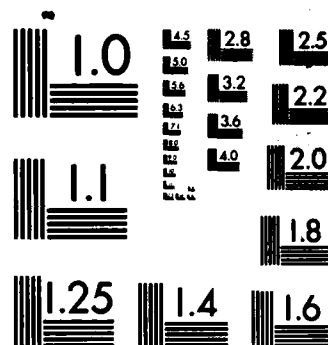


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TECHNICAL REPORT NO. 9

Time Domain Filtering of Two-Dimensional Fluorescence Data

by

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and Isiah M. Warner

Prepared for Publication
in Applied Spectroscopy

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Abstract

Four time domain filtering methods are applied to simulated and experimental two dimensional fluorescence data in order to evaluate their performance. The methods that were evaluated are 1) moving average, 2) Savitsky-Golay polynomial smoothing, 3) Chebyshev filtering, and 4) Bicubic spline filtering. The methods are compared using mean square error analysis and the difference in the amplitudes of the filtered noisy and ideal data. The two dimensional version of the Savitzky-Golay filtering and the spline method produced the best overall results.

Index headings : Time domain data filtering; Image analysis; Digital image processing; Two dimensional data analysis; Two dimensional fluorescence.

INTRODUCTION

The widespread use of microprocessors in analytical chemistry has greatly increased the capabilities for processing data in the laboratory.¹ Even the simplest set of data will require some processing since data will contain random errors, i.e. noise, that are superimposed upon and indistinguishable from the signal. Of fundamental importance to the experimenter is the reduction of this noise without unduly degrading the signal information. A large variety of mathematical methods, i.e. digital filters, have been developed to computationally reduce the noise in experimental data. Such filtering techniques are particularly amenable to spectral data such as fluorescence and absorbance. This is true because fluorescence and absorbance spectra are generally broad band (low frequency) while the random noise content of the data is usually sharp (high frequency).² Thus some of the high frequency distortion in a data set can be removed while minimizing the effects on the low frequency information using digital filters.

Data filtering can be performed in the object domain or in the frequency domain. In the object domain, which is also known as the spatial or time domain, the object and the filter are described in x-y coordinates or as dynamic functions varying in time, t. In the frequency domain, the object and filter are represented by Fourier series of time frequencies. Fourier transform filtering (FTF) and its time domain filtering (TDF) counterpart will yield mathematically identical results, because of the mathematical equivalence between the frequency domain and object domain representation of the data and the filter function.^{2,3}

As new analytical instruments and techniques emerge producing data with increased dimensionality, there exists a corresponding need for evaluating two dimensional filtering techniques. In this paper, four types of time domain filtering techniques are applied and compared for two dimensional fluorescence data. Frequency domain filtering is one of the oldest techniques and is still very popular. Since the development of the fast Fourier transform algorithms,² FTF has been considered more convenient, because it generally requires fewer mathematical operations. However, time domain filtering techniques have improved significantly in recent years and are now widely used in one and two dimensional signal processing. In many cases, time domain smoothing may require fewer mathematical operations than frequency domain filtering depending on the size of the digital image and on the nature of the filter. Moreover, finding the optimal filter, in the case of FTF, by a trial-and-error method usually requires more efforts, than in TDF.⁴ The reason for this is that FTF consists of three steps (performing the Fourier transform, multiplying by a filter function, then inverse transformation of the data), while in TDF the filter is directly applied to the data.

The FTF of two dimensional fluorescence data obtained by the videofluorometer(VF) has been used in our laboratory for several years.⁴ We developed the TDF methods as alternative filtering techniques which can be especially useful in cases when the optimum FTF is unknown. The purpose of the present study is to demonstrate the usefulness of time domain filtering in enhancing the information presented in two dimensional fluorescence data while providing a comparison of the different TDF methods.

This comparison can help in the selection of the optimal filtering method with regard to the special needs of the experimenter.

In the first section, a brief description of the methods (neighborhood averaging, polynomial convolution, Chebyshev, spline smoothing) is given. The next two sections of the report present the simulated and real data sets and the established criteria used in the comparison of the methods. The last section provides a comparison and discussion of the results.

DESCRIPTION OF SMOOTHING ALGORITHMS

Given an $N \times N$ digital image $f(x,y)$, our goal is to generate a smoothed image $g(x,y)$. A digital filter can be considered as convoluting (*) the raw data with some filter function $h(x,y)$.² For discrete functions

$$g(x,y) = h * f = \sum_{i=1}^{M_1} \sum_{j=1}^{M_2} h_{ij} f(x-i, y-j),$$

where the filter size is $(2M_1+1) \times (2M_2+1)$.

Least squares is the most common criterion for finding the proper filter function. The criterion may be defined as follows: a filter function $h(x,y)$ is selected such that the sum of the squares of the differences between the smoothed data, $g(x,y)$ and the experimental data, $f(x,y)$ is a minimum over the interval being considered.

At this point, it is useful to define the four time domain smoothing methods studied in this paper. We assume that the data are equally spaced, which is the case for the fluorescence data presented in this manuscript.

(i) Moving (neighborhood) average method (MA)

One of the simplest filters is the moving average (MA). In this procedure, the filtered image at every point (x,y) is obtained by averaging

the intensity values of the data points representing the function $f(x,y)$ in a predefined neighborhood (so called mask). The mask could be for example the $(2M+1) \times (2M+1)$ nearest neighbor points around a center point (x,y) displaced in both the x and y directions. Then one can assign to the central point (x,y) the average value of the intensities for the maskpoints (by simply adding the intensity at each of these points and then dividing by the number of points). The filter mask is then shifted one row or column to a new point (x,y) and applied to the next $(2M+1) \times (2M+1)$ nearest neighbor data points. Proceeding in this manner, the data in the first and last M rows and columns remains unfiltered since filtering these extreme rows and columns would require the mask to extend outside the region of the data. The moving average filter is equivalent to the convolution of the data set with a two dimensional function of constant value 1. Geometrically, it is the same as using a planar surface as a smoothing function.

(ii) Polynomial convolution method (PC)

A more accurate smoothing surface can be estimated by least square fitting of a two dimensional polynomial of order n to the $(2M+1) \times (2M+1)$ mask points. Using the value of the least squares polynomial over the mask around the (x,y) point, we assign the weighted average value to the (x,y) point. The method is then applied in a manner similar to the MA. This is the two dimensional version of the one dimensional Savitzky-Golay convolution method.^{5,6} The values of the integer coefficients necessary for using the PC method are given in reference 6.

(iii) Chebyshev method (CHEB)

This algorithm uses a small filter mask that is passed repeatedly over the image. The image is multiplied by successive filter coefficients,

$h_1, h_2, \dots$ after each pass and added together to obtain the filtered image. The h_i parameters are the one-dimensional filter coefficients with the same frequency-response characteristics as those of the desired two-dimensional filter (in our case, low pass filter coefficients). One set of the coefficients is given in Table I. The filtering scheme uses the recursion relation between the Chebyshev polynomials.⁷ More detailed information on Chebyshev methods and programs for obtaining the filter coefficients are available in references.^{3,8}

(iv) Spline method (SP)

A bivariate spline function, $s(x,y)$ is fitted to the measured data set by the method of least squares.⁹ A bivariate spline function is a piecewise two variable polynomial satisfying continuity conditions regarding the function and its derivatives. The definition of the bivariate spline and the fundamentals of the method are given in Table II. The number and the position of the knots of the smoothing spline are determined automatically by an iteration procedure. In general, $s(x,y)$ is given by different polynomials in adjoining intervals. In addition to the datapoints and the degree of the spline, a smoothing parameter has to be given to control the tradeoff between closeness of fit and smoothness of fit (Table II).

The Savitzky-Golay convolution and the spline method may seem to be identical since both use least squares fitted polynomials. However, the two methods are very different. The SP method fits splines (more general functions than polynomials) to a set of automatically determined (x,y) points (as in Table II) while the PC method fits a polynomial to the raw data points in a mask.

Table I. The coefficients for the Chebyshev method

the coefficients of F low pass filter coefficients (Wiener filter)

for 3×3 mask:

1 2 1

2 -4 2

1 2 1

the h_i parameter values:

$h_1 = 0.454$, $h_2 = 0.307$,

$h_3 = 0.027$, $h_4 = -0.116$,

$h_5 = -0.074$, $h_6 = 0.059$.

Table II. The mathematical definition of the bivariate spline function

Let $D=[a,b] \times [c,d]$ be a rectangular domain. Consider the strictly increasing sequences of real numbers

$$a = r(0) < r(1) < \dots < r(g+1) = b; \quad c = p(0) < p(1) < \dots < p(h+1) = d.$$

Then the function $s(x,y)$ is called a spline of degree k in x and l in y , with knots $r(i)$, $i=1,2,\dots,g$ in the x -direction and $p(j)$, $j=1,2,\dots,h$ in the y direction, if :

- (i) On any subrectangle $D(i,j)=[r(i),r(i+1)] \times [p(j),p(j+1)]$, $i=0,1,\dots,g$; $j=0,1,\dots,h$, $s(x,y)$ is a given polynomial of degree k in x and l in y ;
- (ii) All partial derivatives $\partial^{i+j}s(x,y)/\partial x^i \partial y^j$ for $0 \leq i \leq k-1$ and $0 \leq j \leq l-1$ are continuous in D .

The algorithms were programmed in Fortran and an IBM 3030 was used for the computations. The experimental data was collected from the VF by a Hewlett Packard 9845B minicomputer and transferred to the mainframe using terminal emulation software.

TEST DATA

In order to compare the performance of the various filters we tested them on simulated and experimental two-dimensional data.

(i) Data Type I: simulated data with known signal/noise (S/N) ratios

Since the typical fluorescence matrix often can be approximated as a combination of Gaussian peaks in both the x and y directions¹⁰, we simulate the ideal data with known signal/noise ratios as surfaces, $z(x,y)$, produced from two or four Gaussian peaks with a noise function added at each data point. The noise function, generated from a random variable, RND, is: $RND \times (\sqrt{z} + c)$. The random variable RND, is equally distributed on the interval (-1,1) and $\sqrt{z}$ denotes the square root of the noiseless function, c is a constant approximating the mean value of the background noise. The term $RND \times \sqrt{z}$ approximates the photon statistical noise present in the data. Figure 1a shows an ideal dataset, while in Figure 1b the corresponding noisy surface is presented. A 64x64 digital image size was used since that matrix size (or smaller) is conveniently used in fluorescence studies.⁶

(ii) Data Type II: experimental data acquired by the VF

Since the FTF of two dimensional fluorescence data has been used in our laboratory for several years,⁶ it seemed natural to demonstrate the usefulness of the TDF techniques on similar types of experimental data. The VF collects data in matrix format. The elements of the matrix represent

the fluorescence intensity of the sample at a unique excitation and emission wavelength pair. This data is referred to as an excitation-emission matrix (EEM). The VF and its operating parameters have been documented elsewhere.¹¹ Figure 2a shows the EEM of rubrene and Figure 3a is the EEM of anthracene. Both samples were obtained from Aldrich Chemical Co. at 98+% purity and used without further purification. Glass-distilled cyclohexane (Burdick and Jackson) was used as the solvent.

COMPARISON CRITERIA

Two quantitative measures of the suitability of a filter have been used for comparison of the different smoothing algorithms. Our first criterion for effective data smoothing is a minimal mean square error (MSE) between the filtered data and the desired result (ideal data in the case of the simulated data sets). The smaller the MSE, the better the filter. In addition to using MSE, we compare the absolute differences of the ideal and smoothed noisy functions at the peak maxima to estimate the influence of the smoothing operation on the amplitude of the signal.

The mean square error between the filtered data, $g(x,y)$, and the ideal data, $z(x,y)$, is defined as

$$MSE = 1/nr \times nr \left(\sqrt{\sum_{x=1}^{nr} \sum_{y=1}^{nr} [z(x,y) - g(x,y)]^2} \right) \quad (1)$$

where $nr \times nr$ is the number of filtered data points (for the PC and MA algorithms, $nr = N - M$; otherwise $nr = N$). Before computing the MSE and the absolute differences at the maximum peak value we normalize the ideal and the smoothed noisy simulated data (Type I). For the experimental data (Type II), a smoothed matrix may be compared to a relatively noise-free matrix of the

same component.* Let us assume that $g(x,y)$ is the filtered version of the EEM, $f(x,y)$. We then define $z(x,y)$ to be the ideal result of the filtering operation performed on $f(x,y)$. When we are smoothing $f(x,y)$, $z(x,y)$ may be approximated by acquiring the matrix of a more concentrated sample that has been subjected to signal averaging of replicate matrices, $z'(x,y)$. Since $z(x,y)$ and $f(x,y)$ must have the same overall intensity for comparison, it will be necessary to scale down $z'(x,y)$ using Equation 2:

$$z(x,y) = z'(x,y) \times f_{\max}(x,y) / z'_{\max}(x,y) \quad (2)$$

where $z'_{\max}(x,y)$ is the maximum of the "ideal" data matrix and $f_{\max}(x,y)$ is the corresponding point in the filtered data matrix.

By substitution of Equation 2 into Equation 1, we obtain the final usable form of the MSE for our real data set, i.e.:

$$MSE = 1/N^2 \sum_{x=1}^{nr} \sum_{y=1}^{nr} \{g(x,y) - [z'(x,y) \times f_{\max}(x,y) / z'_{\max}(x,y)]\}^2$$

This MSE is not necessarily the minimum MSE of the two data sets. The calculations required to generate the minimum MSE are more complicated than the ones presented here, and since our purpose is to compare the methods, the values generated using this method will suffice. In a practical sense, $z(x,y)$ is the best (most noise free, best resolved, etc.) version of $f(x,y)$ which we can conveniently obtain using the VF. However, since this data is obtained with a real instrument, it is not a true $z(x,y)$.

The second measure of filter performance is the difference in the absolute maxima of the filtered and ideal data set. Of course, this value is only available for synthetic data. However, the experimenter will be able to approximate this value for familiar data sets. These values have

been included in Table III which also lists the mean square errors for the simulated data set shown in Figure 1.

RESULTS AND DISCUSSION

The results of applying the four TDF methods to the simulated data are presented in Figures 1c, d, e, f and Table III. The results of the filtering of the experimental data are illustrated in Figures 2c, d, e, f and Table IV for rubrene and Figures 3c, d, e, f and Table V for anthracene. The comparison of their effectiveness is based on the value of the MSE and the absolute difference between the maximum value of the ideal and smoothed noisy data. The values of the MSE in Table IV indicate that all four methods can be successfully used for filtering. However, the MA method will often oversmooth the data, resulting in an unacceptable loss in the maximum peak value. The PC and SPLINE methods with properly chosen parameters produce a well smoothed image and a good approximation of the maximum values of the peak of the ideal data. The CHEB filtering preserves the maximum value of the peak better than the MA, but not as well as the PC and SPLINE methods.

The selection of the proper parameters for the different methods was based on both theoretical considerations and the performance of the method in practice. For the MA and PC methods, the mask size can be regarded as a parameter. In evaluating each method for the best parameter values, we found the following information. For our data sets, the desired smoothing was obtained using 5×5 and 7×7 masks. These two methods are identical for the 3×3 masks³. The high resolution can be lost in the case of PC filters if masks greater than 7×7 are used. This is probably because the bandwidth of our data is usually less than 7 points. The MSE is monotonically decreasing for PC as the mask size is increased. However, for the MA the use

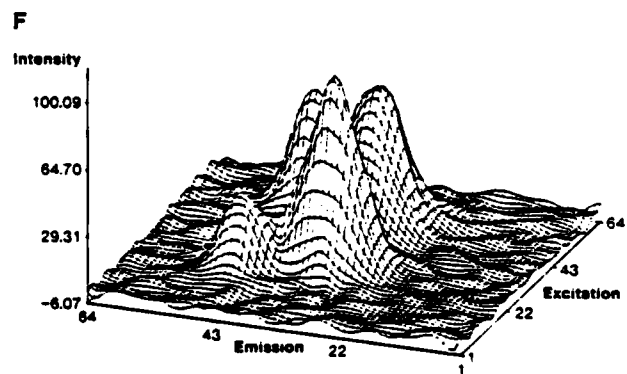
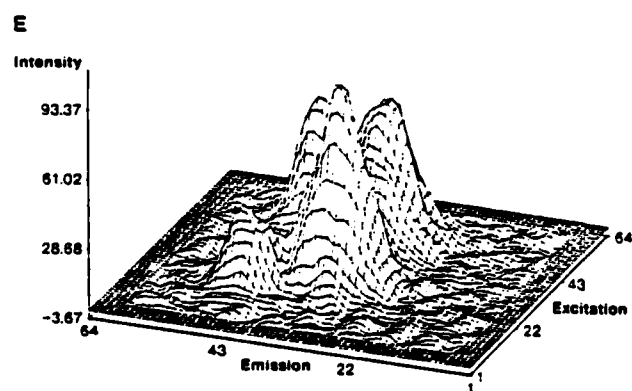
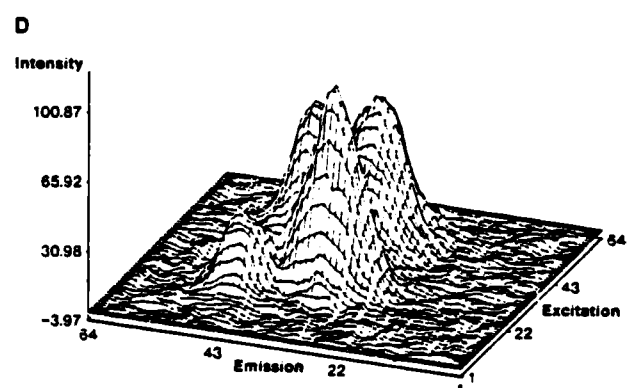
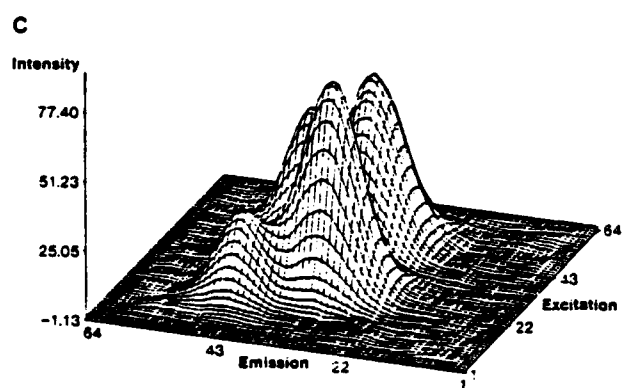
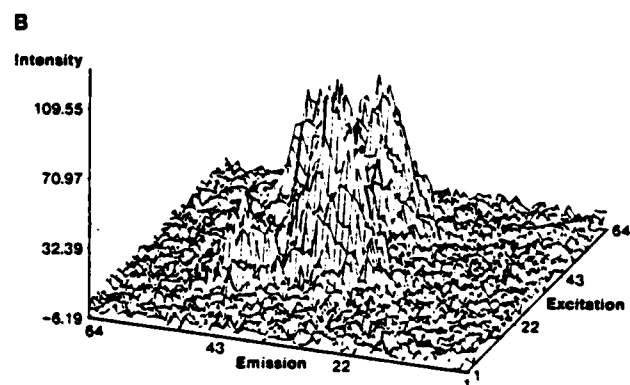
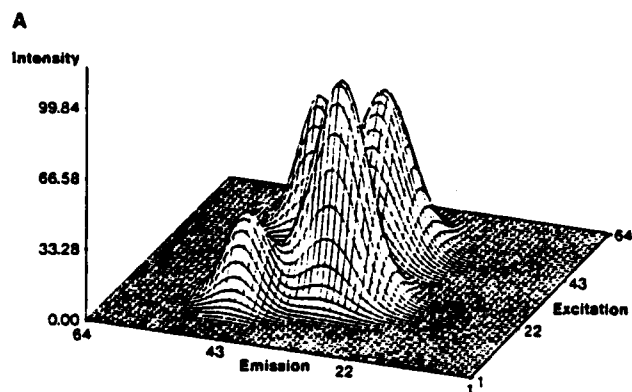


Figure 1

Table III. The mean square errors and the absolute difference in
maxima for the simulated data

METHOD	MSE	DIFF. IN MAX.
MA 5×5	3.5	11.0
MA 7×7	11.0	22.0
PC 5×5	3.3	1.0
PC 7×7	15.0	3.0
CHEB	4.4	7.0
SP (S=70000)	2.5	2.0

Table IV. The mean square errors for Rubrene

METHOD	MSE
MA 5×5	14.0
MA 7×7	30.0
PC 5×5	2.2
PC 7×7	3.5
CHEB	4.8
SPLINE (S=8500)	2.3

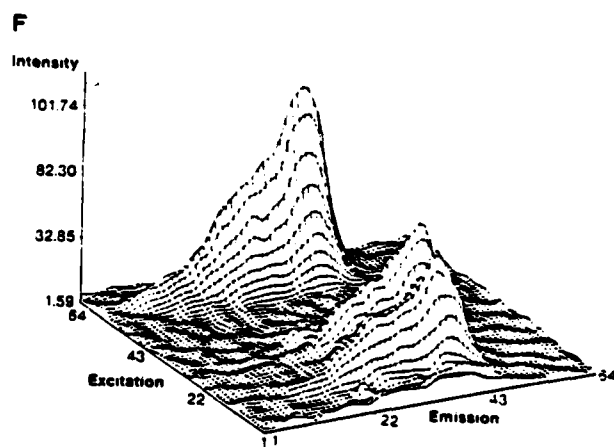
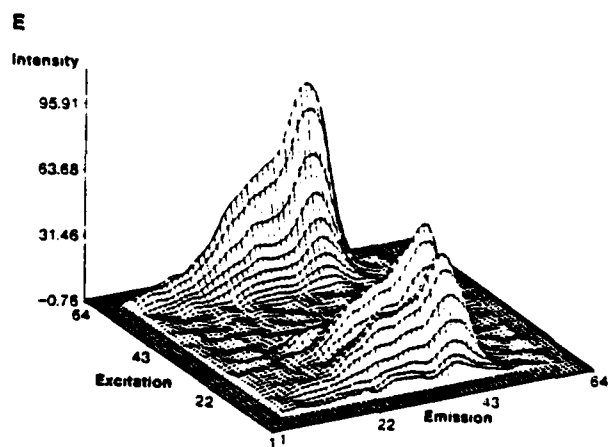
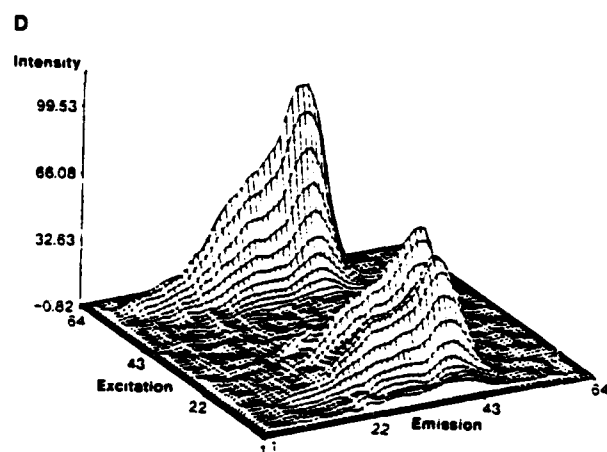
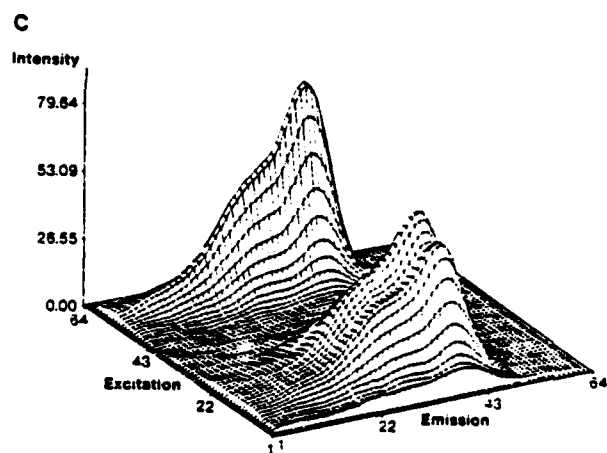
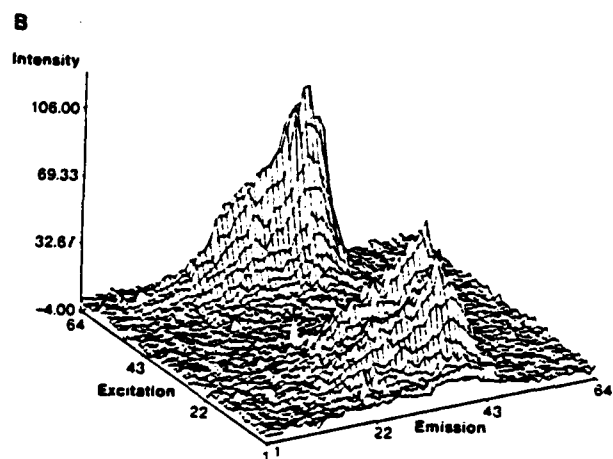
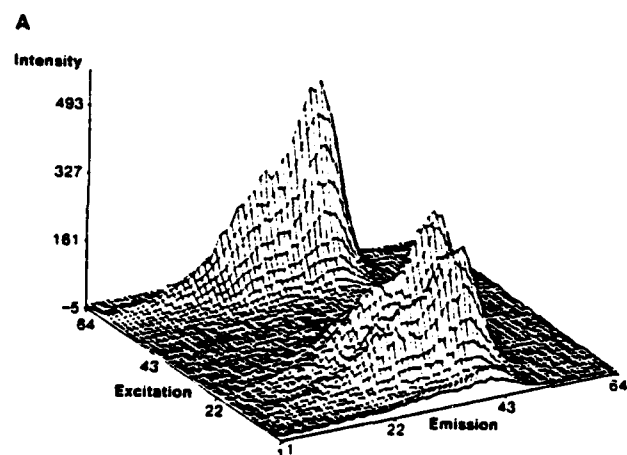


Figure 2

Table V. The mean square errors for anthracene

METHOD	MSE
MA 5×5	6.5
MA 7×7	8.4
PC 5×5	4.1
PC 7×7	4.8
CHEB	2.4
SPLINE (S=8000)	3.5

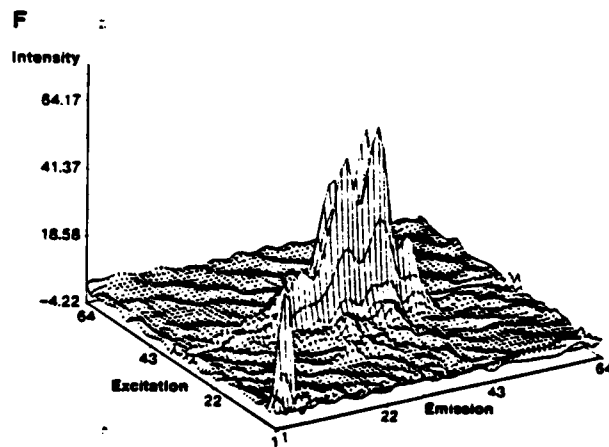
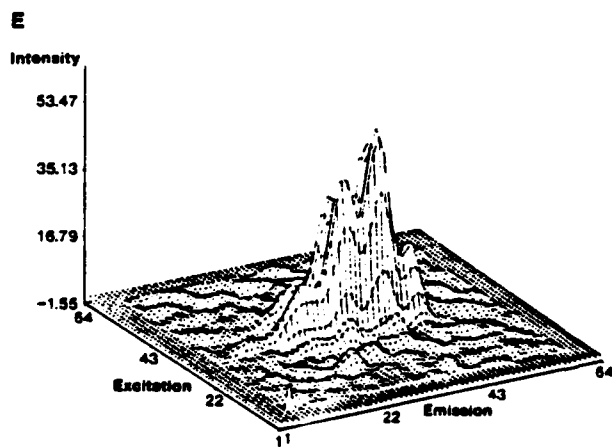
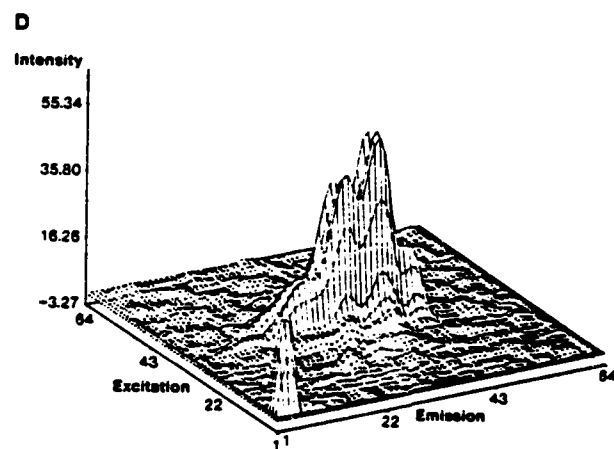
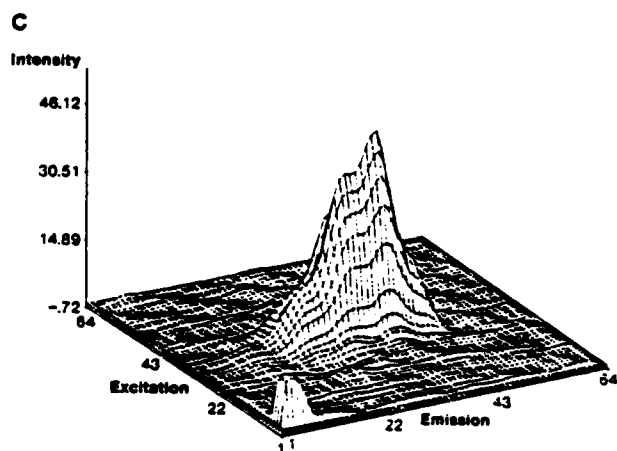
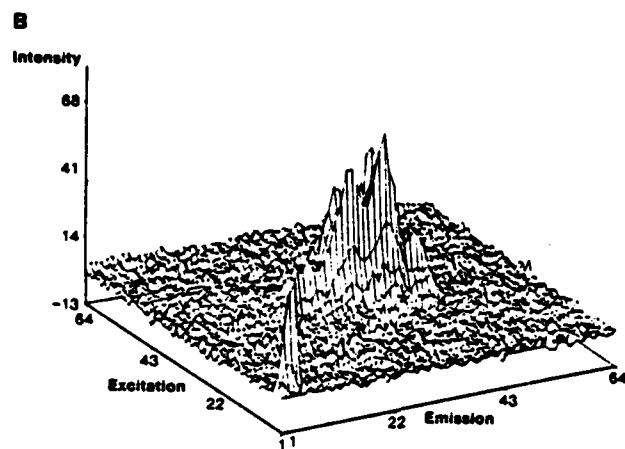
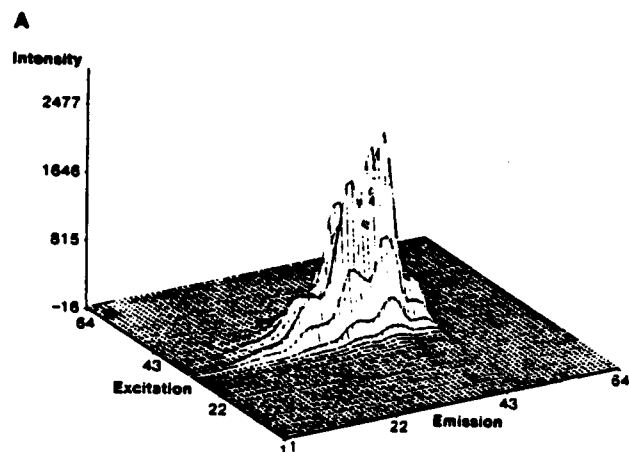


Figure 3

of a larger mask size resulted in an increased MSE. As we discussed in the description of the methods, when a mask's size is $(2M+1) \times (2M+1)$, the data in the first and last M rows and columns remains unfiltered. This may also affect the choice of the mask size. For our 64×64 fluorescence data, the 5×5 and 7×7 masks provide the desired smoothing. In the case of the PC method, higher order surfaces usually filter with less degradation in resolution. However, in all cases considered the second and third order surfaces give relatively good results.

Several sets of low pass filter coefficients have been tested for the CHEB method. Table I provides those values of the coefficients for which the filtering has been satisfactory. A large number of CHEB filter coefficients can be generated corresponding to the special requirements of the user by the algorithms in the references.^{3,8}

In the case of the SPLINE method, we found that the bicubic splines produce a good fit to the data. The value of the parameter controlling the filtering was, as expected around the $N \times N \times V^2$,⁹ where N is the size of the image in one direction and V is the statistical error of the data. If the control parameter S is too small, then the spline approximation picks up too much noise (under-smoothing); if it is too large, the filtered image is too smooth and the signal can be lost (over-smoothing). For the simulated data the estimation of the control smoothing parameter was relatively easy, because the value of the statistical error in the data was accurately known. If nothing is known about the statistical errors in the raw data, then S has to be determined by trial and error. We think that a further development of the method could include an automatic determination of the smoothing parameter while maintaining the possibility of selection. In our

laboratory, we found that the PC and SPLINE methods give the best fit, i.e. for these two methods the MSE between the ideal and smoothed noisy data were the smallest and the maximum value of the "ideal" data was well approximated.

Since in many cases the speed of computation can be important we include Table VI containing the number of multiplications for the different filtering methods. On most minicomputers, multiplication is much slower than addition, therefore the number of multiplication steps may be used to estimate the speed of the computation. However, it is difficult to make unqualified statements about the computing time required for different filtering methods, because of variations in processor speed and other hardware features of different computers. But in most cases, for example, the TDF filtering is faster than the other algorithms up to mask sizes of about 11×11 . This relatively large TDF filter is adequate for many applications. For larger filter sizes, the Fourier transform method using the fast Fourier transform is probably preferable.

CONCLUSION

This study has demonstrated the usefulness of the time domain filtering techniques for enhancing the information of two dimensional fluorescence data. We have found that the TDF methods are easily implemented and work satisfactorily in most applications of interest on fluorescence data. The PC and SPLINE methods produced the overall best results based on the criteria for "best-fit" established in this manuscript. However, the SPLINE method needs an input parameter which has to be found by trial and error and in actual applications may require additional efforts.

Table VI. The number of multiplications for the different filtering methods for NxN images (N=64)*

	<u>Time domain filtering</u>	<u>Fourier filtering</u>
Conv.	$N \times N \times (2 \times M + 1) \times (2 \times M + 1)$	
	M=2: 102400	
	M=3: 200700	
Sym. conv.	$0.6 \times N \times N \times (M + 1) \times (M + 1)$	$N \times N \times (4 \times \log_2 N + 1)$
	M=2: 22100	102400
	M=3: 39300	
CHEB	$N \times N \times (4 \times M + 1)$	
	M=1: 24600	

* the numbers in brackets show the actual number of multiplications for our case (rounded)

Future studies will concentrate on the use of high pass and band pass filtering of fluorescence data using TDF methods and on the further investigation and development of TDF techniques. For example, we have assumed identical resolution in the x and y dimensions of our data. Since this is not always the case, it would be useful to examine the effects of nonequivalent resolution on the performance of time domain smoothing methods. We also hope to develop an algorithm which will evaluate the spline smoothing control parameter which will make the method much more practical.

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FIGURE CAPTIONS

Figure 1. Filtering of the simulated data

- (a) noiseless data; (b) noisy data; (c) moving average 7×7 mask;
- (d) polynomial convolution 5×5 mask; (e) Chebyshev method;
- (f) spline method (S=70000)

Figure 2. Filtering of rubrene

- (a) 1.3×10^{-6} M rubrene; (b) 6.7×10^{-8} M rubrene;
- (c) moving average 5×5 mask; (d) polynomial convolution 5×5 mask
- (e) Chebyshev method; (f) spline method (S=8500)

Figure 3. Filtering of anthracene

- (a) 7.0×10^{-5} M anthracene; (b) 1.4×10^{-6} M anthracene;
- (c) moving average 5×5 mask; (d) polynomial convolution 5×5 mask
- (e) Chebyshev method; (f) spline method (S=8000)

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