency Doubler, and the associated Light Stabilizer. This system in combination with the Spectra Physics Model 2000 argon laser provided by the Chemistry Department allowed generation of CW 257 nm laser light needed for the micro-Raman experiment. For a period of one year beginning in December 1989 while we waited for matching funds to complete our microscope this laser was used by Dr. Marcus Peter of the Dana-Farber Cancer Institute. During that period he came to our laboratories weekly to study GTP-binding proteins.

As outlined in the March 31, 1990 revised budget approximately \$10,000.00 was spent on parts for the UV microscope. This material was purchased from Ealing Optics. A second major budget revision occurred December 1, 1990 detailing many individual items needed to complete the micro Raman system and to allow the processing of spectral data. The final detailed list of expenditures is attached.

F. List of Publications and Technical Reports Published October 1988- present

1. "The Steady State and Decay Characteristics of Protein Tryptophan Fluorescence from Algae", M. Baek, W.H. Nelson, P.E. Hargraves, J.F. Tanguay and S.L. Suib, *Appl. Spectrosc.* 42, 1405-1411 (1988).
2. "UV Excited Resonance Raman Spectra of Heat Denatured Lysozyme and Staphylococcus epidermidis", M. Baek, W.H. Nelson, D. Britt and J.F. Sperry, *Appl. Spectrosc.* 42, 1312-1314 (1988).
3. "Ultraviolet Resonance Raman Spectra of Live Cyanobacteria with 222.5-251.0 nm Pulsed Laser Excitation", M. Baek, W.H. Nelson and P.E. Hargraves, *Appl. Spectrosc.* 43, 159-162 (1989).
4. "Detection and Identification of Bacteria by Means of UV Excited Resonance Raman Spectra", W.H. Nelson, R.A. Dalterio and J.F. Sperry, U.S. Patent 4847198 issued July 11, 1989.
5. "Ultraviolet Resonance Raman Spectra of Bacteria, Bacterial Spores, Protoplasts and Calcium Dipicolinate", R. Manoharan, R.A. Dalterio, K.A. Britton, E. Ghiamati, W.H. Nelson and J.F. Sperry, *J. Microbiol. Meth.* 11, 1-15 (1990).
6. "UV Resonance Raman Spectra of Aqueous Pollen Suspensions with 222-242 nm Pulsed Laser Excitation", K.A. Britton, E. Ghiamati and W.H. Nelson, *J. Appl. Spectrosc.* 45, 307-311 (1991).
7. "UV Resonance Raman Spectroscopic Detection and Identification of Bacteria and Other Microorganisms", W.H. Nelson and J.F. Sperry, Chapter 4 in Modern Techniques in Rapid Microorganism Analysis, W.H. Nelson, ed., VCH Publishers, NY, 1991.
8. "UV Resonance Raman Spectra of Bacillus Spores", E. Ghiamati, R. Manoharan, W.H. Nelson and J.F. Sperry, *Appl. Spectrosc.* 46, 357 (1992).
9. "UV Resonance Raman Studies of Bacteria", W.H. Nelson, R. Manoharan and J.F. Sperry, *Appl. Spectrosc. Revs.* 27, 67-124 (1992).
10. "Deep UV Resonance Raman Detection of Bacterial Cells, Spores, Taxonomic Markers and Agent Simulants", W.H. Nelson, Proceedings 1988 US Army CRDEC Scientific Conference on Chemical Defense Research.
11. "The Effect of Cultural Conditions on Deep UV Resonance Raman Spectra of Bacteria", W.H. Nelson and J.F. Sperry, Proceedings 1989 US Army CRDEC Scientific Conference on Chemical Defense Research.
12. "Rapid Sensitive Spectroscopy, Bacterial ID", W.H. Nelson and J.F. Sperry, Proceedings 1990 US Army Scientific Conference on Chemical Defense Research.
13. "UV Resonance Raman Rapid Detection and Identification of Single Bacterial Cells", S. Chadha, W.H. Nelson and J.F. Sperry, Proceedings 1991 US Army CRDEC Scientific Conference on Chemical Defense Research.

14. "The Resonance Raman Detection and Identification of Bacteria", W.H. Nelson, Proceedings 1991 Troia Conference on Pyrolysis Mass Spectroscopy and Biosensors sponsored by the US Army.

15. "The Resonance Raman Detection and Identification of Bacteria", W.H. Nelson, Proceedings USARO Workshop on Spectrometry and Spectroscopy for Biologicals", Cashiers, NC 1991.

Figure Captions

Figure 1. A Schematic Representation of The UV Micro-Raman System.

Figure 2. The Micro-Raman Optical System.

Figure 3. Resonance Raman Spectra of 30 Cells, Flavobacterium capsulatum, Excited 257 nm, 45 seconds.

Figure 4. Resonance Raman Spectra of 50 Cells, Bacillus subtilis, Excited 257 nm, 60 seconds.

Budget Submitted September 13, 1988

A.	Spectra Physics Model 395B Ion Laser Cavity Frequency Doubler	\$35,000.00
B.	Light Stabilizer for Model 395B	1,000.00
C.	Modified Leitz Orthoplan UV microscope with incident light quartz optics including beam splitters.	\$29,744.00
D.	UV quartz microscope objective	\$ 3,300.00
E.	Vidicon detector for microscope	\$ 2,500.00
F.	High-resolution grating for Triplemate Spex 24009 HC 2400 g/mm holographic grating	\$ 3,400.00
G.	Spex Model 1461 polarization scrambler	\$ 611.00
H.	Spex Model 14620 polarization analyzer	\$ 509.00
	Total	----- \$76,064.00

The \$50,000.00 contributed by DOD will be used to purchase items A and B and parts of items C and D. The remainder will be purchased by funds supplied by the University of Rhode Island. Prices are based upon quotes supplied May 1988.

Actual Expenditures through December 11, 1990

1.	Spectra Physics Model 395B Frequency Doubler	\$32,260.00
2.	Model 295B Light Stabilizer	\$ 3,740.00
3.	Ealing Micro-spectroscopy System (parts for Micro-Raman system)	\$ 9,687.00
4.	High Resolution Holographic Grating for Spex Triplemate	\$ 3,600.00
5.	Quartz Plates (10)	\$ 50.00
6.	Lens and Mirrors	\$ 166.00
7.	Polished Quartz Windows(3)	\$ 69.00
8.	Transverse Travel Slide	\$ 220.00
	Sub Total	----- \$49,792.00

Revised Budget of December 11. 1990

Describes the remainder of expenditures to the end of the grant.

1.	Raman shifter, Spectra Physics Model RS-1	\$ 8,800.00
2.	Sensorlok, BFS-3 thermal controlled microscope stage	\$ 1,300.00
3.	Ealing 52X reflecting microscope objective coated for maximum reflection at 257 nm.	\$ 3,270.00
4.	257 nm half-wave plate	\$ 495.00
5.	Fibre optic microscope illuminator	\$ 259.00
6.	IBM PS/2 Model 55SX with 2Mb RAM and 60 Mb fixed disk	\$ 2,799.00
7.	External disk drive to accept 5.25" floppy disks	\$ 300.00
8.	Laser Printer	\$ 1,039.00
9.	Lab Calc software	\$ 1,192.00
10.	Software to read Spex DM1B disks	\$ 1,100.00
11.	Additional memory for DM1B	\$ 2,100.00
12.	High-resolution Hitachi color video camera	\$ 1,120.00
13.	Video relay lens	\$ 197.00
14.	High-resolution Hitachi color monitor	\$ 339.00
15.	BNC connector cables for monitor	\$ 24.00
16.	Research grade 1000x microscope with stage	\$ 575.00
17.	Optical parts for beam-steering of Raman shifter input and output: mirrors, supports	\$ 1,264.00

	Additional Total	\$26,183.00

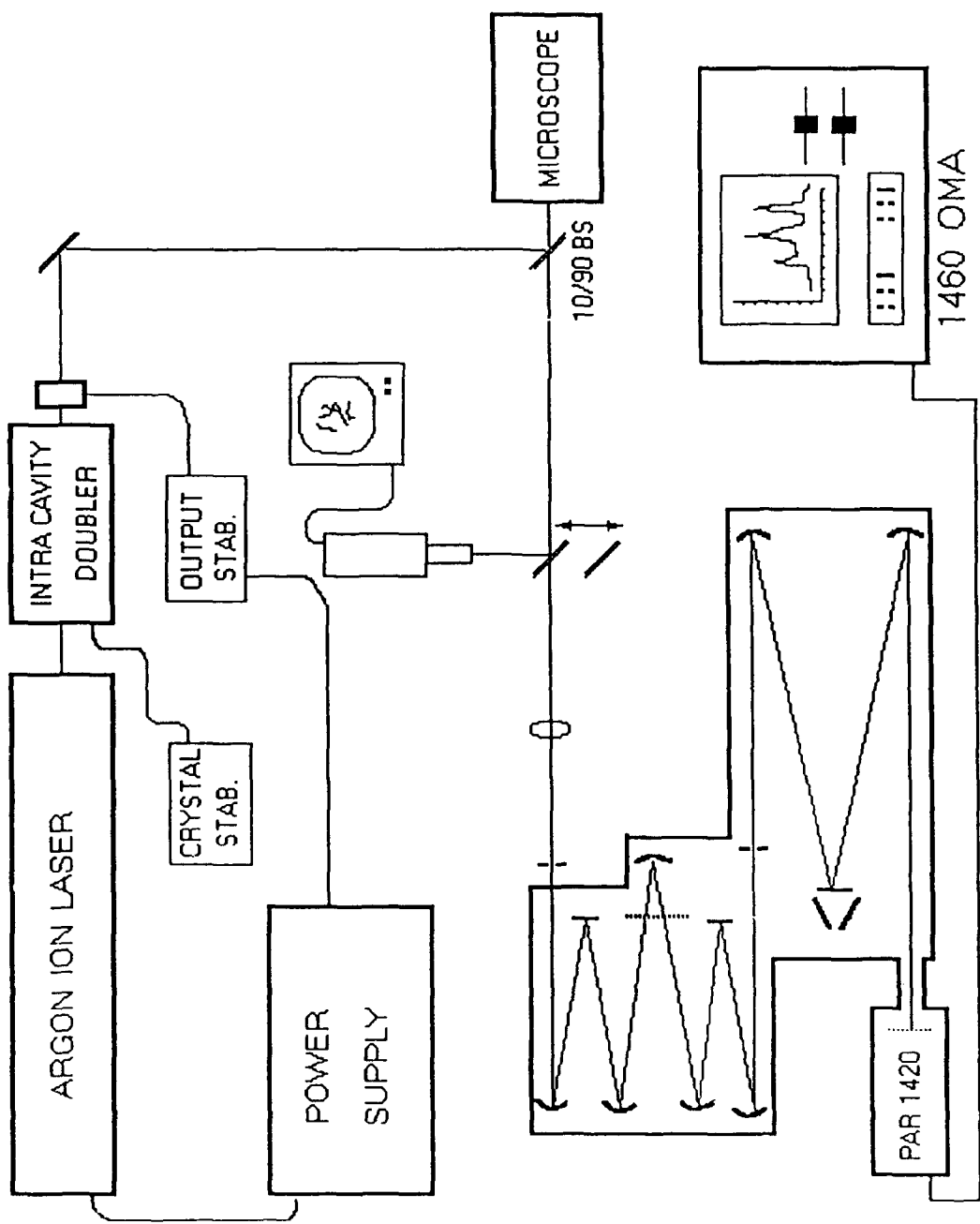


Figure 1

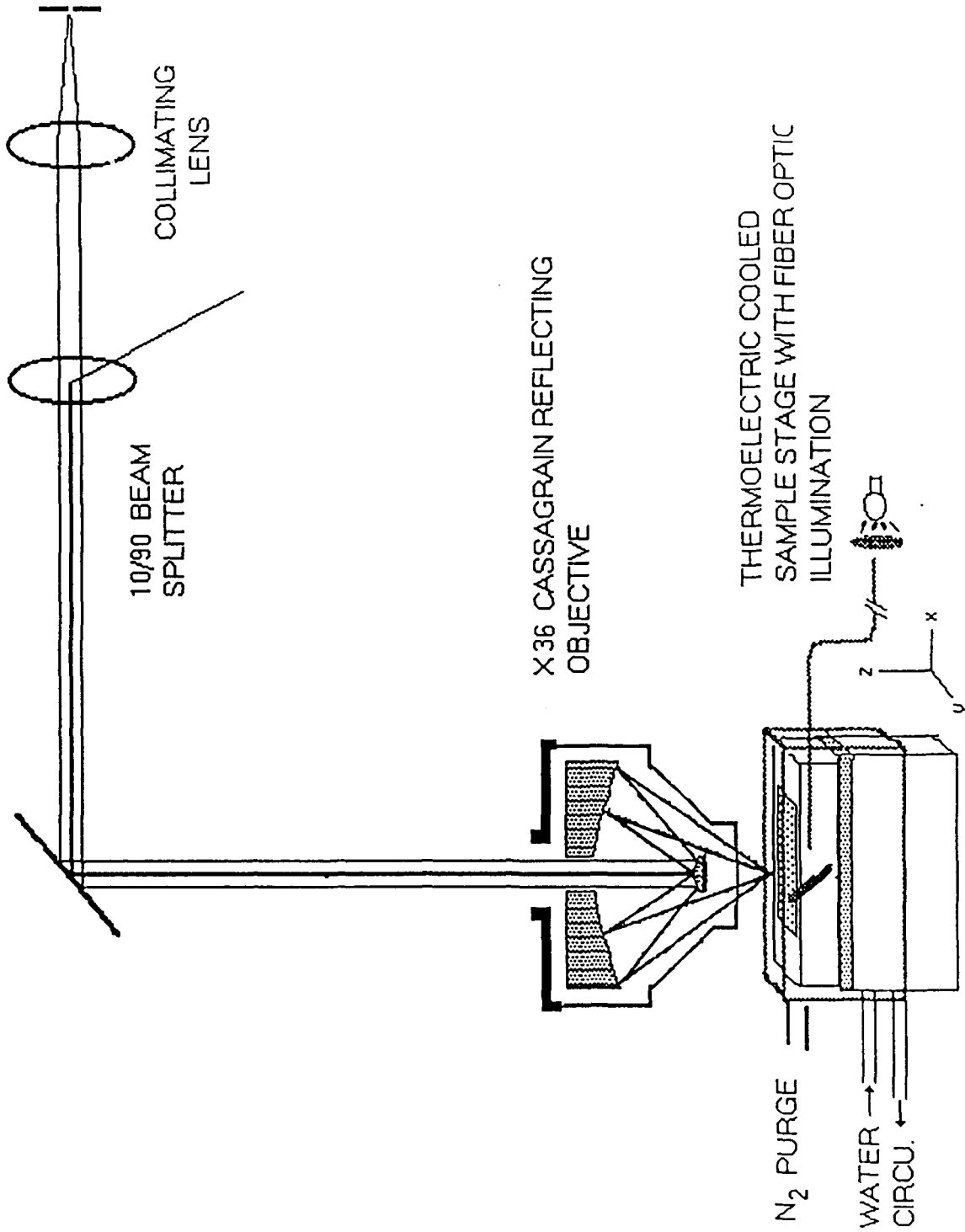


Figure 2

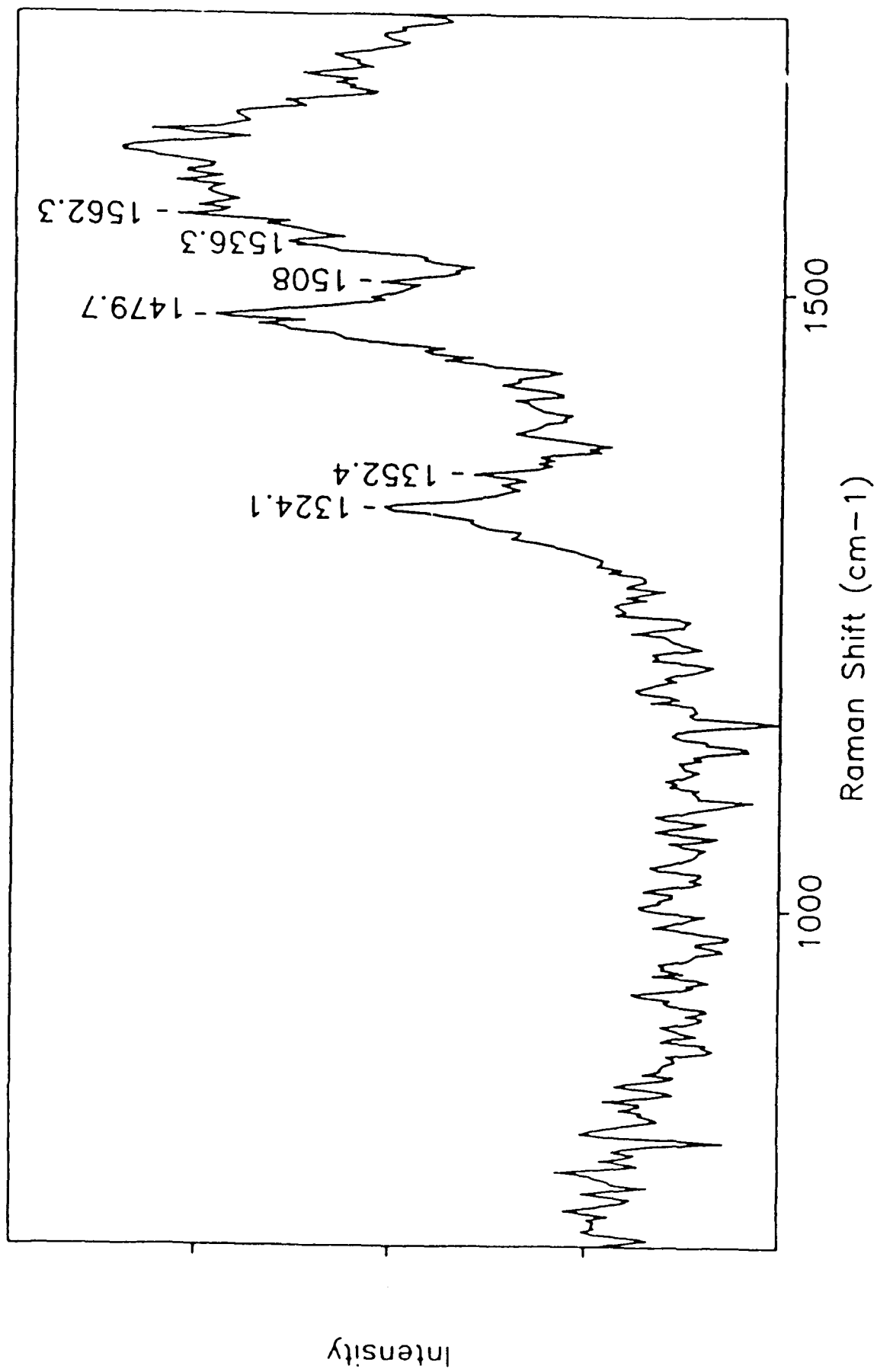


Figure 3

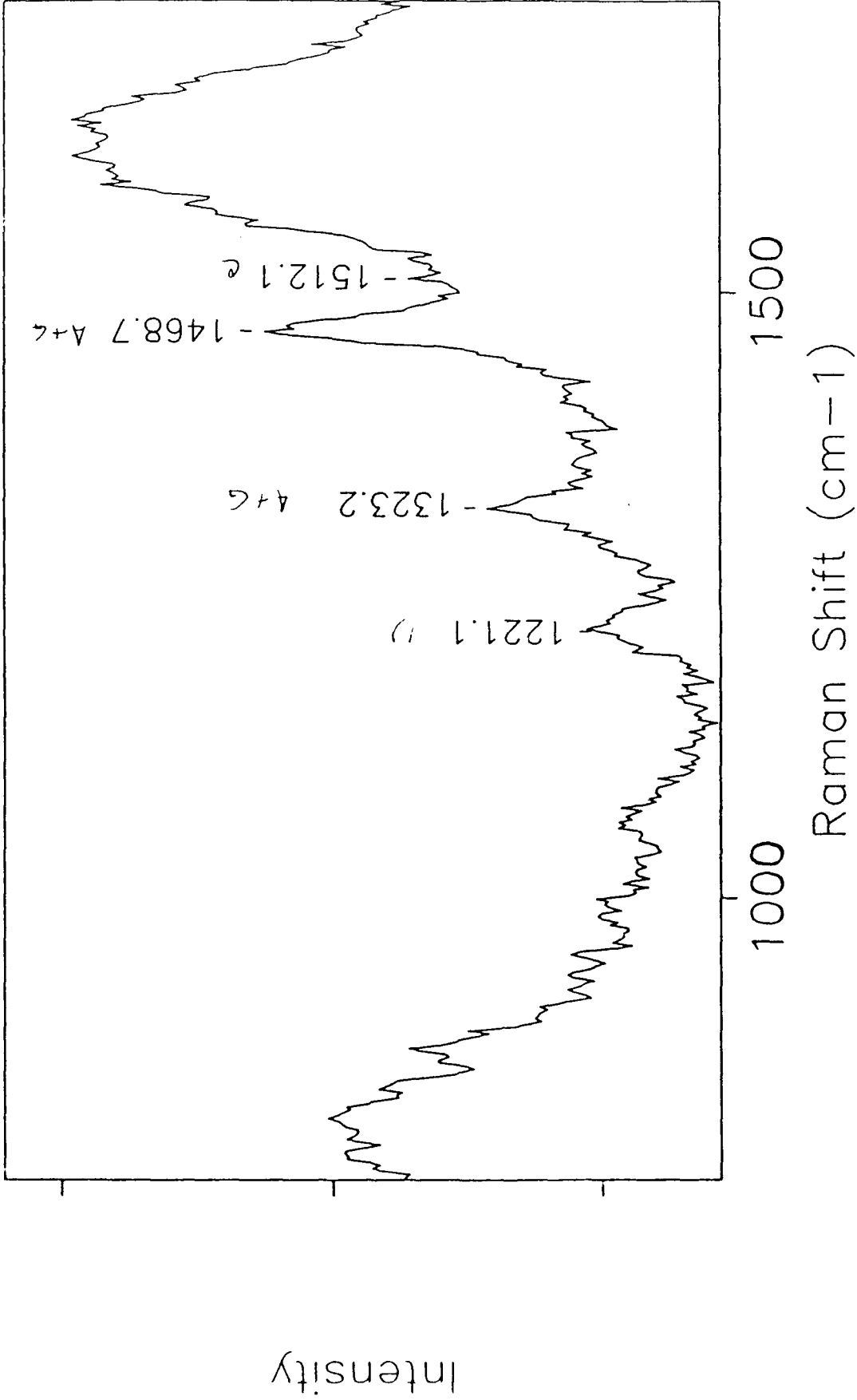


Figure 4