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"INVESTIGATIONS RELATED TO
EVALUATION OF ULTRAMICROFLUOROMETER"

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I. INTRODUCTION

During 1980, the viral Monitoring instrument was moved to the Desert Research Institute's (DRI) Stead location from the Ames Research Center so that DRI personnel could work with the instrument more efficiently. The desirability of several mechanical modifications became apparent and during the course of the year many of those modifications were implemented. High resolution emission and excitation fluorescent spectra were obtained for several samples in an effort to determine the optimum operational design for the instrument. The instrument was used to determine the required nature of a sample which could be detected, and in so doing, several different sample preparation techniques were considered. Numerous experiments were performed to determine the capabilities of the instrument with regard to the detection of suitably prepared virus specimens.

Significant results were obtained in several areas. The fluorescent spectra indicated that substantial changes in the laser might be used advantageously to greatly improve the performance of the instrument. In the existing configuration, the instrument was shown to be capable of detecting the presence of suitably prepared virus samples.

II. MECHANICAL MODIFICATIONS

The instrument was installed in the Laser Laboratory at the Atmospheric Science Center of the DRI, at the Stead Facility north of Reno. Several mechanical modifications were made to the instrument in order to improve the ease and consistency of working with the instrument.

A new "fast flush" drain line was added. This line is not intended for continuous use, but substantially reduces the time required to flush the system by allowing it to be quickly drained. During routine operation of the instrument this line is kept closed and is only opened for the brief time required to drain the sample chamber.

The horizontal position of the sample column is determined by means of two micrometers. The micrometers originally supplied with the instrument were of low resolution, the minimum increment being 0.01 mm. Under optimum operating conditions, the cross section of the sample column is typically 10 μm, and this column must be centered in a laser beam which is focussed to 25 μm and simultaneously centered in slits which are about 10 μm in aperture. Thus, resolution of the same order of magnitude as the required positioning precision made that positioning difficult. The micrometers were replaced with dual resolution micrometers from Newport Research Corp. The new micrometers have coarse adjustment of 0.01 mm and fine adjustment in increments of 0.0005 mm which is easily sufficient precision for positioning the sample core.

The laser beam focussing mount was substantially modified (see Fig. 1). It was found that one of the two horizontal adjustment knobs

was virtually inaccessible without blocking the laser beam, which made focussing difficult. A means was devised of mechanically connecting the knob to a more accessible control, thus allowing the required adjustment without blocking the incident laser beam. Further it was discovered that one of the principle problems incurred in the focussing of the laser beam was that the lens frequently was not perpendicular to the incident laser beam. This problem derives from the fact that the beam steering mirrors are not mechanically coupled in such a way that the beam is steered parallel to the original beam. Thus, the laser beam is incident on the lens at an angle which varies slightly each time the beam is realigned. A temporary remedy was effected by modifying the lens mount slightly to incorporate a small amount of angular rotation to the base of the mount. This solution is not ideal since the lens is not centered over the axis of rotation, but a preferred solution was not feasible due to severe limitations of available space.

A laser safety cage was made to eliminate the possibility of accidental observation of the laser beam. The beam is now totally enclosed from its exit from the laser until it must be exposed to provide access to the positioning controls. In the small region where it is exposed, it is only with conscious effort that one could observe reflections of strength greater than those due to aerosol scattering.

The tubing throughout the low pressure hydraulic system is not normally secured at its connections. Following extensive operation, the tubing begins to fatigue at some of those connections and has exhibited a tendency to leak. In those connections where leaks have

occurred, the connections have been replaced with a syringe-type quick connect in which the tubing can be secured to the connector by a very small clamp or by wiring. In those places where the connections have been so modified, there has been no further evidence of leakage.

It was determined that the principal reason for frequent breakage of the glass capillary was that during flush sequences, the fluid flow is not coaxial with the capillary, thus snapping the extended portion of it. To minimize this effect, a brass sheath was designed to protect all but the last 5 mm of the tip (see Figure 2). The presence of the sheath has virtually eliminated capillary breakage. The presence of the sheath does not affect the quality of the laminar flow surrounding the sample core.

The overall result of the preceeding changes has been the simplification of the daily operation of the instrument, less time spent in repetitive repair, and increased consistency in the aligning and positioning of the sample core and the laser beam.

III. SAMPLE PREPARATION STUDIES

During 1979, extensive testing was performed in an effort to optimize the specimen which would be used in the instrument. Some of the experimental techniques which were considered in those studies are shown in Table I, together with representative results which indicate the relative effectiveness of each potential protocol. During the early part of 1980, the principal effort of the project was making the instrument consistently operational by making the mechanical modifications as described previously.

One the modifications had been completed, samples prepared using the carbonate-bicarbonate buffer technique were tested. A major problem involved removing most, or preferably all of the unbound ethidium bromide from the sample solution with little or no loss of that which had been intercalated. Another problem which was not readily answerable dealt with the actual conditon of the sample: it is suspected that the procedures which seem optimum for the preparation of bulk sample (Table I) may not be optimum for samples to be measured in the fluorometer. Samples prepared by the carbonate-bicarbonate procedure probably show the gratest enhancement because there is the greatest degree of EB intercalation. The technique by which this takes place is likely the following. The carbonate-bicarbonate buffer is a strongly basic solution which seriously weakens the virus-encapsulating protein shell. Some DNA escapes through cracks in the weakened shell, thus providing very ready access for intercalation. In a bulk sample, it is

TABLE 1

SAMPLE TREATMENT TECHNIQUES & RESULTS

TREATMENT	FLUORESCENCE OUTPUT EB ONLY	EB & VIRUS TREATED	ENHANCEMENT
*IN DISTILLED WATER			
No treatment	3.5	4.0	14%
Heat (70^{o}C., 10 min.)	3.7	4.3	16%
Heat (100^{o}C., 60 min.)	3.5	4.2	20%
Sonicate	3.5	3.7	6%
Autoclave	3.5	4.2	20%
Cold (-70^{o}C.)	3.5	3.7	6%
*IN PHOSPHATE BUFFER (pH 7.4)			
No treatment	3.5	4.9	40%
+ $HClO_4$ (Oxidation)	3.5	4.5	29%
+ SDS (Detergent)	3.5	5.6	43%
*IN GLYCINE-HCL BUFFER (pH 3.0)			
No treatment	3.5	3.6	3%
*IN CARBONATE-BICARBONATE BUFFER (pH 10.4)			
No treatment	4.7	7.7	64%

*FURTHER EXPERIMENTS

Titrate EB vs. Constant Virus Concentration

Enhancement quenched at $\geq$ 5 µg/ml

Enhancement $\propto$ EB concentration at $\leq$ 1 µg/ml

Titrate (EB + T2)

% enhancement constant; signal reduced $\propto$ concentration

Titrate Pure DNA vs. Constant EB Concentration

Anomalous results

Dialysis

Intercalated dye reduced in direct proportion to free dye concentration

impossible to distinguish between fluorescence from EB intercalated into whole viruses and that intercalated into escaped pieces of DNA. It is crucial in the microfluorometer, however, that the virus be intact; otherwise, extraneous signals will result. The extent to which the virus is broken by the carbonate-bicarbonate buffer preparation technique can only be determined by high resolution electron-microscopy.

It was found that following centrifugation of the sample and removal of the solvent, the background observed from the resuspended sample was tolerably small, and signal believed due to the virus specimens was observed. These results will be discussed further in later sections of the report.

IV. SPECTROFLUOROSCOPIC STUDIES

The initial spectrofluoroscopic studies were used as a tool to indicate the extent to which the ethidium bromide was intercalated into the viral nucleic acid. These studies provided low spectral resolution, but were quite adequate for the purpose since the characteristics of ethidium bromide are well known. EB is known to be a nucleic acid-specific fluorescent dye which when bound to nucleic acid, exhibits substantially enhanced fluorescence intensity compared to that of its unbound state. Thus, low resolution studies were used to indicate the extent to which different virus preparation techniques have exposed the viral nucleic acid to EB binding.

Uncertainty over the possibility of spectral changes associated with EB intercalation suggested a need for high spectral resolution fluorescence studies. A high resolution spectrofluorometer was made available through the cooperation of Dr. Hubert Caspars of the Naval Oceans Systems Center (NOSC) in San Diego, CA. For the purposes of these studies, eleven samples were prepared. Figures 3 and 4 show the results obtained for five of the samples. The composition of the five samples for which fluorescence curves are shown is given in Table II. These five samples are shown since they demonstrate the extremes in EB intercalation (Sample 1, no DNA, to Sample 5, very high intercalation) and several intermediate degrees.

Figure 3 shows the fluorescence emitted by the five samples. The emission curves were determined with the exciting wavelength set at 515 nm, the wavelength of the argon laser which is used in the microfluorometer. The curves were then obtained by scanning the monochromator through the spectral range of the fluorescence generated

Table II. Composition of samples whose fluorescence spectra are shown in Figures 3 and 4

SAMPLE NO	SOLVENT	EB CONCENTRATION	T2 CONCENTRATION	EXTRACTED DNA CONCENTRATION
1	C-B*	1 μg/ml	0	0
2	C-B*		10^8 pfu/ml	0
3	C-B*	1 μg/ml	10^{10} pfu/ml	0
4	H_2O	1 μg/ml	0	10 μg/ml
5	H_2O	1 μg/ml	0	100 μg/ml

* Carbonate - Bicarbonate buffer solution, discussed further in Section III.

by the sample. It can be readily seen that as the amount of DNA avai - able for intercalation is increased (Samples 2 and 3) the intensity of the fluorescence is also increased. In these samples, identical sample preparation protocols were used, thus the only difference between them is the concentration of the virus and hence the amount of DNA accessible for intercalation. As a check on these results, samples were also prepared using commercially available extracted calf thymus DNA. Samples 4 and 5 thus have no protein coats surrounding the DNA through which the EB must penetrate. The extreme enhancement observed in Sample 5 is conclusive evidence that the amount of enhancement can be used as an indication of the extent of intercalation of the EB. Unfortunately, the only significant difference between the curves of these five samples is in the intensity; there is no spectral difference which would allow a distinction to be made that the fluorescent light was generated by bound or unbound EB molecules.

Figure 4 shows the fluorescence excitation curves of the five samples. The excitation curves were obtained by measuring the intensity of the output fluorescence at 605 nm (very near the peak in the emission) while the input exciting wavelength was scanned from 400 nm to 580 nm. Thus, the excitation curves show the fluorescence intensity obtained by exciting the sample with any given wavelength. As was observed in the emission curves, it can be seen that as the concentration of DNA is increased, so is the intensity of the fluorescence. However, unlike the emission curves, the sole difference between the excitation curves is not the intensity. Comparison of the curves for Samples 2 and 3 indicates a change in the shape of the curves: in Sample 3, there is an additional peak at 520 nm which does not exist in the curves of Samples 1 and 2. Further, the excitation cross section is

non-zero at 570 nm in samples where DNA is present and is zero in Sample 1. This fact is further confirmed by comparison of the curves generated using samples 1 and 5. Thus, there are two important distinctions between the excitation curves arising from bound and unbound EB. Not only is the intensity dependent on the extent of intercalation, if a sample is illuminated at 570 to 580 nm, only the bound EB will produce fluorescence.

The instrument in its present configuration is incapable of utilizing the advantage that could be gained by illuminating the samples at 570 nm since the argon laser is capable of emitting high power radiation only at 488 and 515 nm. The other wavelengths to which the argon laser is tunable are all available only at significantly reduced power levels and, with the exception of a line at 529 nm, are all of shorter wavelength than 515 nm.

V. INSTUMENTAL DETECTION STUDIES

A. Introduction

Numerous experiments were performed in an attempt to determine precisely the instrument's abilities. The modifications described in Section II allowed the instrument to operate for hours at a time with little wander of the laser beam once the laser was warmed up; some thermal instability remains in the laser.

The final configuration of the instrument is shown in Figure 5. This illustration shows the optic path followed by the laser beam, and provides a good reference for understanding the instrument's operation. The majority of the components shown are self-explanatory. The laser beam is steered by means of two mirrors through the beam focusing lens into the sample observation region. Fluorescent light is collected through a microscope objective lens, passes through a changeable filter, is steered by a mirror, passes through both vertical and horizontal slits (the vertical slits are adjustable), through a second filter, and is incident on the photomultiplier tube.

Following experiments using scattering spheres to determine the optimum combination of filters which would transmit the maximum fluorescent signal and effectively block all scattered light, the use of a yellow filter (Corning 3-69) at the objective and an orange filter (Corning 3-66) at the PMT was shown to be preferred. Two filters appear to be necessary since the intensity of the light at the objective is sufficient that significant amounts leak through the initial filter. The transmission curves for the two filters and for a third red filter (Corning 2-63) are shown in Figure 6.

The EB emission curve of Figure 3 is shown for comparison. It is apparent that the yellow filter transmits nearly all the fluorescent light and the orange filter significantly reduces the signal only in the spectral region 550 to 580 nm which is where the fluorescent intensity is increasing. The combination of the two filters, however, reduces the scattered light intensity to a level less than that of other noise which could not be eliminated.

The basic configuration of the electronic data accumulation and analysis instrumentation is shown in Figure 7. The fluorescent light is incident on a photomultiplier tube, the resulting signal is amplified and fed through a sampling strobe generator to a pulse height analyzer. The accumulated data can then be plotted on an X - Y - plotter. The signal from the PMT can also be continuously monitored on a storage oscilloscope fitted with a Polaroid camera.

The instrument in the configuration described above was used to perform experiments with a variety of different samples. The results of some of those experiments will be described in the following sections.

B. Studies using scattering spheres

One very brief set of experiments was conducted using 1.1 μm diameter, commercially available latex scattering spheres. Typical results of those tests are shown in Figure 8. There are three important results which are obvious in this figure. The inset figure is the oscilloscope trace of two of the scattering spheres traversing the laser beam in the microfluorometer. It can readily be seen that

the time required for transit is relatively consistent at approximately 40 μsec. The height of the peaks varies because, due to the high intensity of scattered light, the slits were narrowed to less than the width of the sample column.

The large plot is the result accumulated in the Pulse Height Analyzer (PHA). The peak occurring at 1.48 V is due to the scattering spheres. The position of the peak, at 1.48 V, is the result of a number of experimental factors, including the size and reflectivity of the spheres, the intensity of the laser beam, the slit width, the voltage applied to the photomultiplier and the amplification of the PMT signal. It should be noted that the high voltage side of the PHA peak is very sharp: the upper limit of the scattered light intensity is well defined by the instrument. The lower-voltage side of the peak, however, demonstrates more of an exponential - like onset: there is less definition to the lower limit of the peak. Ideally the peak would be a very narrow spike, occupying only one bin in the PHA, which corresponds to a voltage range of 0.004 V. The width of the peak can be attributed to fluctuations in the size and reflectivity of the spheres as well as possible minor fluctuations in the laser power. The slope of the leading edge of the peak is interpreted to be the result of the slit width being less than the width of the sample column so that in some cases, the spheres may be only partially exposed in the slits. The signal observed at $\lesssim$ 0.4 V is typical of all runs on the instrument and is observed regardless of the presence of sample; both in terms of number of counts and of its voltage, it is dependent principally upon the PMT voltage and the amount of subsequent amplification. A substantial

part of the signal is definitely noise, not related to the sample, but it is this signal which will cause significant difficulty in later tests.

The fact that the instrument successfully detected the scattering spheres is not, in itself, significant. Of importance, however, is the pulse width (consistent transit time) and the understanding of the shape of the PHA output.

C. Studies using Ethidium Bromide - stained T2 Virus.

A variety of tests were performed using EB-stained T2 virus samples. Typical results of the tests are shown in Figures 9 and 10. The sample used in the tests represented by Figure 9 was prepared by the optimized technique discussed in Section III. The virus was suspended in a carbonate-bicarboante buffer solution with the ethidium bromide dye. No further treatment was applied to the sample. The final concentration of the virus was approximately 10^8 pfu/ml. Other experimental conditions are note in the figure.

There are two important features to be noted in the oscilloscope trace shown in the inset. The trace is interpreted as first a virus traversing the laser beam, followed by a noise spike. The virus is identified by the duration of the pulse: as previously noted a 40 μsec pulse is typical of the signal seen for the scattering spheres; the noise spike is much faster. (The duration of the noise spike and the signal pulse both depend on the amplification of the PMT signal; the pulse is integrated more with increasing amplification causing the exponential - like tail of the noise spike to be extended as the amplification is increased, thus making distinction by pulse duration increasingly difficult.) The amplitude of the signal attributed to the virus is 0.4 V,

not greatly different from the amplitude of the noise spike. In fact, as noted in the scattering sphere studies, the noise appears on the PHA output in significant amount at voltages up to about 0.4 V.

Comparison of many oscilloscope traces indicates that a maximum signal of about 0.5 V is typical for the virus prepared in this manner and the amplitude of that signal varies greatly, from very small up to the maximum value. The extreme variation in the signal is reflected in the PHA output: there is no distinct peak associated with the presence of the virus; instead, the viral signal is indistinguishable from noise in the PHA.

The extreme variation in the amplitude of the viral signal reflects a possible variation in the sample itself. As discussed in Section III, it is very likely that viruses are being effectively broken apart by the basic buffer so the sample being observed is not composed of uniform viruses. If, instead, the sample is composed of pieces of DNA with intercalated EB, it is unlikely that those pieces would be of consistent size leading to large variations in the observed amplitude. It is also possible that the viruses are clumped in groups of various numbers also accounting for the extreme inconsistency of the signal.

In an effort to reduce the amount of unbound EB that remained in the sample, a specimen was prepared as noted above. This specimen was centrifuged to remove the EB-bound virus from suspension; the pellet thus obtained was resuspended in distilled water. Typical results of tests with samples of this type are shown in Figure 10. As in the previous figure, the transit of the viral sample is clearly evident in

the inset. The intensity of the transit which is shown is somewhat anomalous; there was again extreme variation, probably resulting from variation in the size of the pieces of stained DNA or in the numbers of viruses associated in the existing clumps. No procedure was applied in an effort to resuspend the pelleted virus since it was felt that sonication or any other vibratory technique would only cause further deterioration of the integrity of the virus. Visually, the background was reduced when centrifuged, although this result is not evidenced in the PHA plots.

In the expanded scale portion of Figure 10, there is evidence of a signal at $\sim$ 1.6 V. It is believed that this signal is directly indicative of the viral sample; however, this result was not reproduceable, thus its reliability must be questioned.

The extreme variation in the intensity of the signal resulting from the virus samples can be seen in Figure 11. In this figure, the time scale has been changed so that the shape of the pulses associated with the presence of virus can no longer be seen. Further, there has been an additional step in the preparation of the sample: once the virus and EB were mixed in the carbonate buffer, the sample was then neutralized before being centrifuged and resuspended in distilled water. It was hoped that the added step might stop further degradation of the virus once the EB had been intercalated; this was not observed to a significant extent. As can be seen in the figure, there is no consistent pulse height that can be associated with the virus. Results such as that illustrated here were common to all the tests using virus: there was no available identification of the virus from the intensity of the fluorescent signal; the only reliable identification was made from the duration of the signal pulse.

There is little doubt that virus sample was actually observed using the instrument in the configuration described. Signal pulses such as those shown in the inserts in Figures 9 and 10 were seen only when there was sample flowing in the instrument. Such pulses were not observed when the sample flow was stopped; the intensity of the pulses was directly related to the intensity of the incident laser beam and to the voltage and amplification of the PMT. With the single possible exception shown in Figure 10 and mentioned above, the fact that the presence of viruses was not clearly observed in the PHA plots is attributed to the variation in the nature of the fluorescing bodies within the samples.

D. Studies using Herpes Simplex Virus (HSV) and fluorescent antibody.

In a meeting at Ames Research Center, Dr. Glen Funk of San Jose State University suggested that the instrument might be used to detect specific virus that were tagged with fluorescent antibody. This technique would be applicable to a search for the presence of a particular virus since the antibody is specific to only one strain of virus. The technique alleviates certain sample preparation problems and, as it will be seen, introduces others. For some virus strains, antibody which is already tagged with the fluorescent dye, fluorescein isothiocyanate, is available commercially. Fluorescein exhibits an emission spectrum similar to that of EB, however without the enhancement effect. It is capable of being excited by an argon laser operating at either 488 or 515 nm. Although Dr. Funk's suggestion was to use polio virus and its fluorescein-tagged antibody, that antibody is not commercially available. Due to availability of both the virus and the antibody, experiments were performed to test this technique using Herpes Simplex Virus.

Sample preparation is considerably simplified from the EB-staining process. The virus and tagged antibody are mixed in distilled water, then centrifuged and resuspended. There is no need to use a buffer to attempt to break the virus' protein coat and, in fact, the virus remains whole throughout this procedure.

Typical results of experiments of this type are shown in Figure 12. It can be seen in the insert that the virus signal is still characterized by a transit time of approximately 40 μsec, which provides a means of distinguishing virus signal from the noise spike which also appears in the inset. Apparently, however, there is not a pulse amplitude which is sufficiently consistent to be considered characteristic of the antibody-bound virus. There are two potential reasons for this problem: the virus may remain clumped within a case of antibodies; or the antibodies, which are effectively bivalent, may act as bridges attaching several viruses together in clumps of varying sizes. The latter possibility could be solved by the use of specially prepared monovalent antibody, thus making attachment of a single antibody to more than one virus impossible. The former problem might be solved by more extensive treatment of the virus prior to mixing it with the antibody.

One further test was performed in an attempt to demonstrate that a concentration dependence could be observed. For this test, since the PHA output is incapable even of resolving the presence of virus much less of generating a representative count of viruses, the data acquisition system was slightly modified as shown in Figure 13. The PHA is operative in the same manner as before the

modification. The change allows any pulse of amplitude greater than a predetermined value to be counted, since the single channel analyzer generates an 8 V pulse for each incident pulse greater than the preset discrimination level. For the purpose of this experiment, the discrimination was arbitrarily set at 0.4 V. It was felt that 0.4 V would allow a reasonable differentiation between signal and noise. The verification of this discrimination was provided by the oscilloscope: it was set to trigger on only those pulses which were counted. It was observed visually by utilizing this technique that the counted pulses included very few noise pulses, estimated to be only a few percent. It must be pointed out that this technique allows only a single discrimination in pulse height: every pulse of intensity greater than the arbitrary value of 0.4 V is counted.

For the concentration experiment, four samples were prepared, three of which were successive dilutions of the first. The results of that experiment are summarized in Table III. It is obvious from this table that there is a detectable concentration variation, however the relationship between the virus concentration and the number of counts which are recorded is not direct. It was suspected that there was a significant amount of clumping of the viruses, and this was confirmed by subsequent electron - microscopy. The size of the clumps was found to be inconsistent although it was impossible to determine any details of the size distribution of the clumps.

There are several potential explanations for the apparent non-linearity of the counting efficiency. Each of those explanations, how-

ever, is purely conjectural and determination of the veracity of each is impossible lacking electron-microscopic examination of each of the four samples. Due to lack of available time on the electron microscope, the desired examination was impossible.

As with the EB-intercalated virus samples, there is little doubt that the antibody-bound virus was actually observed.

Table III. Results of concentration dependence experiment.

Concentration of Sample	Recorded Counts	Actual no. of particles*
Original	125,000	800,000
x 0.5	30,000	400,000
x 0.2	9,500	160,00
x 0.1	2,900	80,000

All counts recorded in 80-min runs, total volume sampled 85 µl per run.

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* Number of particles that should have been observed based on electron-microscopic count of the original concentration.

VI. CONCLUSIONS AND RECOMMENDATIONS

It has been demonstrated that the Total Viral Monitoring Instrument is capable of detecting viruses. It is not, however, presently capable of any size discrimination due to a combination of the inconsistency of the virus sample, the large amount of electronic noise arising from the PMT, and possibly excessive resolution in the PHA. There are several areas in which work should be continued in an effort to improve the instrument.

Substantial further work is required in the area of sample preparation. A protocol must be developed which satisfies the requirements of the instrument in order to make the instrument optimally functional. Requirements for the technique which will be finally accepted are:

A. Maximum intercalation of EB.

B. Consistent amount of EB intercalated, proportional to the size (contentof nucleic acid) of the virus.

C. Integrity of the viral structure must be preserved.

D. Elimination or neutralization of unbound EB.

E. Individual suspension of virus in sample medium, no clumping.

The relative importance of the above requirements is nearly equal. It is important to obtain the maximum possible signal strength which requires the maximum possible intercalation of EB. However, if size discrimination is ever to be accomplished, the intercalation must be extremely uniform between virus of a particular strain, and must be proportional to the specimen size reflected in the amount of nucleic acid contained between viruses of different types. The necessity of the other requirements is obvious from the work and results described in this report.

An alternative approach to the use of the instrument is based on the detection of antibody-bound virus. It has been demonstrated that samples prepared using fluorescein-tagged antibody bound to virus are detectable. If this technique is to be adopted, similar requirements for the optimum protocol must be fulfilled:

A. Maximum binding of antibody to each virus.

B. Consistent amount of antibody bound to each virus.

C. Elimination of unbound antibody.

D. Individual suspension of virus in sample medium, no clumping.

The success of any possible preparation technique must ultimately rest in its performance in the instrument. However, it is equally necessary that the condition of the virus specimens prepared by that technique be thoroughly investigated; and for this, high resolution electron microscopy will be necessary.

Improvements involving major modification to the instrument itself should also be considered. The spectrofluoroscopic studies indicate that consideration should be given to illuminating EB-stained viruses samples at 575 nm, a wavelength at which only DNA-bound EB-stained virus will be stimulated to fluoresce. It is true that the fluorescence intensity of bound EB excited at such wavelengths is much less than when excited at nearly 500 nm, however the increase in the ability to discriminate between bound and unbound EB as the source of the fluorescence should make the reduction in overall intensity a small sacrifice. Wavelengths near 575 nm are not available from the argon laser which is currently the illumination source in the instrument. Dye lasers, operating in the cw mode,

are commercially available which are tunable throughout this region and which emit sufficient power. Further, if the instrument is to be used as a detector for specific virus by means of the antibody technique, other wavelengths than those available with the argon laser may also be desireable.

In addition to the sample preparation problems encountered, many times the signal associated with the virus was of similar intensity as the amplified noise arising in the photomultiplier. Newer PMTs are available which exhibit greatly improved noise characteristics than the RCA 8850 which was supplied with the instrument. Further noise reduction is available by application of a refrigerated housing for the PMT. Installation of a cooled PMT, such as the Varian VPM-192MB, would provide similar signal response with lower noise levels and more applicable spectral sensitivity.

It is apparent that if the instrument is to become routinely operational, further development is needed. The instrument is a logical continuation in the development of fluorescent flow cytometry, for which a commercial market has already been established. It has been demonstrated that the Total Viral Monitoring Instrument is successfully operational in a limited sense; further development should be pursued in an effort to make the instrument fully operational.

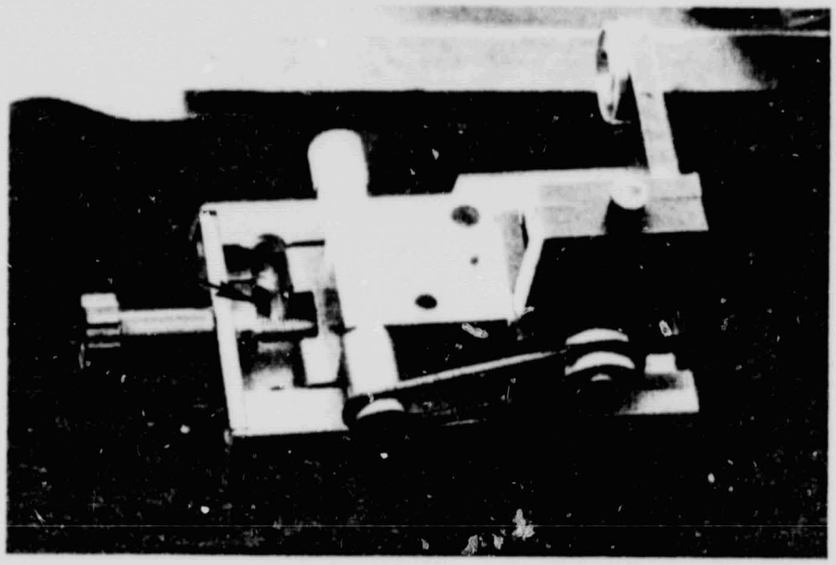

Figure 1. Laser beam focussing lens mount. Modifications indicated with arrows are the remote positioning mechanism and the slot sllowing a small rotational positioning correction.

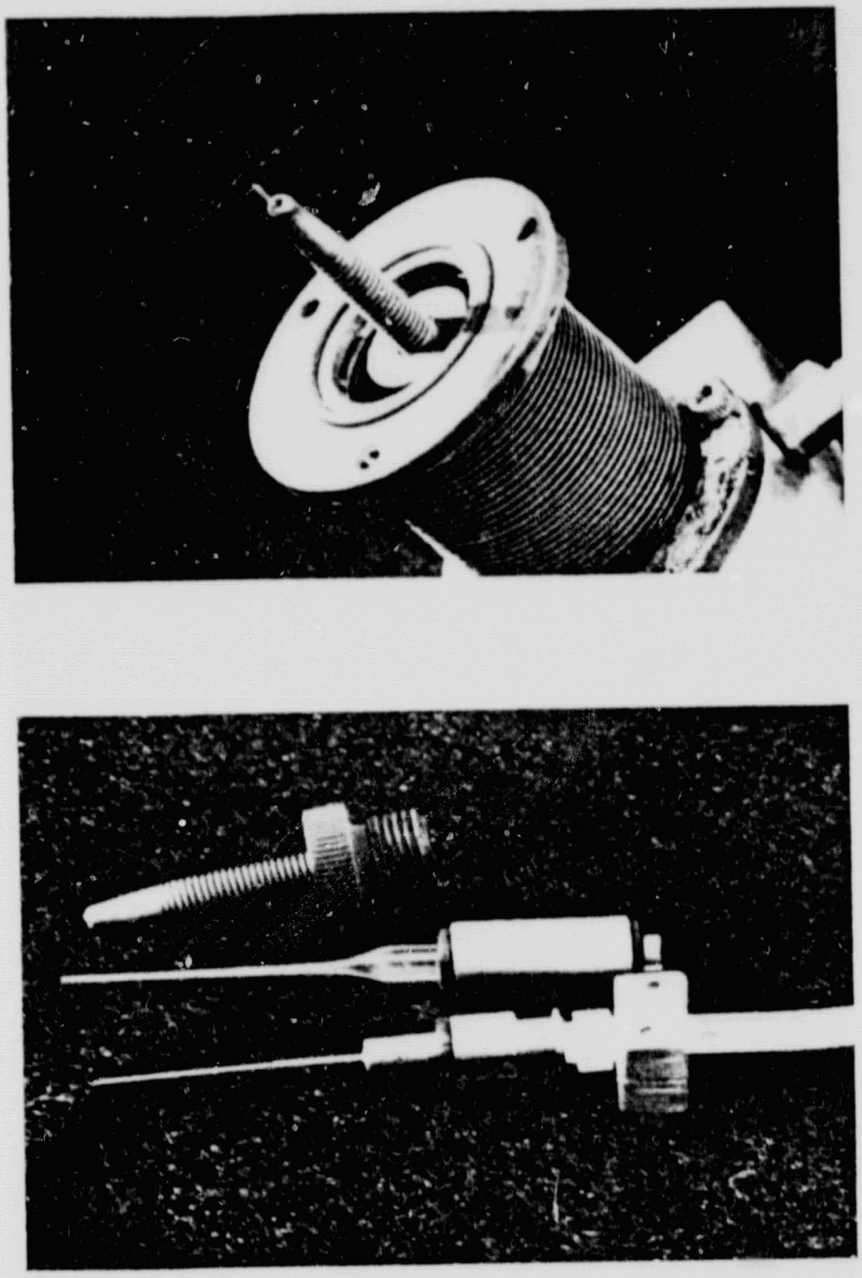

Figure 2. Detail of the sample injection system. The brass sheath was added to reduce the breakage of the glass capillary.

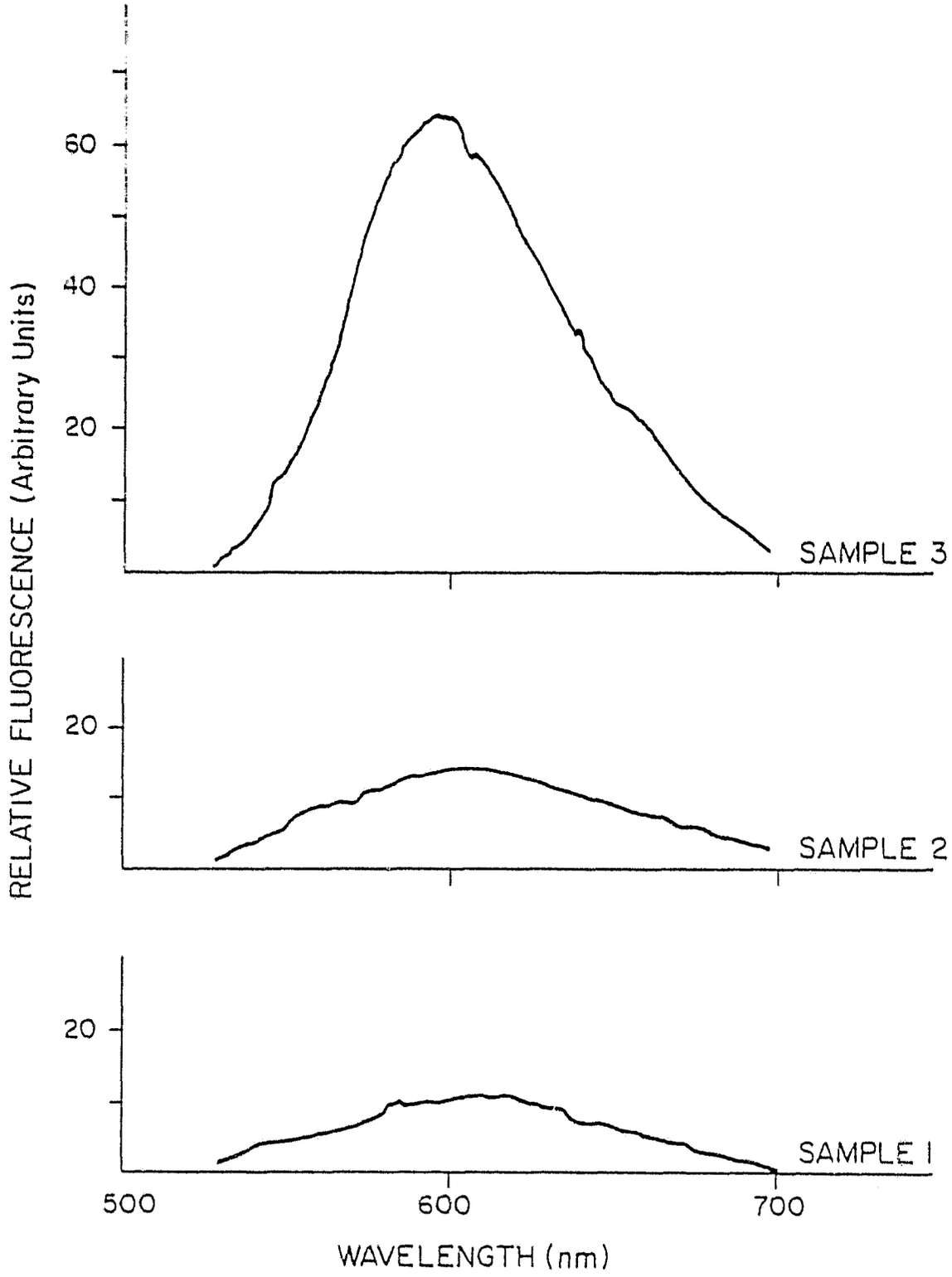

Figure 3. Fluorescent emission spectra of Samples 1, 2, and 3 whose compositions are given in Table II. All samples were illuminated at 515 nm.

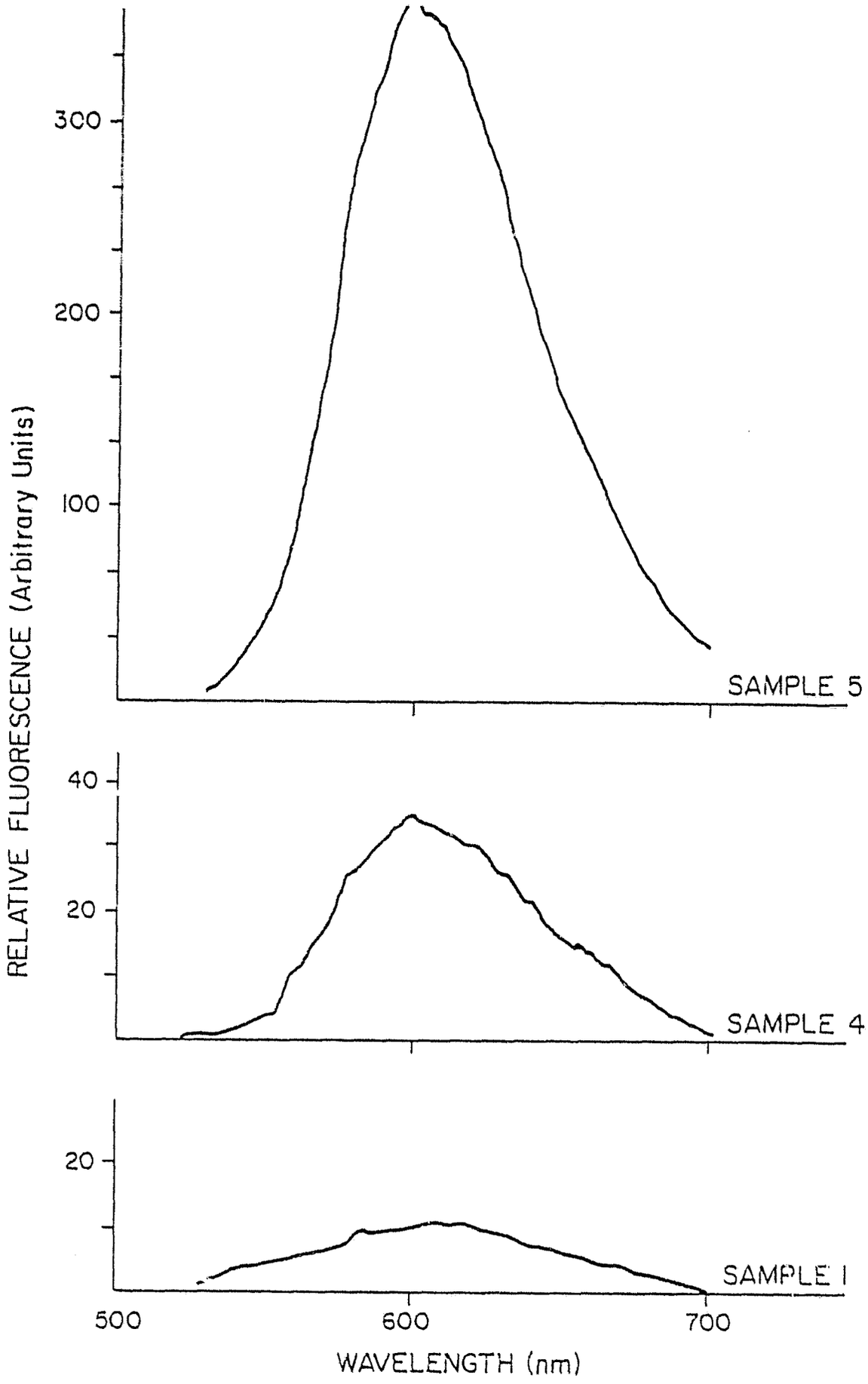

Figure 3, cont. Fluorescent emission spectra of Samples 1, 4, and 5 whose compositions are given in Table II. (Spectrum for Sample 1 is repeated for comparison). All samples were illuminated at 515 nm.

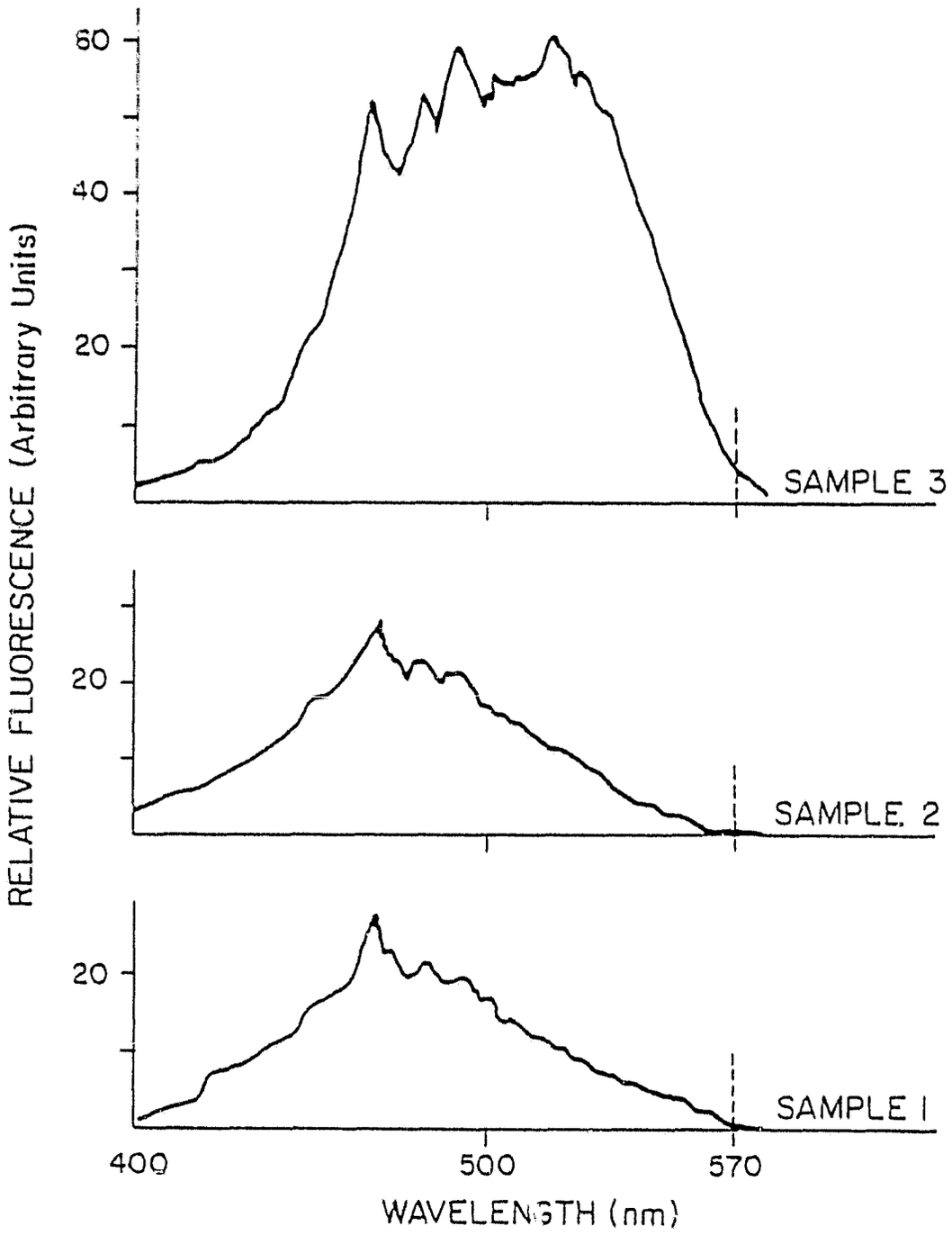

Figure 4. Fluorescent excitation spectra of Samples 1, 2, and 3 whose compositions are given in Table II. The emitted fluorescent intensity for all samples was observed at 605 nm.

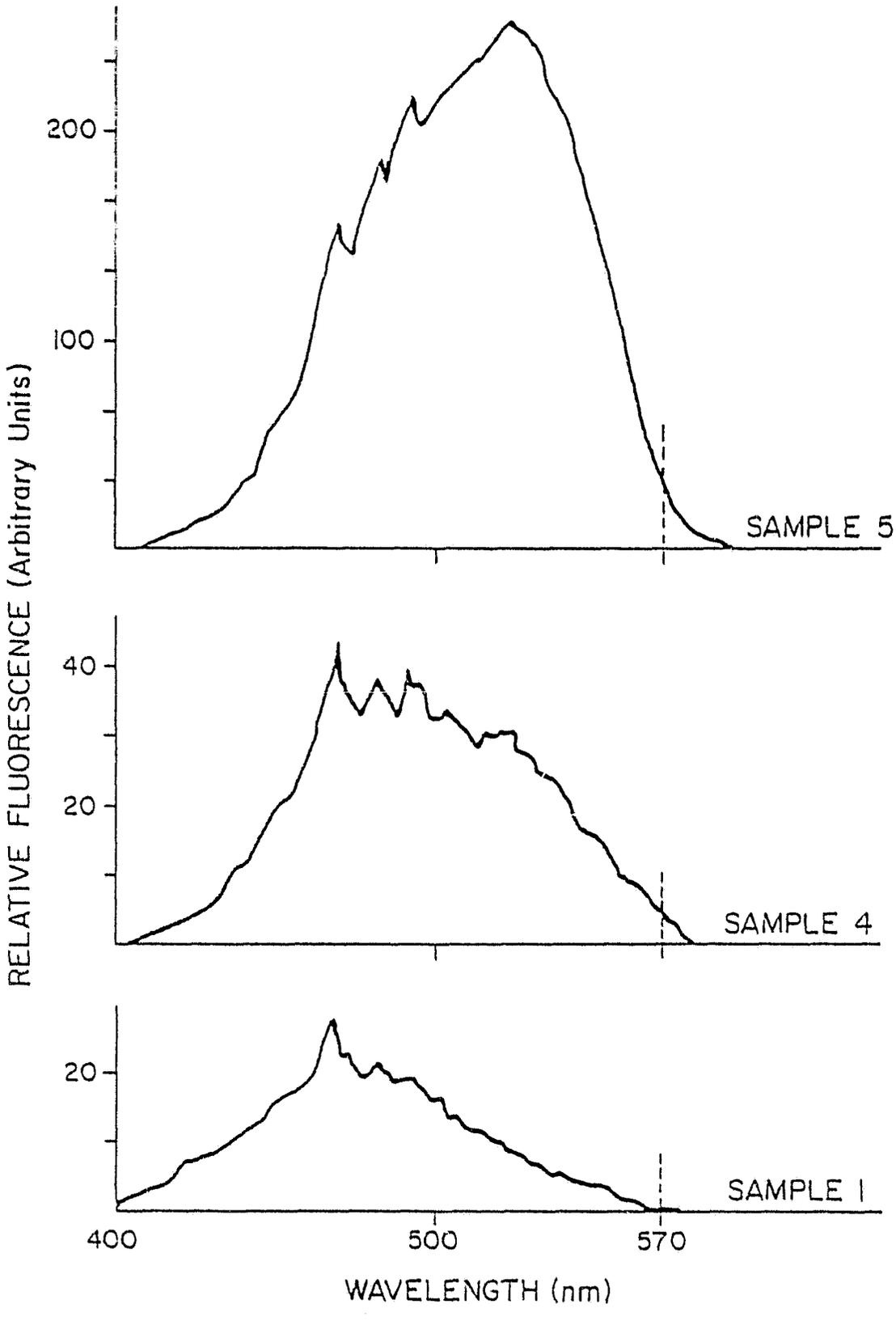

Figure 4, cont. Fluorescent excitation spectra of Samples 1, 4, and 5 whose compositions are given in Table II. (Spectrum for Sample 1 is repeated for comparison). The emitted fluorescent intensity for all samples was observed at 605 nm.

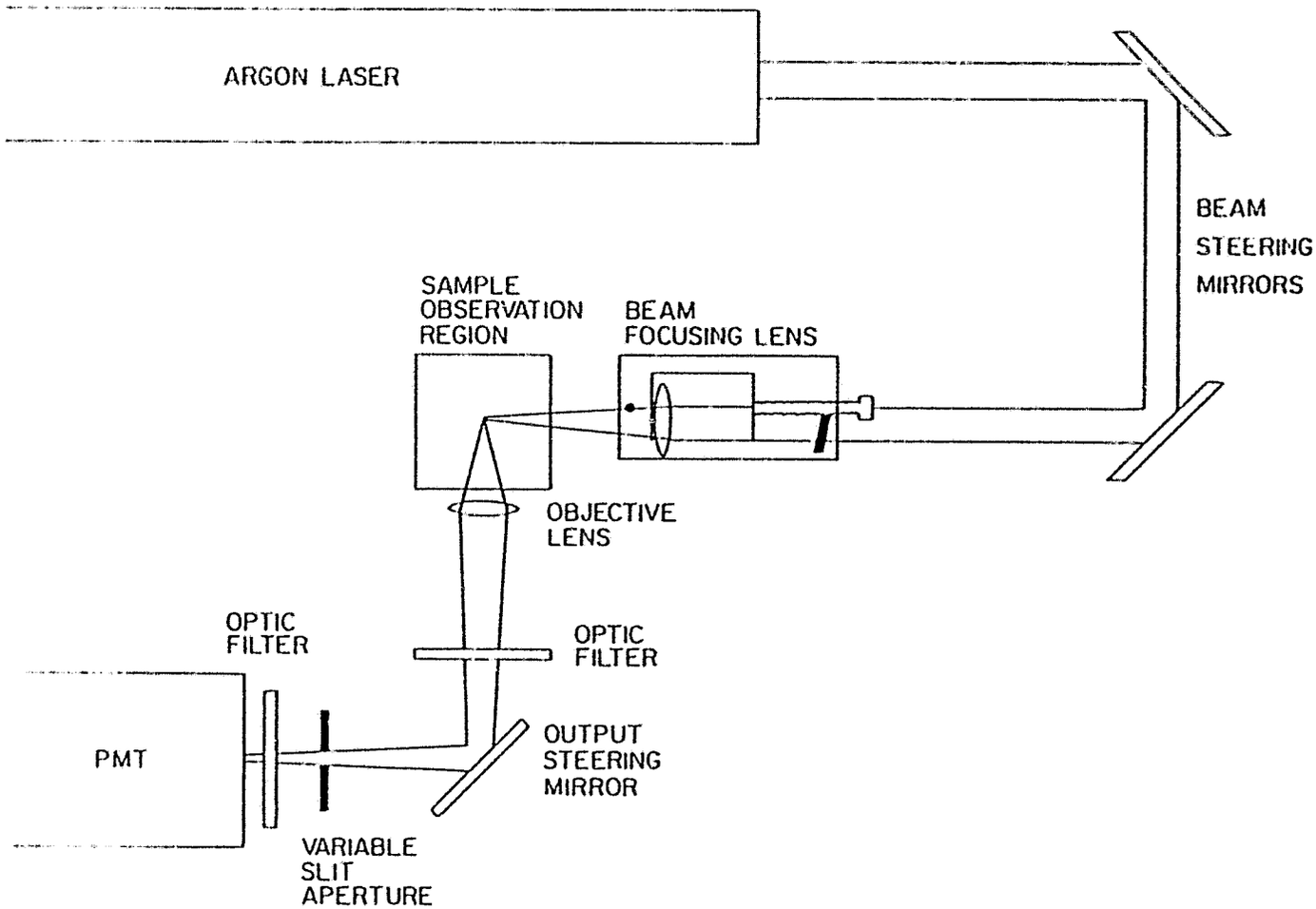

Figure 5. Final operation configuration of the microfluorometer, with emphasis on the optic path.

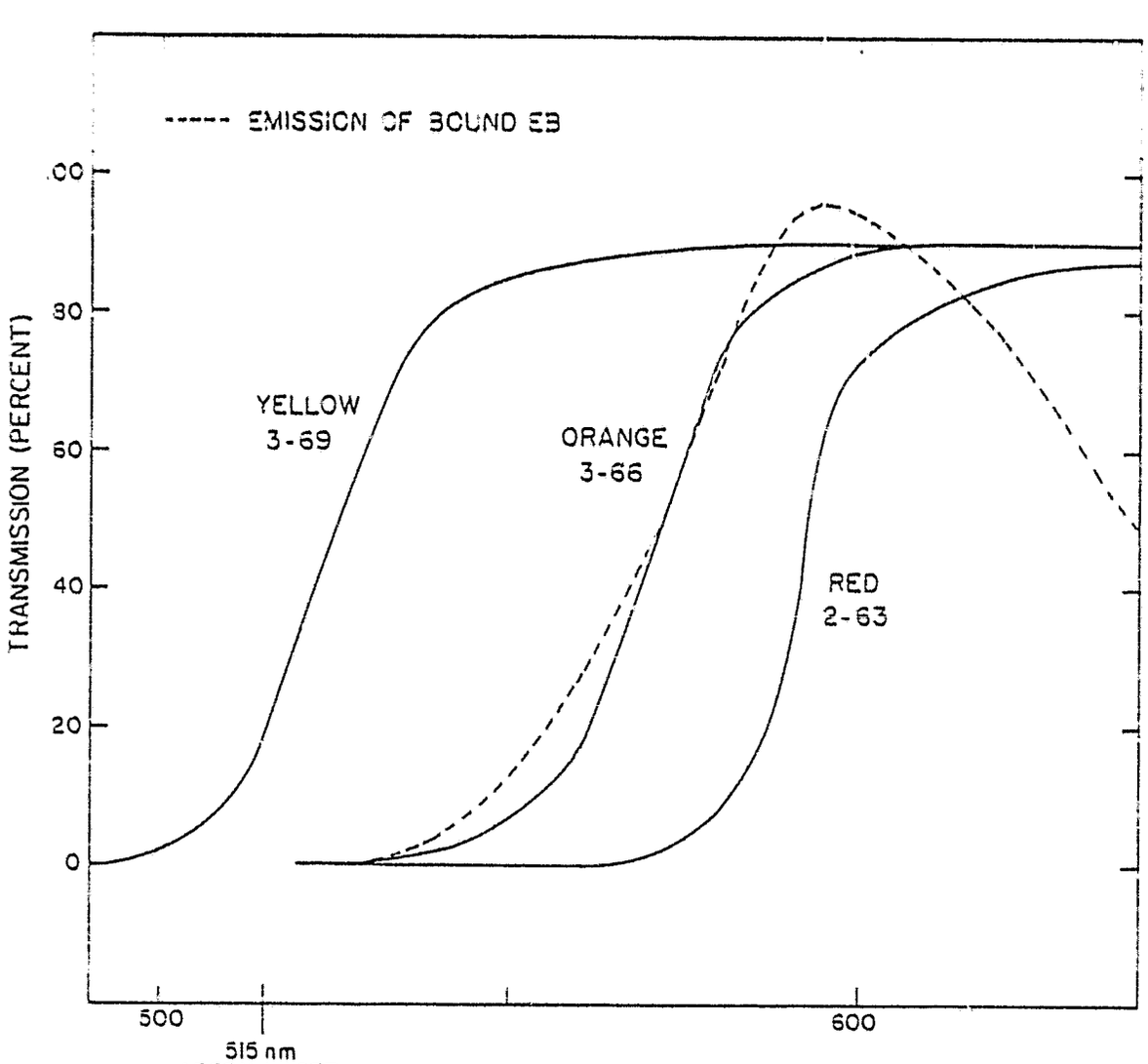

Figure 6. Transmission curves of the filters used in the microfluorometer. (Fluorescent emission of DNA-bound ethidium bromide, included for comparison).

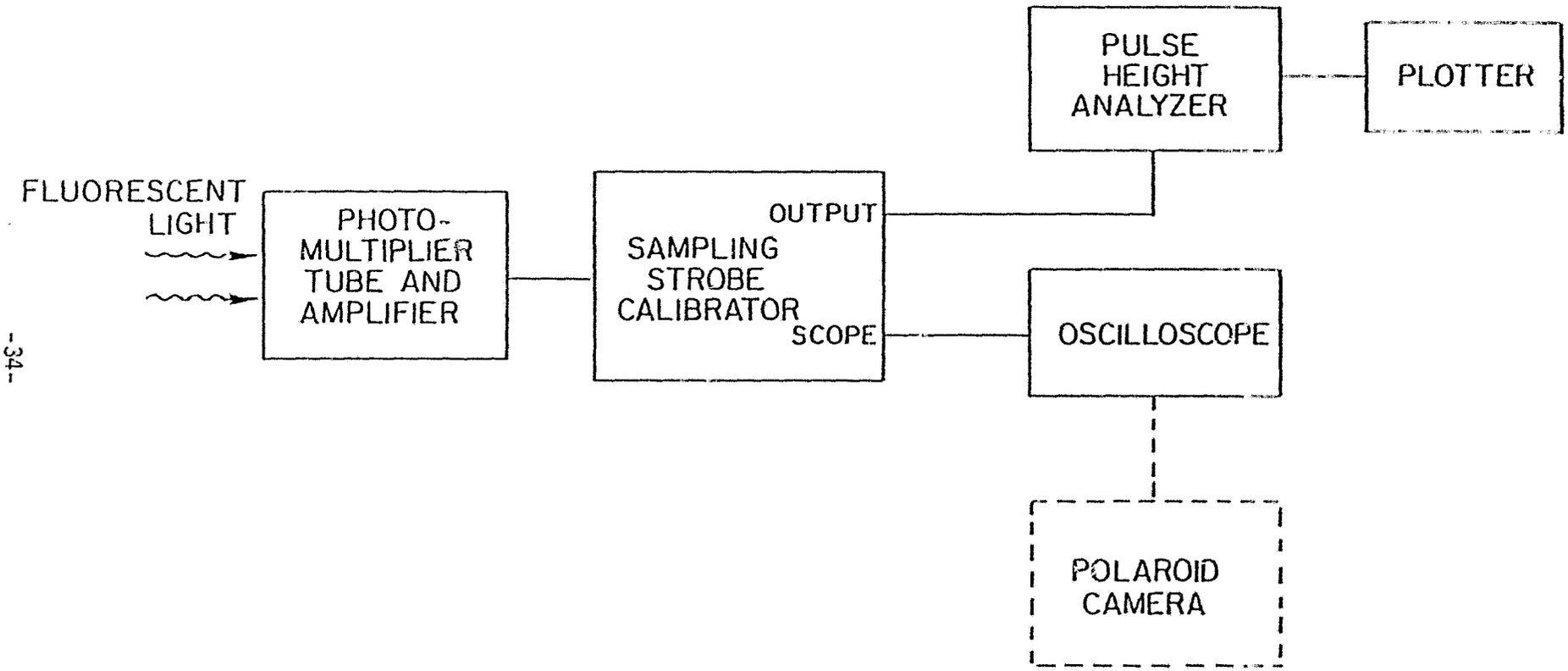

Figure 7. Schematic of the electronic data accumulation and analysis instrumentation.

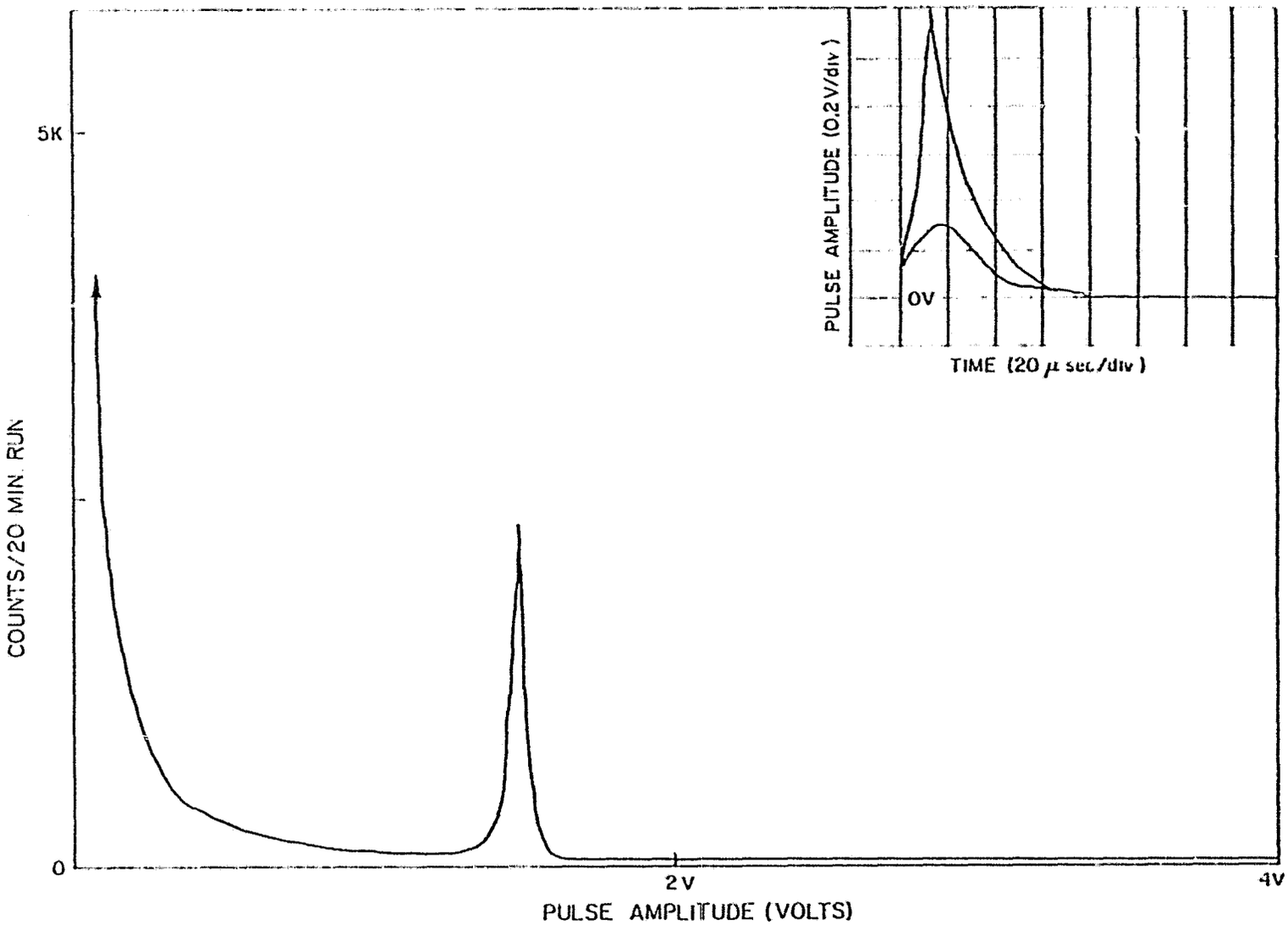

Figure 8. Typical result of experiments using sample of 1.1-μm diameter scattering spheres. The large plot is the output of the PHA, the inset is the oscilloscope trace of the transit of two spheres through the laser beam

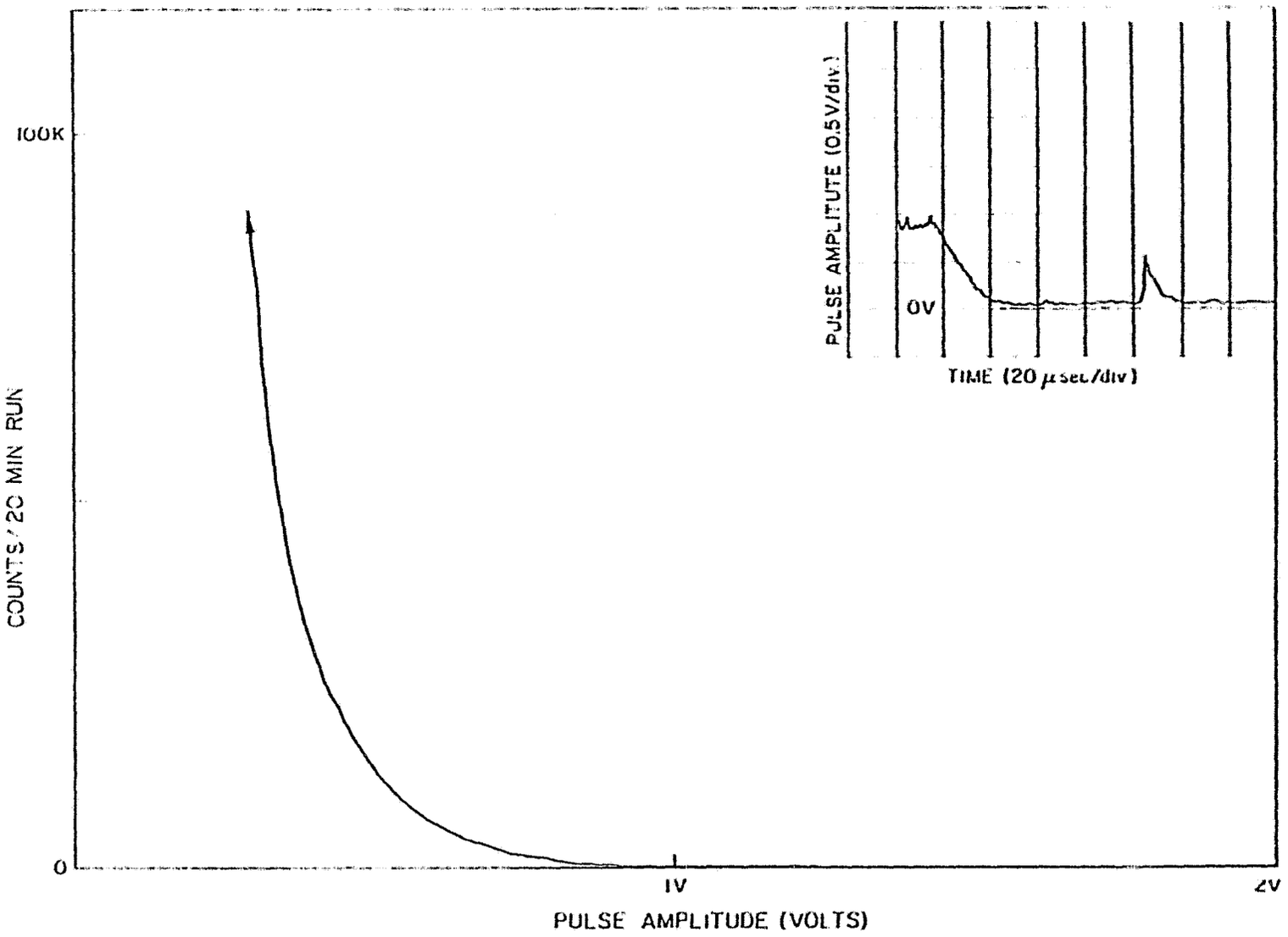

Figure 9. Typical result of experiments using EB-stained T2 virus. The large plot is the output of the PHA, the inset is the oscilloscope trace of the transit of a virus through the laser beam.

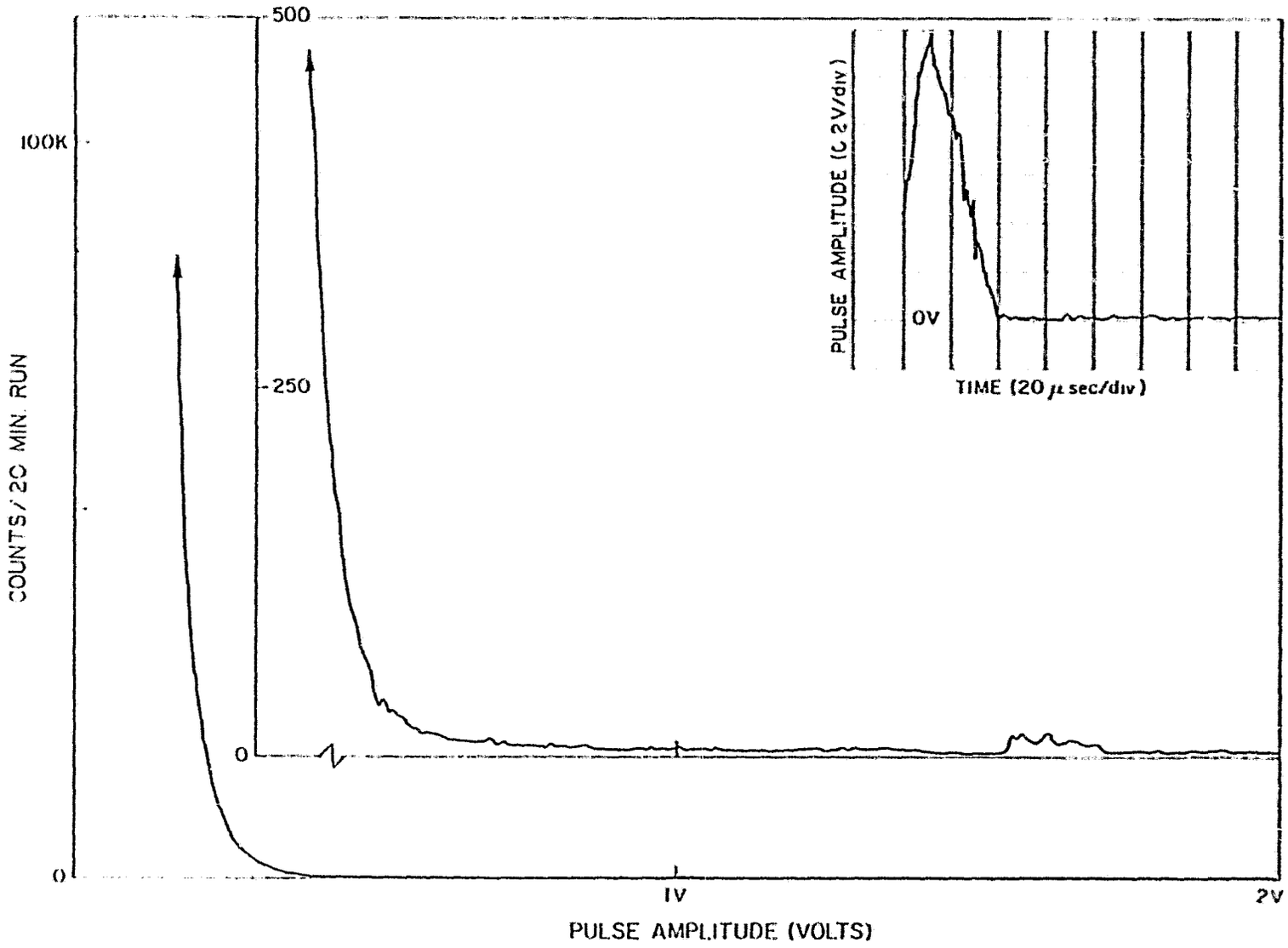

Figure 10. Typical result of experiments using EB-stained T2 virus. Sample was centrifuged and resuspended before testing. The large plots are the output of the PHA, the inset is the oscilloscope trace of the transit of a virus through the laser beam. Of the two large plots, the inner one is the same as the outer except that the ordinate is expanded to show detail at voltages greater than the typical noise level.

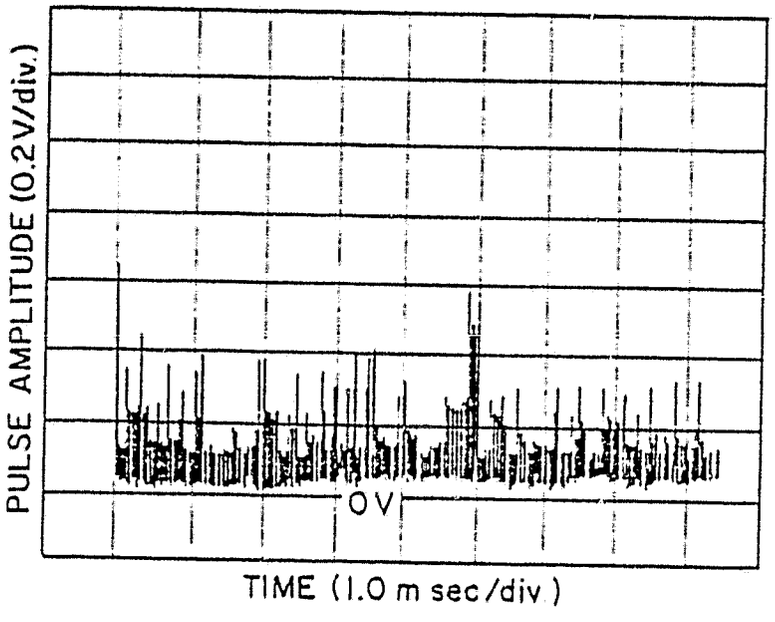

Figure 11. Oscilloscope trace of the transits of many EB-stained T2 viruses through the laser beam. The time scale is condensed to emphasize the extreme variation in the signal pulse amplitudes.

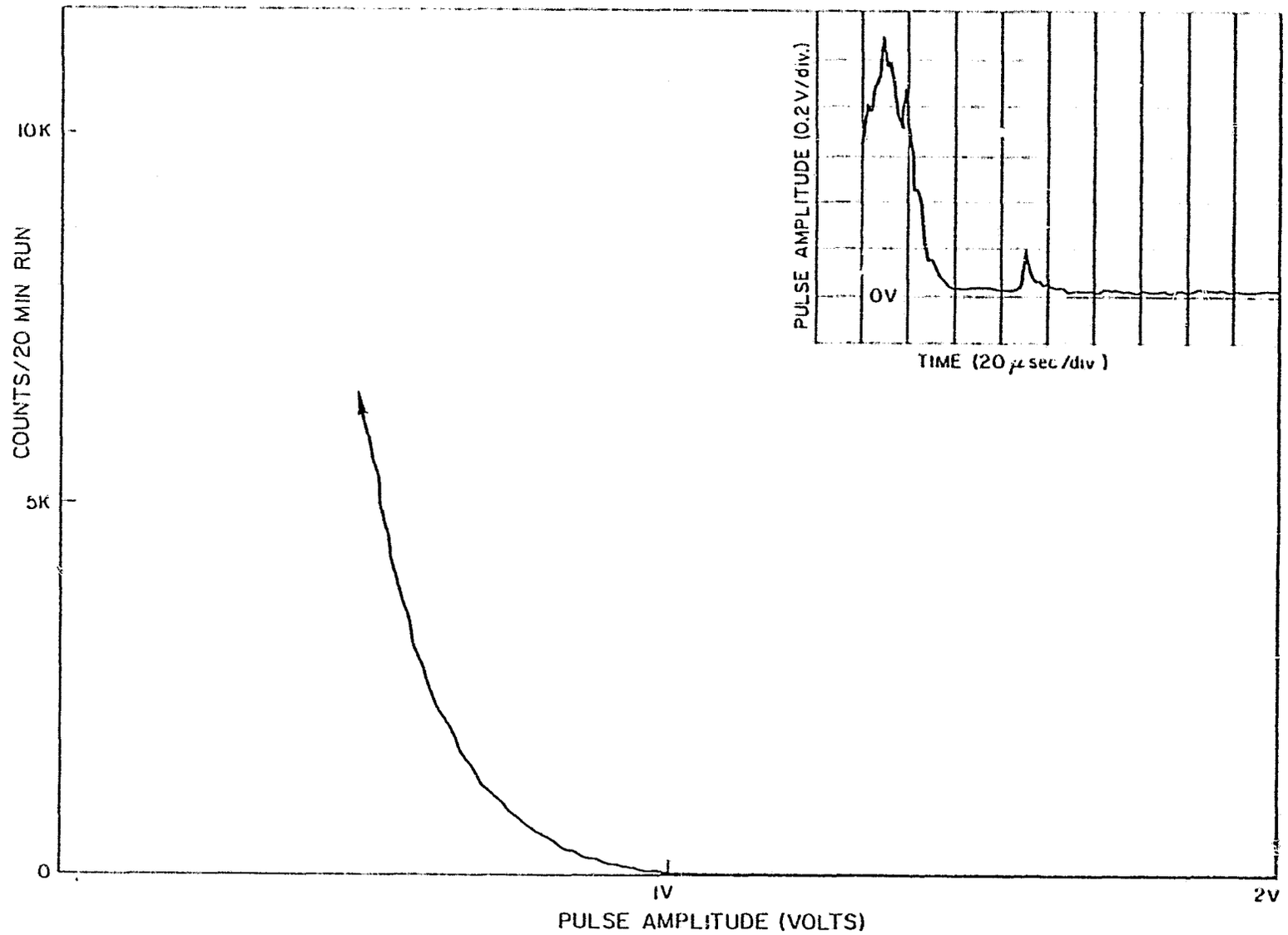

Figure 12. Typical result of experiments using Herpes Simplex Virus and commercially available fluorescein-tagged antibody. The large plot is the output of the PHA, the inset is the oscilloscope trace of the transit of a virus through the laser beam.

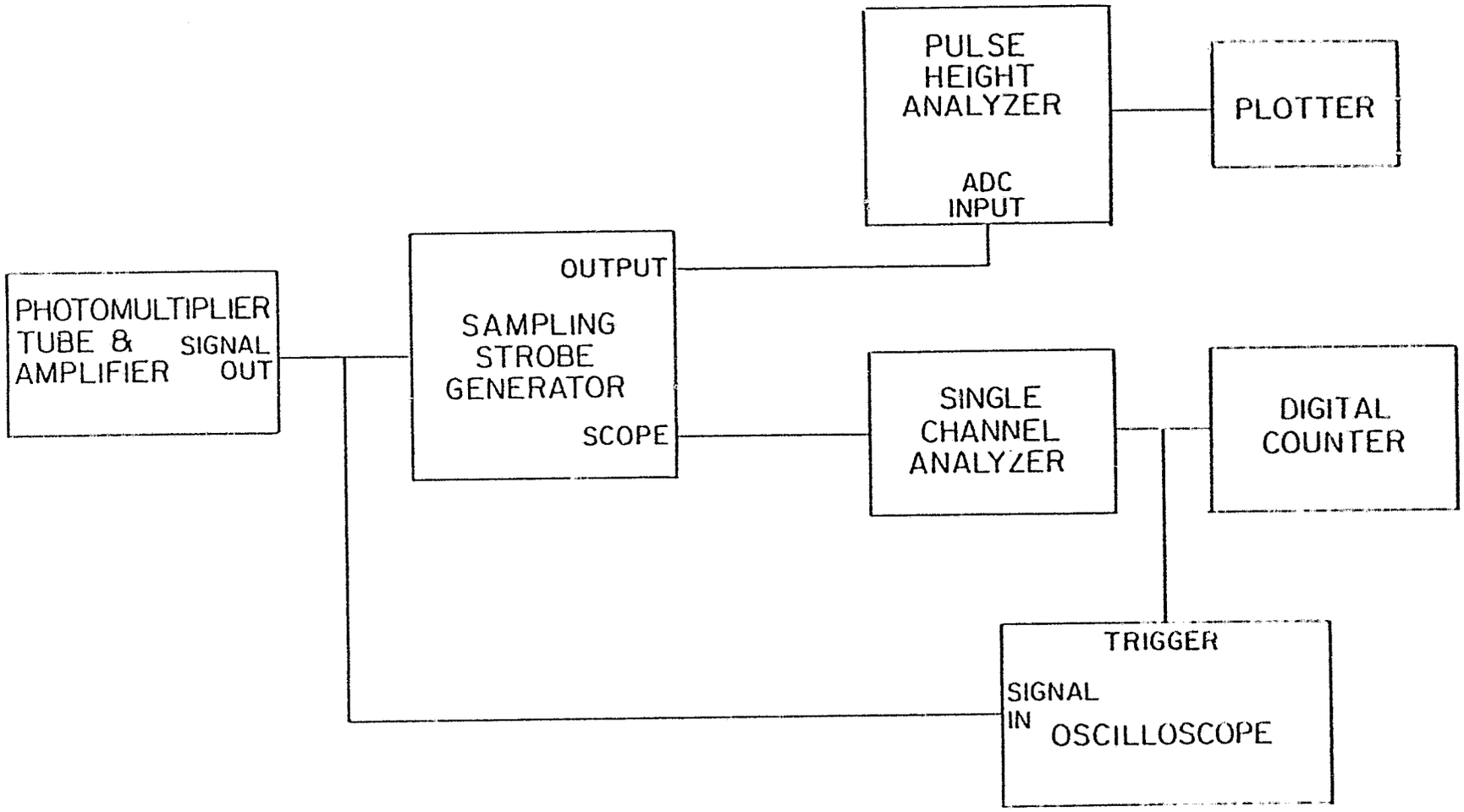

Figure 13. Schematic of the electronic data accumulation and analysis instrumentation, modified to obtain a total count of the signal pulses.