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An electronic device for the automatic correction of fluorescence emission spectra

G. MAZZINI, G. BOTTIROLI *and* G. PRENNA

Centro di Studio per l'Istochimica del C.N.R.,
Istituto di Anatomia Comparata dell'Università, Pavia, Italy

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Synopsis. An electronic device is described for the automatic correction of fluorescence emission spectra obtained by digital microspectrofluorometry based on multichannel scaling and single photon detection as described previously. This device consists of: (*a*) an arithmetic unit for the correction of the spectral values and for curve integration processes; (*b*) a circuit that operates directly on data in the memory of the multi-channel analyser by subtracting from them a pre-established value corresponding to the background; and (*c*) an averaging unit for calculating a mean for the spectral value.

Introduction

The microspectrofluorometers available commercially provide emission spectra that are distorted by the non-uniformity along the optical spectrum of the characteristics of the optical and electronic components. It is, therefore, necessary to correct this by using correction factors that are a function of λ, which take into account the characteristics of:

(*a*) the optical components (lenses, intermediate optics, mirrors, barrier filters, a monochromator and, in some cases, dichromatic mirror), and;

(*b*) the electronic components (non-uniform spectral sensitivity of the photomultipliers or photodiodes).

Normally, spectral data are corrected by using correction factors obtained by comparing the spectrum standard of a tungsten lamp and the spectrum of the same lamp recorded on the instrument under the same conditions as those used in the measurement. The correction procedure is usually carried out manually or with the help of computers; the first procedure involves a considerable amount of time, while the use of computers is not always possible.

The purpose of this paper is to describe a fairly simple and inexpensive electronic device, capable of performing spectral corrections and other operations associated with them (subtraction of the background, averaging of the data, and the integration of the curves in question) in a short space of time. It is, therefore, particularly useful in laboratories where many microspectrofluorometric measurements are made. Previously, we

described a digital microspectrofluorometer for single photon counting based on the use of multichannel analysers (Cova *et al.*, 1972, 1974). The device described here represents a part of it.

The digital microspectrofluorometer

Various components of the microspectrofluorometer, to which the device described here is connected, has been described previously (Cova *et al.*, 1974; Prenna *et al.*, 1974). However, it is useful to recall briefly certain basic features, particularly those concerning the use of multichannel analysers as instruments for measuring the spectral distribution of fluorescence emissions.

The optical part consists of the Leitz microspectrograph (Pearse & Rost, 1969; Ruch, 1960) composed mainly of: (*a*) a source of excitation (usually a mercury or xenon lamp, with a monochromator or interference filters; for special applications, a laser can also be used (Sacchi *et al.*, 1974; Cova *et al.*, 1974); (*b*) a microscope for observing and centring the preparation and equipped with a system for epillumination according to Ploem (Ploem, 1967); and (*c*) a spectrographic arm, including a slit, a dispersion prism, and an oscillating mirror for a time-varying deflection of the beam directed to the output-slit.

The electronic part consists of: (*a*) a system for single photon counting (Cova *et al.*, 1973), including a fast amplifier and discriminator, having a 10 nsec resolving-time (SSR 1120), for the pulse-height selection and standardization of current pulses from an EMI 9558 QA photomultiplier; (*b*) a multichannel analyser (MCA Laben 1024), connected to the discriminator output by a logic interface circuit and operated in 'multi-scaler mode', i.e. a scaler associated to a multichannel digital memory. The number of counts stored in the scaler corresponds to a given position of the oscillating mirror (i.e. to a given λ value) and is transferred to a channel of the memory, the scaler being reset and ready for the next measurement in a few microseconds. By means of a reference signal taken from the oscillating mirror, the mechanical scanning of the wavelengths is synchronized with the electronic scanning of the channels in the memory of the MCA.

Calibration of the channel-wavelength scale and determination of the correction factors $f(\lambda)$

The first operation is to calibrate the channels in the memory of the MCA in wavelengths; a channel-wavelength scale is obtained. The operation is performed by interposing a monochromator, calibrated so as to obtain a series of line spectra, with positions along the channel scale determined by the setting of the monochromator. In keeping with the commonest conditions in which the instrument is used (type of prism, speed of the oscillating mirror, channel-time), channel-wavelength conversion scales are, therefore, constructed which make it possible to associate immediately a given value of the wavelength with the number of each channel. The next operation consists of calculating the normalized correction factors [$f(\lambda) = 1$ indicates no correction] which are obtained by measuring with the instrument the spectrum of a lamp whose spectrum standard (S) is available. By comparing these data with those obtained with the same lamp under the experimental conditions employed (S'), it is possible to obtain $f(\lambda)$ from the following equation: $f(\lambda) = S(\lambda)/S'(\lambda)$.

The values of $f(\lambda)$ thus obtained are recorded on a punched tape, which is used in the subsequent operation of correcting the spectrum of measured fluorescence that is performed immediately by means of the electronic device described here. The true fluorescence spectrum (E) is obtained from the measured spectrum (E') by multiplying the values of the latter by the respective values of $f(\lambda)$:

$$E(\lambda) = E'(\lambda) \cdot f(\lambda).$$

The electronic device

Fig. 1 represents the block diagram of the electronic device for the automatic correction of emission spectra. The device consists of circuits formed entirely with solid electronic components and is composed of the following parts:

(*a*) The arithmetic unit, which performs the actual correcting operations; connected to it are a few synchronizing circuits for the whole system. The multichannel analyser is connected directly to the teletype, for the printing of uncorrected spectral data, and

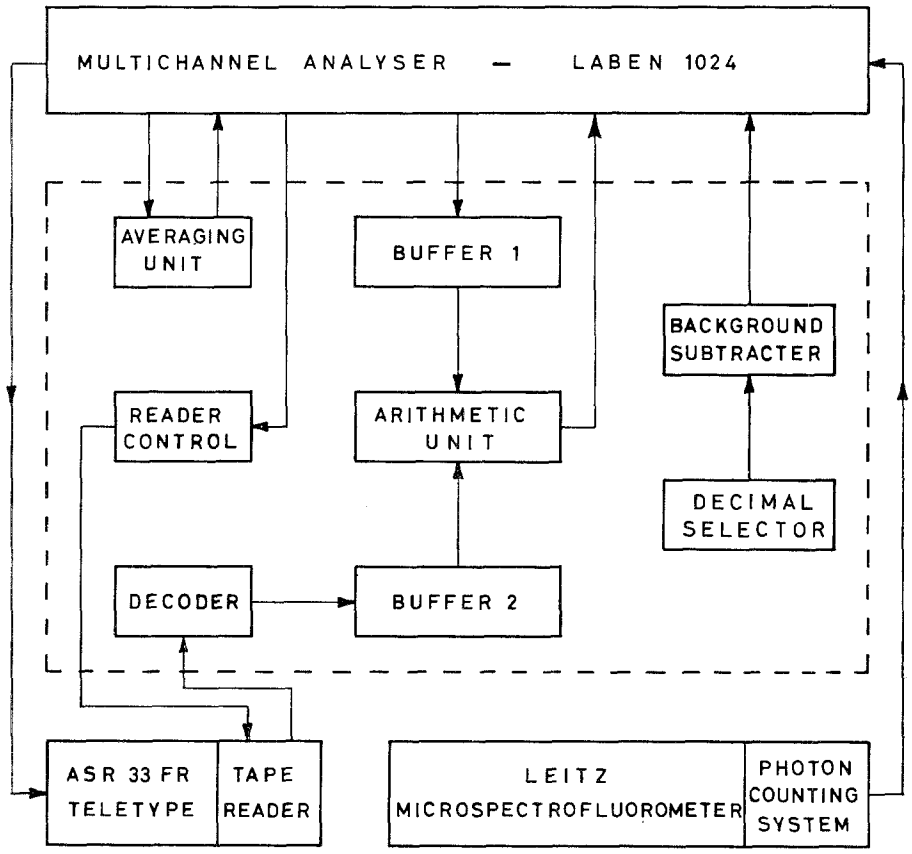

Figure 1. Block diagram of the microspectrofluorometer, the photon counting system by means of a multichannel analyser, and electronic device for the automatic correction of emission spectra (in detail).

to the arithmetic unit. The latter is, in turn, connected to the memory of the MCA on one side, and to the punched-tape reader on the other, buffers being interposed on both parts. Between the teletype reader and the buffer, a decoder is interposed for the data recorded on the tape. The arithmetic unit is also able to calculate the integral of the spectral curve;

(*b*) a circuit that operates directly on the data of the analyser's memory by subtracting from them a pre-established value corresponding to the background; and

(*c*) an averaging unit. This calculates an average of the spectral values contained in the channels of the MCA. It is used in order to improve the dispersion of the data in the case of counts involving few photons.

Correction procedure

The correction procedure may be subdivided into three parts.

(1) *Background subtraction.* By means of a synchronizing signal, the scanning of the wavelength falling on the oscillating mirror is synchronized with the counting times of the MCA; however, it is possible to regulate the position in time of the synchronizing signal along the oscillation of the mirror, and the total scanning time of the MCA memory in such a way that a small initial portion and a final portion of the memory are not occupied by signals corresponding to the spectrum, but by signals corresponding to the background. In this way both spectral and background values are available. The next step is to print these values, and then to determine the average background value. This value is imposed manually on the decimal selector of the background subtractor, which subtracts it from the spectral values contained in the MCA memory.

(2) *Correction of the uncorrected spectral values.* This takes place fully automatically in accordance with the following pattern:

(*a*) The punched tape containing the $f(\lambda)$ normalized correction factors is in service in the teletype, which is arranged such that it can be controlled from the reader, which, in turn, works from the analyser.

(*b*) The analyser sends the uncorrected spectral data, already subtracted from the background, into a temporary memory (buffer 1). At the same time, the correction factor values coming from the teletype reader, after appropriate decoding, are transmitted to a second temporary memory (buffer 2).

(*c*) The arithmetic unit multiplies the uncorrected spectral values, gradually taking them from buffer 1, by the corresponding values of the correction factor, which have been taken from buffer 2.

(*d*) The results of the multiplication, which constitute the corrected spectrum, return to the memory of the analyser, replacing the corresponding uncorrected spectral values.

The transmission of data by the analyser to buffer 1 is synchronized through the reader control with the data output of the teletype reader. This synchronization makes it possible to make the appropriate correction factor correspond to each channel containing a datum of the spectrum measured, this correction factor being obtained, as described above, at the same wavelength in the spectrum of the standard lamp. Fig. 2 shows some spectral data before and after the correction procedure.

In addition, it is possible to correct spectra obtained previously, as long as they are on the punched tape. In such a case, it will be sufficient simply to insert the punched tape

in the reader which, once the correction circuit has been disconnected, will send it to the analyser memory.

(3) *Averaging*. The averaging procedure is used when the dispersion of data in the memory is of a high degree, i.e. when the signals are very weak, or material is in an advanced state of photodecomposition, so that it is not possible to engage in prolonged counting.

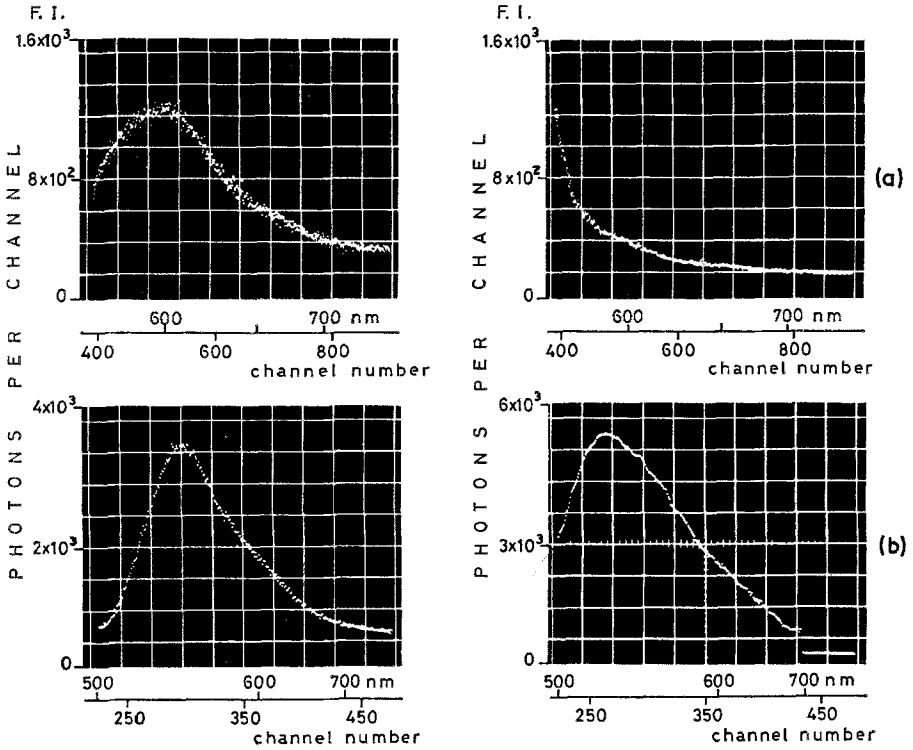

Figure 2. Fluorescence emission spectra (left: uncorrected; right: corrected). (*a*) Nucleus of frog erythrocyte stained with Rhodamine 3GO. (*b*) Nucleus of frog erythrocyte subjected to the Feulgen reaction with Acriflavine-SO_2.

The averaging is carried out on values corresponding to two contiguous channels, with a repeated back-and-forth procedure, including the whole series of channels; i.e. the values of each channel are averaged the first time with the value of the previous channel, and the second time with the value of the successive channel. For example, let us, for the sake of simplicity, consider only three channels; during the first averaging sequence, the value of channel 1 is averaged with the value of channel 2, and the result is stored in channel 1; the value of channel 2 is averaged with that of channel 3, and the result is stored in channel 2; the value of channel 3 represents the beginning of the second averaging sequence, which proceeds in reverse, that is, the value of channel 3 is averaged with that of channel 2, and the result is stored in channel 3; the value of channel 2 is averaged with the value of channel 1, and the value is stored in channel 2. This represents a complete averaging cycle, in which the value of each channel is averaged with that of

the preceding and subsequent one, except for the channels at the extremities, which are averaged one time less. The procedure may be repeated by going through the complete cycle several times. Obviously, the final result obtained is that the content of each channel is a weighted average of the original content of the channel itself and of a number of contiguous channels, with a weight distribution depending on the number of averaging cycles completed. It follows that this is taken into consideration in comparing various spectra; generally speaking, it is advisable to compare averaged spectra with each other with the same number of cycles, since the final form of the averaged spectrum also depends partly on the type of average made. Fig. 3 shows the same curve without the averaging procedure (*a*), after two averaging cycles (*b*), after four averaging cycles (*c*) and after eight averaging cycles (*d*) have been completed.

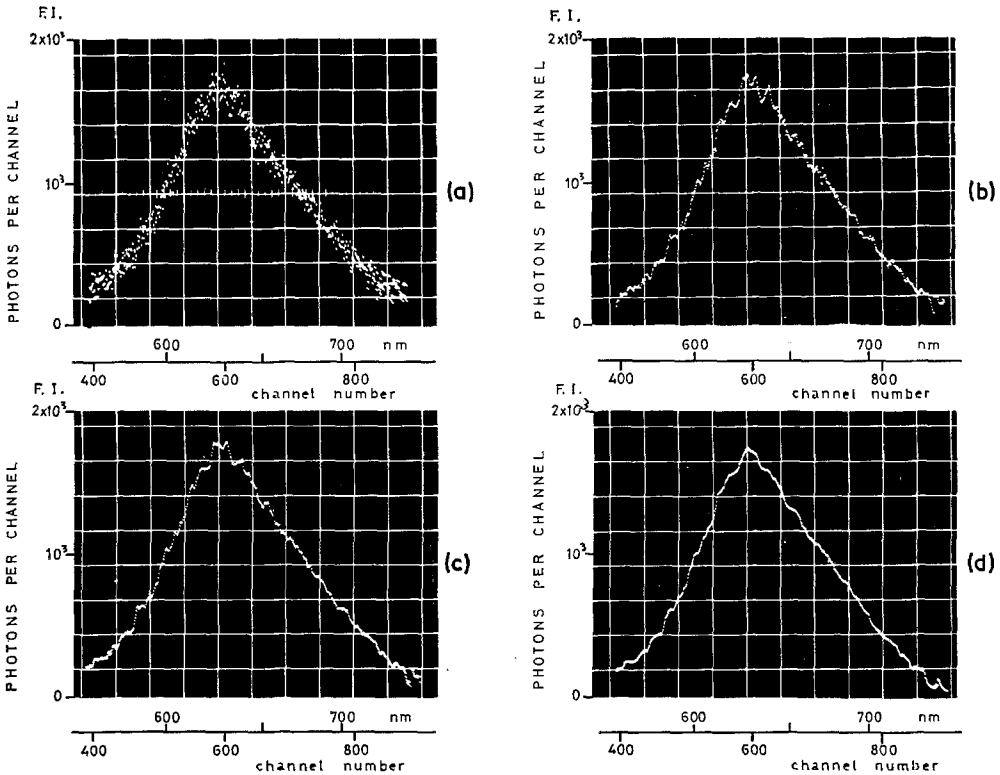

Figure 3. The same, uncorrected spectrum of a frog erythrocyte stained with the conventional Feulgen reaction: (*a*) without the averaging procedure; (*b*) after two averaging cycles; (*c*) after four averaging cycles; (*d*) after eight averaging cycles.

(4) *The integral.* The arithmetic unit is also set out for calculating the integral of the curve. This may be useful when scanning measurements are carried out on the preparation, by synchronizing with this geometrical scanning (instead of a λ scanning as described above) the movement of the oscillating mirror, or of a scanning stage; an example of this is the fluorescence curves of individual chromosome bands, a comparison of which makes it possible to deduce the kariotype (Caspersson, 1973). Knowledge of the integral may also be useful for spectral calculations to establish the extent of inner filter

effect. Indeed, the device makes it possible to calculate the integral itself, not only over the whole spectrum, but also on pre-established wavelength intervals.

Concluding remarks

One of the chief advantages of the device is the short operating time required for correcting spectral data and for averaging, as well as the possibility of performing these operations immediately, in the same place as the experiments itself. The correction time for a single channel is 630 msec, and since the correction factor data are punched on the tape in successive series of eight terms, and the passing from one series to the next involves the reader in a delay of 180 msec, it follows that the total time needed for correcting a spectrum distributed over 1024 channels is about 11 min. Since, normally, the spectrum occupies 512 channels, the total time for correction does not usually exceed 6 min. The averaging procedure is also fairly rapid. Mention should also be made of the limited cost, the simplicity of use (the device may also be operated by a person who has not been specially trained on computers or digital equipment), and the reliability of the system. For the operations previously described, the device is able to take the place of computers, which are not always readily accessible to the experimenter. In addition, it is an integral part of a microspectrofluorometric apparatus, the complete automation of which is gradually being achieved.

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References

CASPERSSON, T. (1973). Fluorometric recognition of chromosomes and chromosome regions. In: *Fluorescence Techniques in Cell Biology* (eds. A. A. Thaer & M. Sernetz), pp. 107–23. Berlin, Heidelberg, New York: Springer-Verlag.

COVA, S., BERTOLACCINI, M. & BUSSOLATI, C. (1973). The measurement of luminescence waveforms by single photon techniques. *Phys. stat. sol.* **18,** 11–62.

COVA, S., PRENNA, G. & MAZZINI, G. (1972). Microspectrofluorometry by single photon multiscaling. *Proceedings of 4th International Congress of Histochemistry and Cytochemistry*, pp. 161–2. Kyoto.

COVA, S., PRENNA, G. & MAZZINI, G. (1974). Digital microspectrofluorometry by multichannel scaling and single photon detection. *Histochem. J.* **6,** 279–99.

COVA, S., PRENNA, G., SACCHI, C. A. & SVELTO, O. (1974). Studies on cellular fluorescences excited by a nanosecond-pulsed tunable laser. In: *Excited States of Biological Molecules* (ed. J. Birks), in press. New York: Interscience.

PEARSE, A. G. E. & ROST, F. W. D. (1969). A microspectrofluorimeter with epi-illumination and photon counting. *J. Microsc.* **89,** 312–28.

PLOEM, J. S. (1967). The use of vertical illuminator with interchangeable dichroic mirrors for fluorescence microscopy with incident light. *Z. wiss. Mikrosk.* **68,** 123–42.

PRENNA, G., MAZZINI, G. & COVA, S. (1974). Methodological and instrumentational aspects of cytofluorometry. *Histochem. J.* **6,** 259–78.

RUCH, F. (1960). Ein Mikrospektrograph für absorptions Messungen im ultravioletten Licht. *Z. wiss. Mikrosk.* **64,** 453–68.

SACCHI, C. A., SVELTO, O. & PRENNA, G. (1974). Pulsed tunable lasers in cytofluorometry. *Histochem. J.* **6,** 251–8.