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Classification of Gynecologic Flow Cytometry Data
A Comparison of Methods

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Several discriminant function methods for automatically classifying flow cytometry data from human cervical material were developed and compared with previously published methods using a sample of 186 specimens. The misclassification rates (approximately 20%) were similar to those of other published techniques for classifying these data. The methods misclassify different cases, however. The apparent system performance appears to be limited by at least three factors: (1) use of too small a sample in constructing classification algorithms, (2) poor "visibility" of small numbers of abnormal cells in the flow histograms and (3) incorrect or inconsistent visual classification of the samples used to construct the classification algorithms. The third factor results in erroneously high estimates of the misclassification rate. Even so, the overall system performance appears to be comparable to that of many cytotechnologists.

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Flow cytometry (FCM) has been extensively investigated[3,6,10,18-20] as a method for screening uterine cervical material for the early detection of malignant and premalignant changes. Although several algorithms have been published for classifying patients on the basis of the two-dimensional flow histograms obtained by FCM,[3,10,18-20] their performance has fallen short of that desired for a practical screening method. It has not been clear whether this apparent deficiency in system performance resulted from deficiencies in the pattern classification algorithms or from limitations in the raw data obtained due to problems of sample preparation and staining or problems inherent in the FCM technique. It is difficult to separate these two sources of error when evaluating the system as a whole. Nevertheless, one would suppose that if several different pattern classification algorithms were to result in the same misclassifications when applied to a constant set of patient data, the errors would probably result from limitations in the information content of the cytometry data. On the other hand, if the algorithms were to misclassify different patients, at least some of the difficulty would probably lie in the pattern classification schemes. In this case, it might be possible to substantially improve system performance by combining features of more than one pattern classification method.

In this study we tested several approaches to the classification of FCM histograms. The results are compared, with the goal of discovering sources of error in the FCM screening system as a whole. Finally, the expected performance of various systems in a "real world" operating environment is discussed.

Materials and Methods

The cases studied were those accumulated by Habbersett et al[10]; specimen preparation and FCM analysis are presented in detail in that paper. Two hundred

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nine samples of cervical material from patients treated at family planning and gynecology clinics were collected on Dacron swabs, suspended in alcohol, stained with propidium iodide and fluorescein isothiocyanate (PI-FITC) and analyzed on a Los Alamos Scientific Laboratory Flow Sorter. This procedure yields two-dimensional 64×64 histograms with red fluorescence on one axis and green fluorescence on the other. Twenty-three specimens consisted of fewer than 10,000 cells; these were considered inadequate for diagnosis and were not analyzed further. The remaining 186 samples were classified into five classes (I = normal; II = negative with atypia; III = dysplasia; IV = carcinoma *in situ;* V = invasive carcinoma) by consensus of three cytologists. These visual classifications were used as the basis for developing automatic classification techniques for the FCM data. The 124 specimens in classes I and II were considered "normal" for this purpose, while the 62 specimens from classes III, IV and V were considered abnormal.

The computer algorithms used in the data analysis were based on the mathematical techniques of discriminant analysis and the singular value decomposition. A brief exposition of the singular value decomposition is contained in Appendix I.

Classification Method 1

In method 1 (Figure 1), a peak at one corner of the flow histograms, corresponding to nonstaining cells, was first set to zero in each case. Next, the flow histograms were partitioned into 64 8×8 blocks; the elements of each block were then summed. This reduced the data for each patient to an 8×8 matrix, which was then normalized so that its elements summed to one. The within-group means of the 64 elements thus obtained were calculated for both normal and abnormal case groups. Visual inspection of these 64 pairs of means showed that only 32 pairs differed significantly. These 32 elements were then used as the basis of a stepwise linear discriminant analysis.[12,13] Only the eight elements that contributed significantly to the discriminant function were retained in the final discriminant function. These seemed to reflect the dispersion of the squamous cell peak in much the same way as earlier studies had.[6,10] The frequency of misclassification was estimated using the "hold-one-out" method.[16] This is perhaps the simplest of all the methods proposed for classification of flow cytometry data, requiring a minimum of computation.

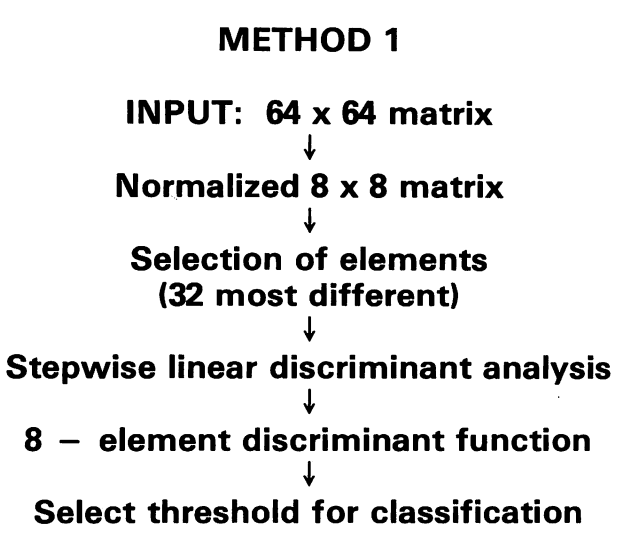

Figure 1
Outline of classification method 1.

Classification Method 2

In method 2 (Figure 2), the original 64×64 histograms were reduced to 32×32 by 2×2 partitioning and summing, similar to method 1, after zeroing the nonstaining cell peak. The left and right singular vectors corresponding to the largest singular values of these matrices were then computed using the Golub-Reinsch algorithm,[9] as implemented in EISPACK.[8] The outer product of these vectors constitutes the best (in a least-squares sense) rank-one approximation of the 32×32 matrix. Examination of the sample data shows that this singular value and singular vector pair generally contains approximately 50% to 60% of the information in the original 32×32 matrix. Following their computation, the elements of these vectors were used in a stepwise linear discriminant analysis, which reduced to ten the number of important features.

Although the singular value decomposition has been used as a tool in image restoration,[1,2] to our knowledge it has not been used directly as part of a pattern classification algorithm.

Classification Method 3

In method 3 (Figure 2), the singular values of the 32×32 matrix obtained in method 2 were normalized to sum to one. These were then analyzed using stepwise linear discriminant analysis. The rationale for classification based on the singular values is as follows. There is a fundamental change in the nature of the cells populating the female genital tract in dysplastic and neoplastic states. Elements of the normal

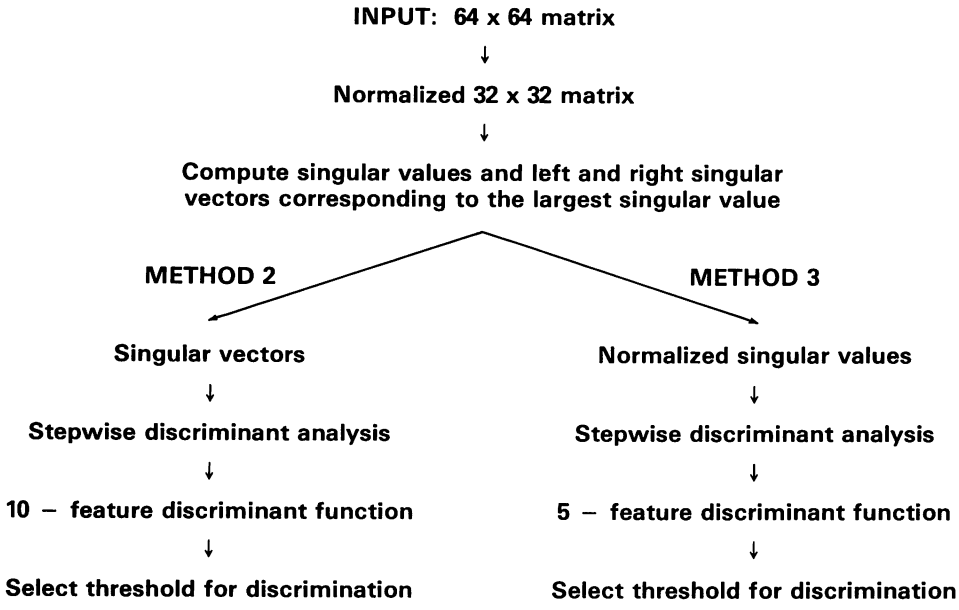

Figure 2
Outline of classification methods 2 and 3.

genital epithelium are still present, but abnormal elements are also present in varying numbers, usually increasing in percentage as the severity of the dysplastic/neoplastic process increases. The rank of the data matrix might be expected to reflect the number of different cellular elements present.[22] If a single cell type were present, the rank of the data matrix would be expected to be less than if two cell types were present. The rank of the data matrix might be expected to increase as more cell types with different fluorescence histograms were added to the specimen.

Other Classification Methods

Several other related classification algorithms were investigated. All began by zeroing of the nonstaining cell peak. Then the histograms were reduced to 8×8 matrices as in method 1. One method summed the rows and columns of the 8×8 matrix, yielding a two-vector characterization of the axes of the flow histogram. The elements of these vectors were then used as features in a linear discriminant analysis. This method is reminiscent of that used by Sprenger et al.[19,20] A second gave a two-vector representation by calculating the singular vectors of the matrix as in method 2. These vectors were then used as the data for discriminant analysis. A third method used the singular values obtained in the above process in a discriminant analysis.

In all of the classification algorithms, the constant of the discriminant function was chosen near the point where the total error of classification is minimized. In several cases it was shifted slightly from this point to reduce the number of false-negative classifications.

Results and Discussion

The results for methods 1, 2 and 3 are summarized in Table I. The performance of the singular value method (method 3) was not as good as expected. Although the general trend we predicted, that the first few singular values of the abnormal specimen matrices would be larger than those of the normal specimen matrices, seems to be true, there are many individual exceptions. Attempts to define an "effective rank" for these matrices, based on the number of singular values greater than some threshold, fail to give good separations of normal and abnormal specimens. This occurs at least in part because there are several different sources of changes in the rank of the matrices. In addition to the increase in rank caused by the presence of cytologically atypical cells, the rank may be increased by the presence of inflammatory cells that, although often seen in specimens thought to be dysplastic or neoplastic, are not always present and may be seen in material from patients with no cytologic abnormality. Errors made by this method (method 3) tended to parallel those of the singular vector method (method 2), although there were more errors overall. Both of these methods gave results that differed significantly from those of method 1, particularly in er-

Table I *Analysis of PI-FITC Data for Screening of Gynecologic Cytology Specimens*

Method	Positive called negative (%)	Negative called positive (%)	Overall (%)
1	31	11	18
2	23	20	21
3	37	19	32
Two-step	10	27	20
Fourier	31	15	20
Combined	18	18	18

rors in assigning visually abnormal specimens.

Although the misclassification rates for the first two methods were nearly the same, analysis of the canonical variable for the methods showed better overall separation of groups for method 2 than for method 1. For this reason method 2 is preferable: selection of the discriminant function constant is easier since finding the optimal constant is less likely to be complicated by sample noise.

None of the algorithms discussed above under "Other Classification Methods" performed at a level comparable to these three. The singular vector method was far superior to the vector-summing method, as would be expected. The singular vector method for the 8×8 matrix was less effective in classification than that based on the 32×32 matrix, however. An attempt to classify using the singular values of this 8×8 matrix failed. These results suggest that data useful in classification are lost when reducing the matrix from 32×32 to 8×8.

Table I also presents data for the two-step method and the Fourier classification method (as described previously by Habbersett et al[10] and Bentley et al,[3] respectively). We see that the error rates are comparable to those of the methods described in the present work. Table II shows the visually assigned classifications for the cases incorrectly classified by any of the five methods. The list tabulated for the two-step method[10] is not strictly comparable to those for methods 1 to 3, however, for the two-step method rejected groups of data if an associated pooled normal control was misclassified (see Habbersett et al[10] for details). Cases that were rejected for this reason are marked with a "§" in Table II. Also, the list of cases misclassified by the Fourier method is incomplete, for the original calculations are not all available. Many cases were misclassified by several methods, but only one was misclassified by all.

The overlap in misclassification between method 1 and the other methods proposed in this paper was small. For this reason we attempted to construct a linear discriminant function based upon those features known to be useful from investigation of methods 1 and 2. The results shown in Table I for this "combined" method were somewhat disappointing. We might have hoped that the total number of misclassifications would be lower than with either of the methods that were combined. There was little improvement, however, and the combined classifier misclassified some cases that were not misclassified by either of the methods that were combined. The combined method, however, did misclassify fewer class IV and V patients that did either method 1 or 2. These results, particularly the misclassification of specimens thought to be severely abnormal on the basis of visual classification, again raise the possibility that some of the classification errors that have been encountered in flow cytometry are due to limitations in the information content of the FCM data and will not be eliminated by the development of new pattern classifiers.

What are some of the possible sources of error in classification? The first, mentioned above, is a fundamental limit in the amount of information contained in the two-dimensional flow histogram. The number of atypical cells may be too small to permit their identification in the midst of a large number of normal cells. Even if the data are present in the flow histogram, they may be impossible to include in a discriminant function of reasonable size constructed on the basis of the relatively small number of cases we have used. Use of a larger number of cases might well permit the inclusion of a larger number of features for discrimination and subsequent improvement of the algorithms.

Second, and equally important, is error in visual classification of the input data. While Lachenbruch[14,15] has shown that small errors in assigning initial classifications result in small changes in the true rate of misclassification using linear discriminant functions, the effect on the apparent rate of misclassification determined by the hold-one-out method is certain to be more pronounced and no less than the rate of initial misclassification. For classification of samples drawn from two multivariate normal distributions, the rates E_N and E_A of misclassification of normal and abnormal specimens, respectively, estimated by the hold-one-out method are nearly the same as those estimated by the formulas

$$E_N = \Phi\left[\frac{-\ln(p_N/p_A)+D^2/2}{D}\right]$$

and

$$E_A = \Phi\left[\frac{\ln(p_N/p_A) - D^2/2}{D}\right]$$

where p_N is the proportion of normal specimens in the sample, p_A is the proportion of abnormal speci-

Table II *Misclassified Cases*

	FCM classification method						Visual
Case	1	2	3	TS	F	C	classification
3			x				I
6		x	x				I
9	x		x		x		I
11			x			x	II
12	x	x	x		x	x	II
13			x				I
14	x						I
17	x						I
20			x				I
23	x	x				x	I
24	x	x	x	x		x	II
25			x				I
30			x				I
31	x	x	x			x	II
32			x				II
33						x	II
34	x						I
35						x	I
36	x		x				I
38			x				I
42	x		x				I
43			x				I
45	x		x			x	I
47					x		I
50			x	§			pooled normal
52				§	x		I
55		x		§			I
56			x	§			I
57			x	§			II*
59		x		§			II*
60			x	§			pooled normal
61			x	§			I
63		x		§		x	II
65		x	x	§		x	II
68		x	x	§	x	x	I
71		x					I
72		x				x	I
73				x	x		II
75		x	x	x	x		I
77						x	I
79		x					II
80					x		I
82			x				pooled normal
83				x	x		II
85	x	x		x		x	II
89			x				pooled normal
90		x				x	I
92				x	x		I*

Table II *(continued)*

	FCM classification method						Visual
Case	1	2	3	TS	F	C	classification
97		x	x	§		x	II*
98				§	x		II*
99			x	§			I
100		x	x	§	x	x	I
103		x		§	x	x	I
104		x	x	§	x	x	II
108				x			I
109		x	x	x	x	x	II
110		x	x	x	x		II
112				x			I
113				x			I
114				x			II
116		x		x			I
117			x	x			I
118		x		x	x	x	I*
119				x			I
120				x			II
121			x	x			I
125		x					IV
126		x			x		IV
131		x	x	x		x	V
134	x	x				x	III
136				x			III
137	x	x	x	x	x	x	III
139			x				V
140		x	x	§	x	x	III
141			x	§			III
142			x	§			III
144				§	x		III
146			x	§			V
147	x			§			IV*
148	x	x	x		x	x	V
151			x				III
152	x		x				III
154	x	x			x		III
155	x						III
156	x		x				V
157			x				V
158			x				V
159	x					x	III
160				x			III
161			x				III
162			x				III
165	x						III
166	x						III
167			x				III
168	x						III
169	x						III
170	x	x	x			x	III
171	x	x	x			x	III
176	x						V
178			x				V
180			x				V
182			x				V
183			x	x			V

TS = two-step method; F = Fourier method; C = combined method; x = misclassified case; § = case not considered because of misclassified pooled normal specimen; and * = significant disagreement in interpretation among the three cytologists.

mens, D^2 is the squared Mahalonobis distance estimated on the basis of the sample used to construct the discriminant function and Φ is the cumulative normal distribution function. The true misclassification rates are properly estimated by

$$E_N = \Phi\left[\frac{-\ln(p_N/p_A) + \delta^2/2}{\delta}\right]$$

and

$$E_A = \Phi\left[\frac{\ln(p_N/p_A) - \delta^2/2}{\delta}\right]$$

where δ is the separation between the "correctly classified" populations. If fractions α_1 and α_2 of the normal and abnormal specimens are initially misclassified, D will tend to $(1-\alpha_1-\alpha_2)^2\delta^2/(1+Z\delta^2)$, where Z is positive, and may thus severely underestimate δ^2 (see Appendix II). The effect of misclassification is illustrated in Tables III and IV, in which the effects of misclassification for the Mahalonobis distances and the apparent error rates are summarized. This estimate of the bias is based on the assumption that input misclassification errors are randomly dispersed in the sample. If there is a nonrandom distribution, which seems likely, the effect on the estimated errors of misclassification may be even more severe, as may be the effects on the calculated discriminant function.[15]

The number of "misclassification errors" in the input data is potentially quite significant. Of the 209 specimens that were visually classified in this study, there was significant disagreement on whether a specimen was normal (class I or II) or abnormal (generally class III) in 21 specimens. Most of these were not "misclassified" by the pattern recognition techniques used here. Those that were are identified with an asterisk in Table II. This rate is consistent with reports in which significant discrepancies in the interpretation of cytologic material have been found in 10% to 20% of the cases.[4,7,11,17] It may well be that many of the "misclassifications" reported in this and other studies of automated cytology techniques should be regarded as no more than "disagreements" *with* the cytopathologist of the sort that occur routinely *between* cytopathologists. If, in fact, the input misclassification rate for our sample were 10%, the actual false-positive and false-negative rates for method 2 and for the combined method could be less than 5%, as compared with about 20% reported in Table I. The fact that several of the misclassified specimens were felt to be severely abnormal (as in cases 156 to 158) suggests that while the difficulties with input classification may account for some of the deficiencies in system performance, they probably do not account for all of them. Nevertheless, even experienced screeners occasionally misclassify cytologically severely abnormal specimens, and sometimes specimens with severe cytologic atypia do not reflect histologically confirmable malignant or premalignant disease. In any event, it seems likely that the error rates estimated in this study and in all other studies of automatic cytologic screening are higher than the true error rates of classification. Only by use of noncytologic criteria, such as clinical course or (less desirably) biopsy, can optimal criteria for cytologic classification and accurate estimates of the error rates of various classification algorithms be determined.

How would these systems perform in the real world? If we accept all cases in the test samples as correctly classified, the combined method achieves a sensitivity of 90% with a specificity of 30% in an operating environment having 10% abnormal specimens (this algorithm has a false-positive rate of 23% at the point on the ROC curve where the false-negative rate is 10%). This performance is comparable to that being achieved with high-resolution image analysis systems.[21] It is also comparable to the performance of many cytotechnologists,[4] though not the best.[5]

Table III *Squared Mahalonobis Distances (D^2) Expected for Populations Separated by Distance δ for Samples Misclassified with Rates $\alpha_1 = \alpha_2 = \alpha$*

δ^2	α=0.05	α=0.10	α=0.15
1.0	0.77	0.59	0.43
4.0	2.72	1.88	1.30
16.0	7.33	4.20	2.57

Table IV *Expected and Apparent Rates of Misclassification (E_N and E_A) for Specimens Drawn from Populations Separated by Distance δ and Initially Misclassified with Rates $\alpha_1 = \alpha_2 = \alpha$*

	α = 0.00		α = 0.05		α = 0.10		α = 0.15	
δ^2	E_A	E_N	E_A	E_N	E_A	E_N	E_A	E_N
1.0	0.12	0.43	0.11	0.64	0.10	0.70	0.08	0.76
4.0	0.09	0.26	0.11	0.34	0.12	0.43	0.12	0.52
16.0	0.02	0.03	0.05	0.14	0.09	0.25	0.11	0.36

Appendix I

The singular value decomposition of an n×n matrix G is a factorization of it as the product of three matrices:

$$G = USV'$$

where the columns of V are eigenvectors of $G'G$, the columns of U are eigenvectors of GG' and S is a diagonal matrix whose element are the square roots of the eigenvalues of $G'G$ (or, equivalently, GG'), arranged in decreasing order. This decomposition has many applications in statistics, but the property we rely on in this work is that a good approximation to G can be obtained by truncating the three matrices. In fact, of all matrix products WTX' where W and X are $n \times r$ matrices and T is an $r \times r$ matrix ($r \leqslant n$), the one that best approximates G in a least-squares sense is $U_1S_1V_1'$, where U_1 is the matrix containing the first r columns of U, V_1 contains the first r columns of V and S_1 is an $r \times r$ diagonal matrix containing the first r diagonal elements of S. The columns of U and V are called the left and right singular vectors, respectively, and the diagonal elements of S are called singular values. The number of nonzero singular values is equal to the rank of the matrix.

Appendix II

Lachenbruch[14] has previously considered the estimation of error rates in classifying by means of discriminant analysis members of samples drawn from two multivariate normal populations with equal covariance matrices Σ when there are errors in initial assignment of samples used to construct the discriminant function. His published result contains an error, so the derivation is repeated here with the correct result.

The optimal discriminant function for classification of a sample is

$$D_T(X) = X - 0.5(\mu_1+\mu_2)'\Sigma^{-1}(\mu_1-\mu_2)$$

where μ_1 and μ_2 are the means of the two populations. The population Mahalonobis distance is given by

$$\delta^2 = (\mu_1-\mu_2)'\Sigma^{-1}(\mu_1-\mu_2).$$

The variance of $D_T(X)$ is δ. For the sample with covariance matrix S, the counterpart to δ found in the discriminant analysis is given by

$$D^2 = (\bar{x}_1-\bar{x}_2)'S^{-1}(\bar{x}_1-\bar{x}_2).$$

If α_1 is the proportion of the n_1 observations from the first sample that really belong to the second population and α_2 is the proportion of the n_2 observations from the second sample that really belong to the first population, then for large n_1 and n_2

$$x_1 = (1-\alpha_1)\mu_1 + \alpha_1\mu_2$$

and

$$x_2 = (1-\alpha_2)\mu_2 + \alpha_2\mu_1.$$

S tends to Σ^* where

$$\Sigma^* = \Sigma + (\mu_1-\mu_2)(\mu_1-\mu_2)'\frac{(c_1+c_2)}{(n_1+n_2)}$$

for $c_i = \alpha_i(1-\alpha_i)n_i$.
Σ^{*-1} is given by

$$\Sigma^{*-1} = \Sigma^{-1} - \frac{\frac{(c_1+c_2)}{(n_1+n_2)}\Sigma^{-1}(\mu_1-\mu_2)(\mu_1-\mu_2)'\Sigma^{-1}}{1 + [(c_1+c_2)/(n_1+n_2)]\delta^2}$$

and D^2 thus tends to

$$\frac{(1-\alpha_1-\alpha_2)^2\delta^2}{1+Z\delta^2}$$

where

$$Z = (c_1+c_2)/(n_1+n_2).$$

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