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DONNER LABORATORY

September 1977

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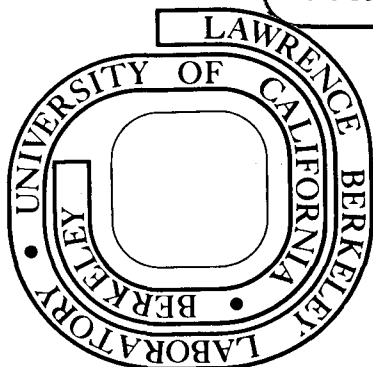
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Pulmonary Macrophage and Epithelial Cells

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Visualization of Fly-Ash Particles in Macrophages Using Color X-Ray Mapping

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ABSTRACT

A new method is described for applying color X-ray mapping to the microanalysis of coal fly ash both before and after ingestion of the fly ash by mouse pulmonary alveolar macrophages. The system uses a scanning electron microscope with energy dispersive X-ray spectrometer and a color television monitor to produce simultaneous X-ray maps of three elements. Because higher average real-time count rates are possible when the sample is scanned at television rate, three element maps of fields containing 100 to 200 particles can be produced in 1 to 5 min, and thus the system is valuable as a screening tool. Particles having cross sections as small as 0.5% of the scanned area can be detected, provided that the element being mapped constitutes at least 10 to 20% of the mass of the particles. Particles inside critical point dried pulmonary alveolar macrophages, previously exposed to respirable polydisperse stack fly ash in vitro, have been mapped successfully.

As the energy industry increases its reliance on coal, there has been a renewed interest in the ultimate fate and possible biological effects of the by-products of coal production and combustion.¹⁻⁴

In 1974, 6×10^8 tons of coal were mined in the United States and, after combustion, 11% of this was ash. In modern utility boilers fired with pulverized coal, 80% of the ash is converted to fly ash. Most of this is removed from the flue gas by electrostatic precipitators or other collection devices, but approximately 10^6 tons is probably released to the atmosphere. The material most likely to escape the precipitators is small [mass median diameter (MMD) $< 5 \mu\text{m}$] and this prolongs its residence time in the atmosphere and also

increases the probability that, once inhaled, the material will be deposited in the lung.⁵ Furthermore, some of the trace elements that are known to be biologically harmful seem to be preferentially concentrated in or on smaller particles.⁶

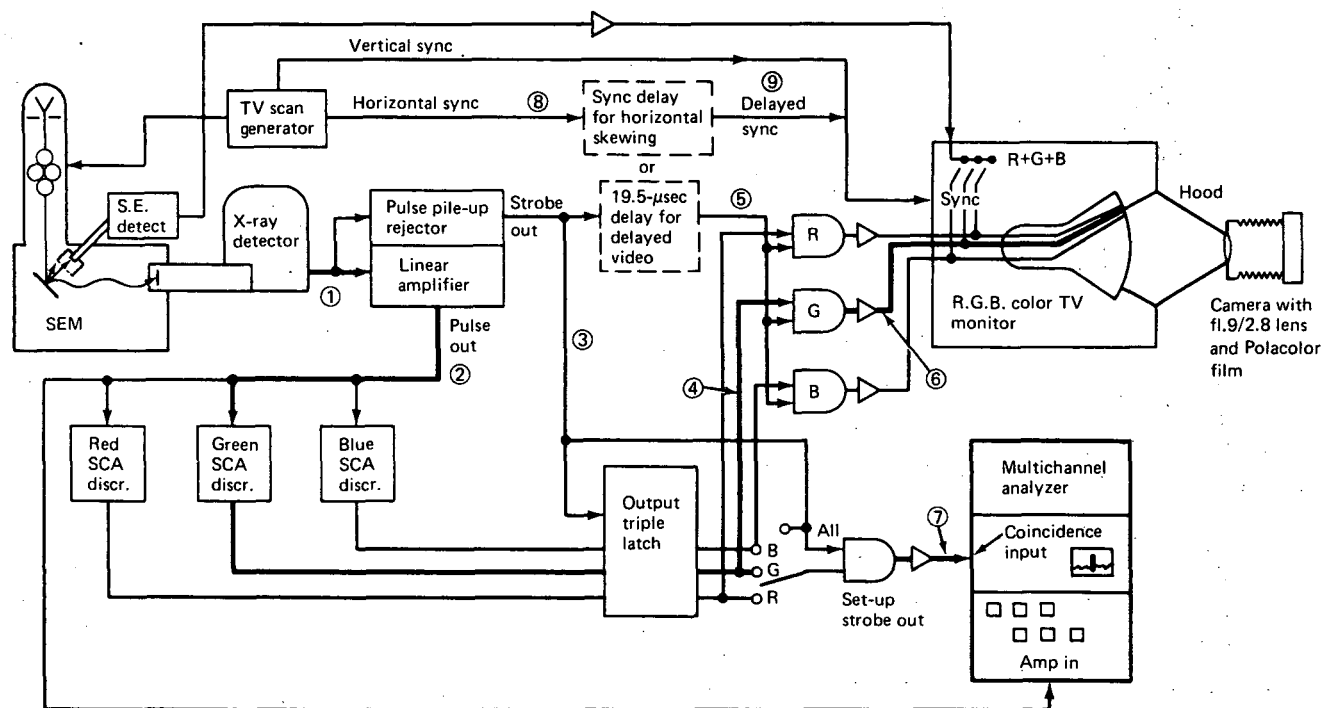
The massive quantity of this material produced must not blind us to the fact that most biological effects will be produced at the cellular level by a small number of particles weighing a few picograms. Because these particles are too small for careful study in the light microscope, the study of fine particulates on a particle-by-particle basis requires such microtechniques as scanning and transmission electron microscopy and electron microprobe analysis. Obtaining information on elemental composition using the microprobe is complicated by the difficulty of obtaining data on a statistically significant number of particles either in their free state or after their interaction with biological targets, such as the pulmonary macrophage.

This paper describes an optimized method of color X-ray mapping which we hope can help alleviate this problem by permitting a large number of particles to be screened for the presence of up to three elements simultaneously. This method differs from previous methods of color X-ray mapping^{7,8} because it is 10 to 30 times more rapid and requires minimal operator involvement.

METHODS

Since a preliminary version of this system has been described⁹ and a more complete description containing examples of colored X-ray maps will soon be available,¹⁰ only a brief outline of the operation of the system is included here.

The sample is scanned at television rate in a scanning electron microscope (SEM) using a current sufficient to produce the maximum possible average real-time count rate from an attached dispersive X-ray spectrometer detector (about 3000 cps for a detector with a linear amplifier time constant, $t_c = 8$ to $10 \mu\text{sec}$). The output of the linear amplifier is applied to three single-channel analyzers [Fig. 1(a)] so arranged that a high logic signal is passed to one of three latches whenever the linear amplifier signal is in that window. A strobe pulse is produced by the linear amplifier at the instant that the linear output reaches a peak, which is exactly $44 \mu\text{sec}$ after the X ray is detected (t_0). This strobe pulse triggers the latches so that their outputs continue to mirror the state of their inputs at the instant that the strobe pulse arrives. The latch outputs are each fed to one input of an AND gate. The strobe pulse is also



(a)

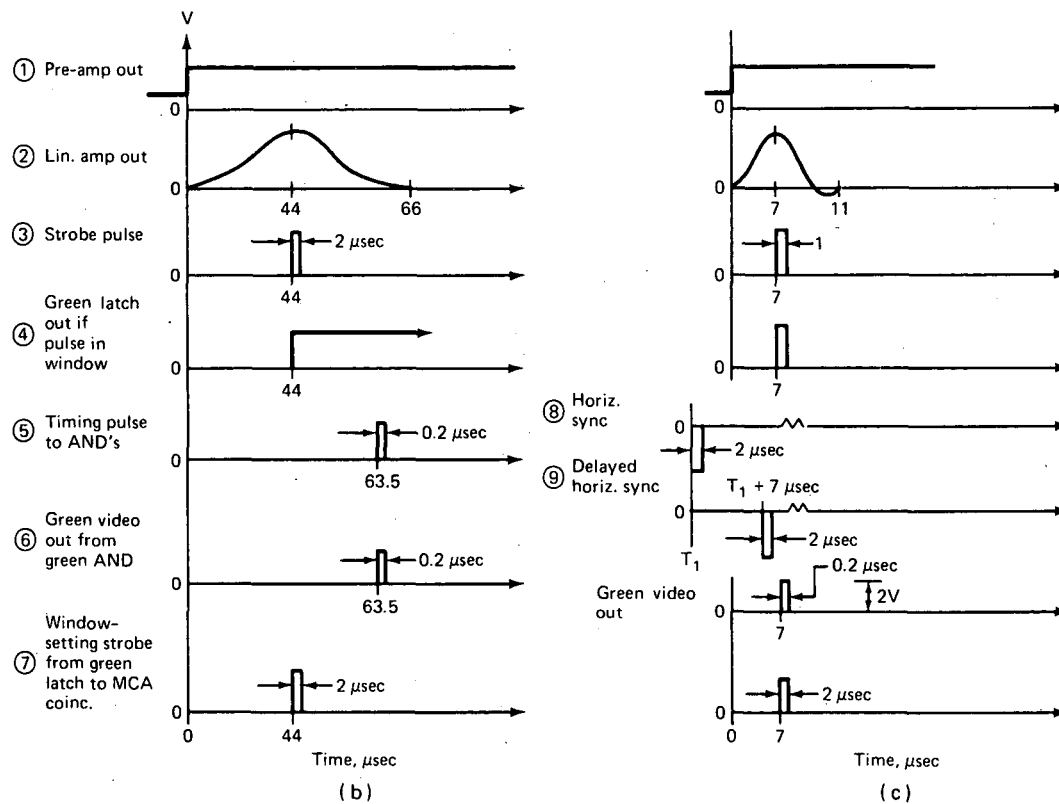


Fig. 1 Block diagram of simultaneous three-element color X-ray mapping system. Signals present in the system, when used in delayed video mode or horizontal scan skewing mode, are diagrammed in parts (b) and (c), respectively.

used to produce a delayed video pulse 0.2 μsec wide and delayed by exactly 19.5 μsec , which is applied to the second input of all three AND gates. These gates will therefore produce a 0.2- μsec pulse a total of $44 + 19.5 = 63.5$ μsec after t_0 only if the linear-amplifier output pulse peak was in the appropriate window. After this pulse is buffered, it is passed to one input of a red-blue-green color television monitor, where it is displayed as a dot of a particular primary color. This dot is then recorded on instant process color film. The delay circuitry is necessary because the time between t_0 and the time that the linear amplifier output can be analyzed (44 μsec) is long compared to television horizontal sweep rate (63.5 $\mu\text{sec}/\text{line}$). The delay procedure has the effect of displaying an X-ray count exactly one line below the point from which it is detected and is called delayed video. The pulses referred to are shown in Fig. 1(b); the circled numbers indicate the parts of the schematic diagram where each pulse will be found.

Delayed video produces a display dead time of 63.5 μsec , which is comparable to the dead time of an amplifier having a $t_c = 8$ μsec . If a linear amplifier having $t_c < 8$ μsec is used to increase the maximum real-time count rate at some loss in energy resolution, a different system may be used to compensate for the delay between t_0 and the linear amplifier peak. This second method is called horizontal scan skewing, and the relevant pulses are shown in Fig. 1(c). The video pulses are not delayed but are passed on immediately after the strobe pulse arrives. Registration is preserved by delaying the horizontal sync pulses sent to the monitor so that the display beam lags the probing beam by the correct amount.

A signal composed of the output of a switch-selected latch ANDed with the strobe pulse is passed to an attached multichannel analyzer (MCA) used in the coincidence mode. This allows a spectrum composed of only the counts in a particular window to be generated on the MCA, and thus the window is easy to set.

The samples used to prepare the maps shown during the symposium, which are not included here because publishing considerations do not permit the reproduction of figures in color, consist of NBS No. SRM-1633* coal fly ash, size-classified stack fly ash, or mouse pulmonary alveolar macrophages exposed to the stack fly ash. The NBS fly ash was deposited onto smooth carbon SEM stubs and coated with carbon by vacuum evaporation. A fine fraction

*National Bureau of Standards, Standard Reference Material, No. 1633, *Trace Elements in Coal Fly Ash*.

(MMD = 2.2 μm) stack fly ash¹ was deposited on Formvar-coated copper EM grids to reduce the bremsstrahlung production in the substrate. The mouse pulmonary alveolar macrophages were obtained by lung lavage and exposed to stack fly ash of 2.2- μm MMD by a modification of the method detailed elsewhere in this volume.^{1,2} After exposure, drops of cell suspension were placed on Formvar-coated EM grids that had first been dipped in 0.1% poly-lysine solution^{1,3} and then blotted dry. The poly-lysine made the cells adhere to the Formvar while the grids were first fixed in cacodylate-buffered glutaraldehyde-formaldehyde and then dehydrated in ethanol before being critical point dried using amyl acetate and liquid CO_2 . After the grids were dried, they were coated with evaporated carbon.

With a 25-kV beam voltage, we recorded maps and corresponding secondary electron images from the television monitor. Exposure times were 3 to 10 min, and 5×10^4 to 3×10^5 counts were recorded at magnifications of 2000 to 5000x (on the print). Elements mapped during the initial studies reported here included aluminum, sulfur, potassium, calcium, titanium, and iron, although future studies will map elements known to have deleterious biological effects, such as cadmium, selenium, arsenic, and lead.

RESULTS AND DISCUSSION

The results showed the method to be effective in producing images in 1 to 5 min which were sufficient to detect particles having high concentrations of a mapped element in fields containing up to 200 particles. The samples of NBS and fine stack fly ash proved to be very heterogeneous, and several maps showed that virtually all of a particular element in a field of over 100 particles was concentrated in a single particle. Conclusions based on such maps regarding the elemental composition of individual particles were verified by spot-mode analysis of the particles involved. Fine particles containing high concentrations of iron, calcium, or titanium which had been phagocytized by pulmonary alveolar macrophages could be clearly visualized, particularly if they were near the cell surface. One map included a part of a copper EM grid in the field of view, and, although this gave rise to a perceptible increase in the background level, this increased background level is in no way comparable to the intensities produced by the intracellular particles.

Our studies are just beginning, but we hope to use this tool to discover whether trace elements of biological interest, such as cadmium, selenium, arsenic, zinc, and lead, are distributed nonuni-

formly, as are the elements mapped in this study. This knowledge will permit a better estimation of the biological toxicity of such particles. It may also provide a test for one theoretical explanation for the predominance of such elements in smaller particles because, if small particles show the increased concentration simply because they provide a greater specific surface area on which volatile elements can condense as the flue gases cool,^{6,14} then we would not expect large variations in concentration between particles of the same size. Finally, by correlating information on the elemental composition of particles within pulmonary alveolar macrophages with the morphological appearance of the cells after various periods of incubation, we hope to determine differential uptake rates and toxicity on a cell-by-cell basis.

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