Potential for Clinical Use of the Analytical Laser Microprobe for Element Measurement

David Glick¹ and Kenneth W. Marich²

Use of the laser microprobe for rapid emission spectroscopic analysis of elements in microscopic samples of biological material is described. The technique depends on vaporization of the microsample with a focused laser beam at a temperature that renders the vapor incandescent for spectrochemical analysis. Spectral line intensities are recorded photographically with densitometry of the negatives or photoelectrically. Current capability permits analysis of about $10^{-8}$ to $10^{-10}$ g of tissue, with detection limits of $10^{-12}$ to $10^{-15}$ g of element. Groups of elements can be simultaneously analyzed. Minimum sample preparation is required, and the analysis is done on the stage of a light microscope, usually on an air-dried sample on a plastic slide. We exemplify the technique in analysis of gold in cultured fibroblasts treated with gold salts and in human skin after treatment with gold salts for rheumatoid arthritis, in element changes in biopsies of transplanted human hearts, and in unique profiles of groups of elements in human cancer tissue.

Additional Keyphrases: trace elements • gold in cells • cancer diagnosis • picotrace analysis • heart transplantation

Development of the laser microprobe for detection and measurement of elements in morphologically defined microscopic samples, by means of emission spectroscopy, has proceeded in our laboratory for over 10 years. Equipment that was commercially available earlier was redesigned and rebuilt to introduce new features required, to study certain fundamental properties of laser beam-induced incandescent vapors or "plasmas" of samples, to define analytical variables and sensitivity, and to demonstrate areas of application.

As with all instrumentation and technology, further development will continue, but present attainments permit certain applications that will be discussed here and the general potentialities of the approach will be indicated.

Principles and Instrumentation

Principles. The analysis is based on the use of emission spectroscopy, which depends on vaporization and atomic excitation of a selected sample on a microscope stage by a Q-switched ruby laser pulse sent through the microscope, transmission of light from the incandescent vapor of the sample to a spectrograph, and photographic or photoelectric recording of the intensities of the spectral lines of chosen elements in the sample.

The photographic technique provides a record of the spectral lines of many elements in the same sample, and, for quantitative work, intensities of selected lines in the photograph are measured densitometrically. This method is particularly useful for preliminary screening work.

The photoelectric method gives a direct and more rapid and sensitive measure of spectral line intensities for analysis of a group of elements in the sample, but it is limited by the number of photomultiplier tubes that can be mounted on the spectrograph. Each tube is mounted at the position of a selected spectral line with a control tube to measure background. With our present equipment, five elements and the background can be measured simultaneously.

Background work. The first unit designed, constructed, and tested in our program was described and discussed in 1968 (1). From experience gained during the subsequent three years, improvements were incorporated into our second unit (2) (Figures 1, 2). Essentially, this unit operates by (a) generation of a single giant laser pulse ("Q-switched") in the laser system...
Fig. 1. Laser microprobe apparatus
P, six-channel polychromator; S, emission spectrometer; RL, retro-alignment laser; R, retro-alignment optics; CO, lens coupling optics; M, binocular microscope; TV, television camera; LM, calibrated laser monitor; BS, laser beam splitter. From Marich et al. (2)

Fig. 2. Diagram of laser microprobe apparatus (solid lines indicate components that have been changed or improved)
BP, Brewster angle roof prism; Q, liquid Q-switch; R/FL, ruby and flashtube; C, air cooling port; T, transverse mode selector; E, two element sapphire etalon reflector; L, liquid attenuator; RP, right angle prism; BS, beam splitter prism; LM, laser monitor; A, microscope and TV viewing system; PL, optical by emitting plasma; CO, coupling optics (mirrors or lens); S, spectrometer; P, six-channel polychromator; E', photoelectric detection electronics; and RL, He/Ne retro-alignment laser. From Marich et al. (2)

Characteristics of various types of coupling optics have been discussed before, and the two systems adopted have particular advantages (2). The first, corrected for spherical aberration and constructed specifically for use with plasmas <1 mm high, consists of an f/1.5 quartz collecting and collimating lens mounted on a calibrated rack and pinion that transmits the light to a 85-mm quartz photographic lens that, in turn, focuses a 4X-magnified image of the plasma onto the entrance slit of the spectrometer. The second, free of astigmatism and chromatic aberration, consists of an off-axis, over-and-under dual-mirror system in a Z configuration, which is used at unity magnification.

Among the special features in the analytical system is the use of photoelectric time differentiation (gating) that, in effect, separates specific spectral emission from nonspecific background continuum (3). This differentiation of the signal depends on the fact that an initial large nonspecific emission occurs during the period of the laser pulse that rapidly decreases, while the specific emission decreases more slowly. The ratio of the specific to nonspecific signal increases to a maximum value at a delay time depending on the laser energy, the particular element, and the nature of the sample itself (Figure 3). The duration of the Q-switched laser pulse is usually <100 ns, and the predominant nonspecific emission occurs within this time, while the light-emitting plasma continues for some microseconds. The photoelectric time differentiation exploits these differences to

Cavity (BP, Q, R/FL, T, E, Figure 2); (b) reflection of the laser beam by a prism (RP) to a beam splitter (BS) that sends approximately 25% of the light to a monitor (LM) to record the energy of the pulse and 75% to a microscope (M), where the beam is focused on the area of the specimen to be analyzed, which has been placed in the optical axis of the microscope; (c) collection of the light from the incandescent plasma of the sample (PL) by coupling optics (CO) and its transmission into the 0.75-m Czerny-Turner (Model 78–490, Jarrell-Ash) spectrograph (S); and, finally, (d) recording of spectral line intensities (P, E').

Ancillary devices shown in Figure 1 are a television camera (TV) and screen for viewing the sample on the microscope stage, a Vicker's binocular microscope (M) equipped for automatic photomicrography, and a retroalignment He/Ne laser (R) for simplified optical alignment of the laser-induced plasma with the spectrometer.
achieve an improvement in the analytical performance such that an increase of the ratio of the specific to nonspecific signal can approach 2000%, with reduction in the specific signal of only about 40%. An integrated measurement is taken to obtain the optimal useful signal.

Another special feature is the six-channel polychromator, which is arranged to house six shielded photomultiplier tubes (C31005C, RCA) on the spectrograph. Each of these tubes is furnished with a unique wedge-shaped light guide, the design of which is included in the description (2) of the improved apparatus (Figure 4). The light guide, an aluminum pipe, diamond-polished internally, collects light from a selected spectral line through a 0.15 × 20 mm slit located in the focal plane of the spectrograph and internally reflects it onto the photomultiplier tube. At 300 nm, about 80% of the light is transmitted. The position of each photomultiplier tube is adjustable laterally to permit setting for different spectral lines. This entire polychromator unit was designed and built by Helen Gustafson, Systems Sciences Group, San Francisco, Calif. Earlier, a two-channel polychromator in which mirrors are used for light reflection to the multiplier tubes had been designed and constructed (4).

Intensification of spectral emission to gain greater sensitivity by the use of electric spark cross-excitation is achieved by positioning charged electrodes above the sample so that the plasma formed between them triggers a spark and excites the atoms in the plasma to yield even greater emission. This technique was refined for sampling in the range of 10- to 25-μm diameters (5) from previous practice employing 50- to 250-μm diameters. However, considerable variability in the sampling, inherent in this technique because of indeterminate burning and charring of the periphery of the sample crater, led us to abandon it. This placed greater demands on the sensitivity of measurement, which were met to some extent by increasing the efficiency of the optical coupling between the plasma and spectrometer and by use of time differentiation in signal recording.

During the evolution of the instrumentation and from the experience gained, it became evident that several safety features should be built into the equipment, to protect the instruments and the operator. Thus protection of photomultiplier tubes from accidental light exposure, and of the substage condenser on the microscope from the focused laser beam, was afforded by suitable electronic interlock switches. The eyes of the operator, when viewing specimens under the microscope, were protected from inadvertent pulsing of the laser by an automatically positioned metal plate barrier (2).

Performance

A particular advantage of this technique is that sample preparation is minimal; an air-dried or freeze-dried cell smear or tissue section on a plastic slide placed on the stage of the microscope is all the preparation required for analysis. (Plastic slides are used because the elements in glass interfere with the analysis.) No element loss occurs in this preparation, and the in vivo element localization is essentially unchanged. Present instrumentation permits rapid simultaneous analyses of a group of elements in 10⁻⁸ to 10⁻¹⁰ g of biological material with detection limits of 10⁻¹² to 10⁻¹⁵ g of element.

Over a range of defined conditions, the diameter of the area sampled can be varied by adjusting the energy output of the laser source, e.g., by use of the CuSO₄ attenuator (A, Figure 1), by changing the size of the aperture in the transverse mode selector (T, Figure 1), or by changing the magnification of the microscope objective. With the first instrument constructed in this program (1), a linear relationship was found over a sampling diameter of 5 to 50 μm with energy delivered from the laser source of 5 to 35 μJ.

Reproducibility of laser sampling depends on the homogeneity and stability of the laser beam and composition of the sample. Coefficients of variation ranging from 5.5 to 8.1% were observed with the improved instrument, for Q-switched single laser pulses ranging in energy from 0.5 to 26 mJ per pulse. This reproducibility is greater than that obtained with comparable instruments in other laboratories, which found coefficients of variation over 10% (6, 7).

Analytical detection limits depend on: (a) spectral emission properties of the element; (b) the matrix material of the sample in which the element occurs; (c) the atmosphere in which the sampling occurs; (d) energy of the sampling laser beam; and (e) the efficiency of the instrumentation by which the light is collected, spectrally dispersed, and its intensity recorded. We have studied these factors and investigated their effects on performance.

The magnitude of the suppression of spectral emission of metallic elements by the matrix material of the sample can be considerable, as demonstrated in a
study of model systems containing organic and inorganic matter (8). For each kind of matrix and each emitting element, a threshold concentration of matrix exists, above which emission decreases. However, the useful signal obtained is still adequate for analytical purposes.

The influence of the atmosphere in which the plasma is generated on spectral emission was investigated using argon, helium, nitrogen, air, oxygen, and vacuum (9). Although some gain in signal-to-background ratio was observed with certain atmospheres or vacuum, these varied with laser energy in an irregular way, and it was concluded that, for most work, the gain was not sufficient to merit the inconvenience in changing from air to a controlled atmosphere.

The influences of the energy of the sampling laser beam, and of the instrumentation used, on analytical sensitivity have been considered in a number of our publications, but a special study was made of the detection limits of a series of elements in albumin or gelatin matrices under various conditions of time differentiation to derive optimal sensitivities of measurement (Table 1, 10).

Applications and Discussion

Relatively few biomedical applications of the analytical laser microprobe have been made to date, but to indicate the capability of the technique, examples from investigations in our laboratory will be given.

Gold Uptake by Mouse Fibroblasts

Gold thioglucose treatment of mice provides a model for observation of cellular effects and clinical consequences. The treatment has been found to produce hyperphagia, hypothalamic lesions, and obesity in the animals, and it has been used to study hunger mechanisms (refs. cited in 11).

The effects of gold thioglucose on cultured mouse strain L fibroblasts were studied by light and electron microscopy, histochemical staining, and laser microprobe analysis (11). Monolayer cell cultures were grown on plastic cover slips, fixed in buffered (pH 6.9) glutaraldehyde, rinsed in double-distilled water, and air-dried. Single cells were analyzed for gold with the laser microprobe, and their gold uptake was found to be a function of the gold concentration in the medium and of the incubation time, as would be expected. A gold content of >2.5 pg/cell was required to produce demonstrable cytopathic effects (Table 2).

Morphological changes accompanying the gold uptake were an increase in the numbers of microfilaments, nuclear pleomorphism, and numerous dense electron-opaque particles, presumably gold, in the lysosomes. The untreated control cells were practically devoid of such electron-opaque particles.

Gold in Human Skin Biopsy Tissue

In a direct clinical application, the laser microprobe was used to identify gold deposits in the skin of patients who had received gold-salt injections for treatment of rheumatoid arthritis (12). Of the six patients studied, two had not had the injections for at least 20 years but, characteristic of the gold treatment, all showed lesions with an increased number of elongated cells, apparently fibroblasts or histiocytes, in the upper and mid-dermis.
Biopsies from these areas were taken for laser microprobe analysis and morphological examination by light and electron microscopy. Formalin-fixed, paraffin-embedded sections, 20 μm thick, were deparaffinized and used for the gold analysis, and sections were also prepared from the same specimen for routine morphological study.

Gold was identified in dermal lysosomes as dense electron-opaque granules up to about 1 μm in diameter (Figure 5), and gold was not demonstrable in areas without the granules.

Elements in Human Heart Biopsy Tissue

A rather different clinical application of the laser microprobe that we have explored is its use for element analysis in evaluation of pathological changes in the human endomyocardium. In a preliminary study, the changes were followed after heart transplantation performed by Dr. Norman E. Shumway and members of his surgical team at the Stanford University Medical Center. Our work was done in collaboration with Dr. Margaret E. Billingham, who did the histopathological studies. The biopsy technique was developed and applied by Dr. Philip K. Caves and coworkers in the Department of Cardiovascular Surgery (13, 14).

Essentially, the technique depends on passing a clamp-form of tissue sampler on the end of a fine cable through a catheter previously passed through the internal jugular vein into the apex of the right ventricle. The clamp is made to bite out a small piece of the endomyocardium with an externally operated hand device. The entire procedure is done under fluoroscopic control. The operation is relatively simple and produces minimal discomfort. It requires 15–20 min, and is usually performed as an outpatient procedure. It has now been used more than 350 times and it has been repeated without complications over a period of months on the same patients.

The laser microprobe was used for the rapid, simultaneous analysis of a battery of elements with the small (1 × 1 × 2 mm) tissue specimens from which fresh-frozen sections, 40 μm thick, were prepared, dried on plastic microscope slides and analyzed. The data (Figure 6) show significant changes in Na, K, Ca, and Cu in relation to the postoperative course of a heart transplant patient suffering rejection. Additional elements could be included in the simultaneous analyses.

More extensive investigations should be made, including comparison of these data with those of the corresponding heart tissue from autopsy cases with normal hearts. Because the data presented are primarily to show the feasibility of undertaking such work, these data were limited to the demonstration that the element changes could be correlated with the pathological events. It is interesting to note that the changes in Na, K, and Cu were parallel over the period from early rejection through the time of no evident specific abnormality to the first acute rejection episode, while, by comparison, the Ca changed inversely. However, from the first to the second state of acute rejection, and on to the second state of no evident specific abnormality, the changes in Ca and Na were parallel and the inverse of those of the K and Cu. There was little change in the Na/K ratio until after the first acute rejection; it was inverted at the time of the second acute rejection, finally approaching unity at the second state of no specific abnormality. The significance of these changes is not apparent now, but their importance should emerge as additional relevant data are obtained.

The foregoing illustrates how the microprobe technique can be employed to measure changes in elements and their ratios in biopsies in relation to physiological and pathological sequelae. After heart transplantation or in various heart diseases, changes that may occur in cell permeability as a result of ischemia, loss of nerve function, and the like may be assessed and perhaps may provide early signs of pathological change.

Element Changes in Tissues in Human Cancer

In another clinical application of the laser microprobe we made a preliminary study of whether certain elements in microscopically defined samples of malignant tissues occur in relative concentrations that are significantly different from those in the corresponding normal tissues, and, if so, whether the el-
Element pattern is specific for tumor type and the organ in which the tumor occurs. The histopathological studies involved were conducted by Dr. Klaus Hou-Jensen, and technical assistance in the microprobe analysis was given by James G. Hawley, David A. Swisher, and John P. Ertel.

Relatively few studies of differences in element content that might characterize human cancer tissue have been reported (e.g., 15, 16), and these have not provided fully definitive data. One of the reasons for this was inadequate morphological definition of the specimens analyzed, because the methods used required relatively gross samples that included indeterminate proportions of noncancerous material along with the cancer cells. Nevertheless, the data have been encouraging, indicating that significant differences probably do exist, even though the proof remains to be more thoroughly established.

Data of element patterns in liver, lung, and colon tumors with reference to nonmalignant tissue in the same organ are presented in Figures 7–10. Figure 7 shows that, of the eight elements analyzed, the significant increases in Na and Ca and the decreases in Fe and Zn appear to be consistent changes in liver cancer. Differentiation of carcinoma from sarcoma based on P and Cu values may be possible, but more data are needed.

The changes in Na, Ca, Fe, and Zn in cases of liver adenocarcinoma and oat cell carcinoma are demonstrated in Figure 8 and, in addition, significant differences between these cancers in Al and P are shown. The element patterns found in cancers of the lung (Figure 9) and colon (Figure 10) are very different from the patterns found in the liver. In lung can-

Fig. 7. Element profile change in human liver cancer
Twelve analyses each in tumor and in normal part of liver per specimen per patient. P values = significance of change, tumor vs. normal.

Fig. 8. Element profile change in human liver carcinomas
Designations as in Figure 7.

Fig. 9. Element profile change in human lung carcinoma
Designations as in Figure 7.

Fig. 10. Element profile change in human colon adenocarcinoma
Designations as in Figure 7.
cancer, Na and Zn do not change compared with normal tissue, and the change in Ca is a decrease rather than the increase seen in liver cancer. Data in Figure 9 raise the possibility of differentiating primary from metastatic lung tumors. In colon cancer, the Na decrease also contrasts with the increase in liver cancer and the lack of any change from the normal in lung cancer. However, the colon cancer shows the same Ca, Fe, and Zn pattern as the lung cancer.

While the reasons remain to be established for these differences in the elements in the cancer, compared to the normal tissue, and in the specificity of some of the element profiles with regard to organ and tumor type, consistencies that have emerged in these data indicate potentials both for increased understanding of the biochemical changes in malignancy and for a diagnostic approach.

The foregoing data illustrating examples of clinical application indicate the clinical usefulness and promise of the analytical laser microprobe.

This work was supported by Stanford Research Institute, and in part by PHS Research Grant GM16181 and Career Award 5K6AM18,513 (to D.G.) from the NIH, USPHS.

References