Spectroscopic Ultramicroanalysis with a Laser

Abstract. *A microbeam probe capable of vaporizing many biological materials is formed by focusing a conventional laser through a microscope objective. The elemental gases which are excited by this demagnified spot are raised to emission levels when sparked between carbon electrodes. Elemental analysis is then performed on the excited material by conventional spectroscopy. The method is simple and rapid. Simultaneous analysis of multiple elements is practical.*

By a versatile new method for the atomic analysis of biological materials under direct microscopic control, one operator may perform simultaneous analyses for ten or more elements in any area at a rate of two targets per minute (*1*).

In principle, the intense energy from a ruby laser is focused through a microscope upon a previously selected target. The sample is vaporized by the laser at a temperature of about 5000°K and the resultant activated atomic gases are sparked between carbon electrodes at 1000 v, thereby raising the energy of the gases to spectral emissive levels. Resultant light is relayed to a spectrograph for recording photographically or photoelectrically. The entire process takes 0.01 seconds. Both the selection of the sample area to be analyzed and the effects of the laser on the sample are observable by a conjugate visual system. With a 20× objective, numerical aperture 0.25, and a laser face of 0.6-cm diameter, the laser beam can be so concentrated that a crater about 50 μ wide is produced by vaporizing the sample. This corresponds to a sample size of about 10^{-7} g. The technique is applicable to living or resected material, sectioned or whole; the primary skills required are those of ordinary microscopy, coupled to familiarity with optical spectra.

Spectra from typical experiments are illustrated in Fig. 1. For the first set, a coronal section of rat forebrain was sliced at 100 μ in a cryotome, dried, and probed by the laser. The actual targets are illustrated for this set in Fig. 2. In the second set of spectra, Fig. 1, a pancreatic islet identified by dithizone staining (*2*), was compared with acinar tissue and with vascular septal tissue. This section was 100 μ thick. In the third set of spectra of Fig. 1, it is apparent that certain soft biological materials, in this case ligamentum nuchae elastin, may be analyzed even when sectioned as thin as 12 μ. Thickness was established by actual measurement. The last set of spectra of Fig. 1 were obtained from unmounted, unfixed, unsectioned, bloody and pearly portions of periodontal calculus. The difference in iron content is presumably due to the presence of blood.

The analytical limits go as far as 10^{-10} mole for some biologically important cations. More sensitive detection systems may be directly superimposed. Although this method is not amenable to quantitative analysis, careful experimental techniques permit estimation of relative concentrations to ±20 percent. The illustrated spectra show that the simplest application is determination of the changes in the *ratio* of one or more elements to some internal base (see Fig. 1).

Heterogeneity of biological target areas is unavoidable, and therefore experiments for the determination of absolute quantities need to be designed

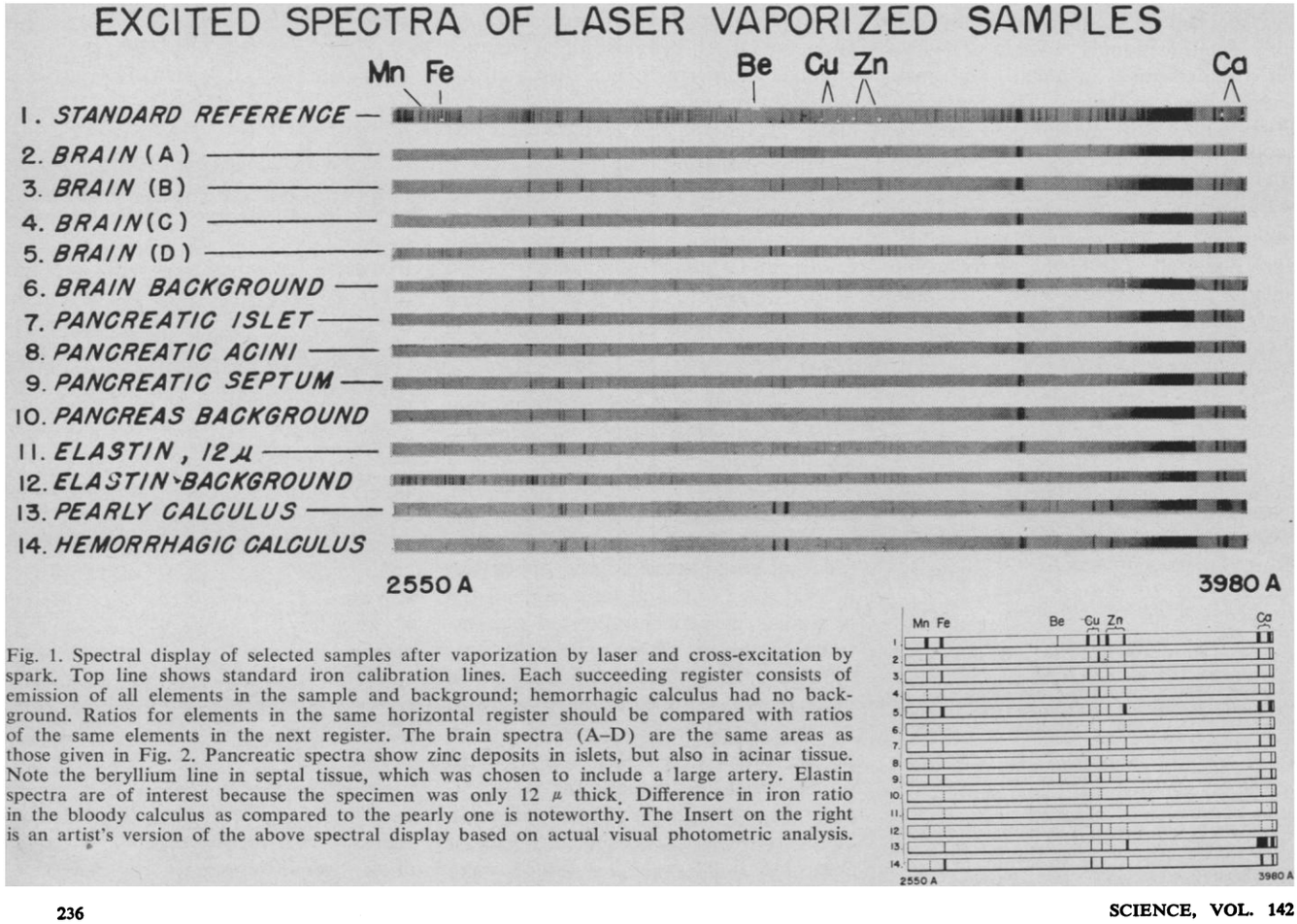

Fig. 1. Spectral display of selected samples after vaporization by laser and cross-excitation by spark. Top line shows standard iron calibration lines. Each succeeding register consists of emission of all elements in the sample and background; hemorrhagic calculus had no background. Ratios for elements in the same horizontal register should be compared with ratios of the same elements in the next register. The brain spectra (A–D) are the same areas as those given in Fig. 2. Pancreatic spectra show zinc deposits in islets, but also in acinar tissue. Note the beryllium line in septal tissue, which was chosen to include a large artery. Elastin spectra are of interest because the specimen was only 12 μ thick. Difference in iron ratio in the bloody calculus as compared to the pearly one is noteworthy. The Insert on the right is an artist's version of the above spectral display based on actual visual photometric analysis.

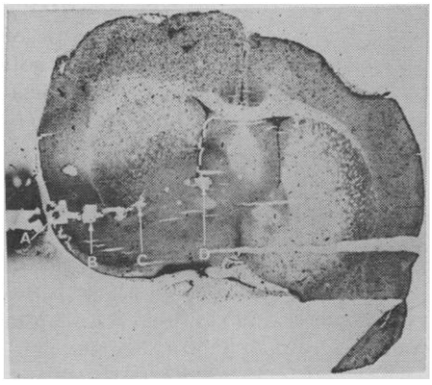

Fig. 2. Coronal section of rat brain after application of probe. Black crater outside brain represents background probe. Some fine dust from the sparking electrodes has fallen on the section. *A*, Outer cortex; *B*, inner cortex; *C*, nucleus caudate-putamen; *D*, tip of ventricle.

with caution. The pure graphite used in spectroscopic electrodes is the best support for samples, as can be seen from examining the spectrum of the "chemically clean" ordinary glass slides in Fig. 1. Direct electronic readout permits rapidity of analysis for the selected elements when many samples of the same matrix are to be analyzed. Photographic spectra used for the illustrations presented here, however, afford a greater versatility in the number of atomic elements that may be identified in each sample.

Until the advent of the laser, no practical method for *in situ* spectroscopic analysis existed because selection from intact sections was not possible without considerable tedious manipulation. The technique described is only an illustration of the possibilities, which include intracellular probes, analysis from frozen sections, and extension of the spectrum to higher and lower frequency ranges (*3*).

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References and Notes

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2. W. F. McNary, Jr., *J. Histochem. Cytochem.* **2**, 185 (1954).
3. The instrument used for these studies was the Jarrel-Ash Laser Microprobe #45-601 with a Wadsworth 1.5 meter grating spectrograph; the apparatus was kindly loaned by R. Jarrell, F. Brech, and associates, who stimulated this work.

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Freezing-Point Depression: New Method for Measuring Ultramicro Quantities of Fluids

Abstract. *Accurate determination of the solute content of millimicroliter samples of biological fluids can conveniently be made with an instrument consisting of a thermoelectric cooler controlled by an electronic proportional servo system. Accuracies to within less than 2 percent have been achieved in determining the osmolarity of body fluids.*

Freezing-point depression of millimicroliter samples of biological fluids can be determined conveniently and accurately by a new method. Although methods now exist for making these determinations, their dependence upon solid refrigerants, large regulated baths, and expanded-scale thermometers makes them cumbersome. By using modern materials and electronic techniques, we have eliminated these inconveniences. Thermoelectric junctions provide direct cooling of the samples, so that freezing is accomplished without external coolants. A high-gain electronic servo system senses the temperature of the samples and provides proportional control of the current to the thermoelectric junctions to obtain precise control of the temperature of the samples. The method is convenient because (i) several determinations are made rapidly during a single run; (ii) samples are frozen and, if necessary, refrozen by flipping a switch; and (iii) temperature is controlled to 0.001°C by turning a dial. Accuracy is achieved by making simultaneous determinations on unknowns and standards, finding values for unknowns by interpolating between accurate values for standards, and eliminating problems of thermal equilibrium through tight feedback control of the temperature of the samples. In addition to finding wide use as a freezing point apparatus, the device can be used to control precisely the temperature of ultramicro quantities of sample and thus is suitable for use in controlled-temperature studies of the physiology of microorganisms and cells.

Measurement of freezing-point depression provides a practical means of determining the effective osmotic activity of biological fluids obtained in studies of diffusion, secretion, and absorption in biological organisms. Often, in such studies, minute regions of the organisms are being analyzed; hence it may be impossible or unwise to take samples of fluid larger than 1 millimicroliter. In physiological studies of renal tubular function, for example, samples of this order are obtained, by means of micropuncture techniques, for several kinds of analyses as well as for the freezing-point determinations. The accuracy of the other chemical and physical determinations made during such studies demands that measurement of osmotic pressure be accurate to within a few milliosmoles (*1*).

With current techniques (*2*) the freezing points of individual, micro samples are determined by observing, on an expanded-scale thermometer, the temperature of a regulated bath as the melting of the last ice crystal in the sample is observed through a microscope. With such methods it is necessary to approach the end point slowly to prevent the building up of thermal gradients between the thermometer, the bath, and the sample. Gradients which contribute less than the acceptable error can be tolerated, but strict adherence to laboratory procedure is required to obtain high accuracy.

The method outlined here was designed for handling multiple samples and standards identically and simultaneously so as to decrease the time required per determination, to achieve greater accuracy through close interpolation of unknowns between standards, and to eliminate the necessity of bringing a large bath to thermal equilibrium. In this method, the values obtained for unknown solutions are interpolated between the values for standard solutions maintained in the same environment, so that the accuracy of the values for the standard solutions determines the accuracy of the calibration curve. Equilibrium between the temperature of the sample and the temperature of the control system is obtained by tight thermal control of the sample stage of the instrument. In this way the end point can be approached rapidly, and the temperature can be controlled to make the final ice crystal of a sample shrink, grow, or remain constant in size in instantaneous response to a change of setting of a few divisions on a 1000-division potentiometer.

The instrument is an electronically controlled thermoelectric cooler capable of controlling the temperature of several samples of millimicroliter quantity to within 0.001°C and small enough to be placed in the substage region of a microscope. The cooler utilizes semiconductor thermoelectric