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Crystalline object evaluation by image processing

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INVESTOR IN PEOPLE

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Abstract

Purpose – The purpose of this paper is to propose a state discrimination for crystallization samples (droplets), the purpose of which is to discriminate between diffractable extracts (crystal) and other objects.

Design/methodology/approach – The line feature from the image of the protein droplet was extracted and the state discriminated using a classifier based on line features. A support vector machine is used as the classifier.

Findings – In order to verify the performance of the proposed method, the growth state was discriminated experimentally using the images taken by TERA, an automated crystallization system. The correction ratio was determined to exceed 80 percent.

Originality/value – Contribution to automated evaluation process of the growth state of protein crystallization samples.

Keywords Crystallization, Image processing

Paper type Research paper

Introduction

Recently, the clarification of protein structures and functions for the purposes drug discovery and clarifying vital activity has been studied extensively in structural genome science, and higher-throughput protein structure analysis (in terms of both quantity and speed) is required. High-throughput X-ray structure analysis is a commonly used method of protein structure analysis. In the crystallization process using X-ray structure analysis, it is necessary to obtain fine-quality protein crystals by crystallization with protein solutions. However, specific crystallization conditions (factors that facilitate the crystallization of protein solution, such as temperature, types of chemicals to be added) of

protein solution are generally unknown. In order to examine the numerous possible combinations of crystallization conditions, numerous experiments under various crystallization conditions must be conducted, and this limitation has prevented the realization of high-throughput X-ray structure analysis. Thus, research and development of a robotic crystallization system is important for the realization of a high-throughput crystallization process.

Currently, the TERA automatic crystallization system is under development at the RIKEN Harima Institute (Sugahara and Miyano, 2002). In this crystallization system, a protein solution-blending unit and a handling unit for administrating the droplets have been already realized. This system is also equipped with a camera unit and can automatically capture images of crystallization droplets. Figure 1 shows the TERA system and a number of sample images captured using the TERA system. The acquisition image size is $1,392 \times 1,040$ pixels, and the pixel size is $4.65 \times 4.65 \mu\text{m}$.

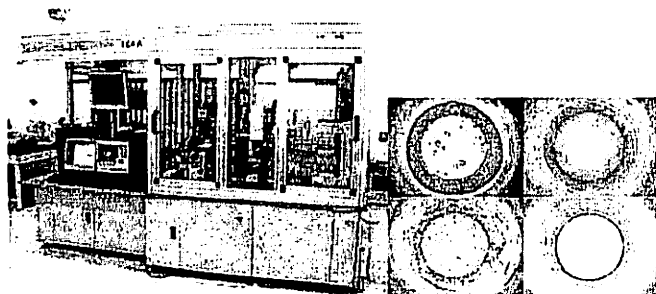
However, at present, the task of identifying samples that contain subjects for X-ray diffraction experiments is

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Figure 1 Automatic crystallization system – TERA – and examples of autographed images



performed manually. Thus, the images have been evaluated through observation by human experts (observers). The score decision is also conducted in order to evaluate and record the growth condition of each crystallization droplet by matching crystals with the score decision criteria. TERA can take 500,000 images per month, and it is difficult to process all of the images by observers. Therefore, an efficient protein structure analysis experiment to realize automation of the score decision work is needed.

Previous studies on automated growth evaluation of crystallization solutions employed various methods, including the utilization of polarized filters (Bodenstaff *et al.*, 2002), a rotating-polarizing filter (Echalier *et al.*, 2004), and image processing (Cumbaa *et al.*, 2003, Zuk and Ward, 1991). Rupp (2003) used phase congruency to detect a large number of small crystals and Gester *et al.* (2003) automated the counting of the number of crystals to generate three-dimensional surface plots of the crystals and to determine crystal size based on the length of the perimeter of the crystals. Miyatake *et al.* (2005) developed an automated crystallization/observation robotic system: HTS-80, which was reported to be able to categorize the crystallization droplet status into four stages based on extracted contour information. Most of these previous studies focused on the existence or absence of a crystal. No detailed study to determine whether detected crystals are suitable for X-ray diffraction experiments has yet been conducted. Nonetheless, for efficient high-throughput protein structure analysis, it is important to determine whether a specific crystal from among a large number of crystallization droplets is suitable for X-ray diffraction analysis.

Thus, in previous studies, the determination of whether a detected crystal is actually a subject for the X-ray diffraction experiment was not discussed in any detail, despite its importance to efficient protein structure analysis. In order to realize a high-throughput crystallization process, a large number of crystallization samples should be treated, and it is important to judge whether there is sufficient subject crystal for the X-ray diffraction experiment.

The reason why it is not easy to automate such observation processes is thought to be that there is no definition of detailed criteria for evaluation. In the present paper, we attempt to evaluate the incipient growth states of protein crystallization droplets by image processing and statistical analysis in order to achieve a categorization accuracy of more than 80 percent in individual classes, whereas the evaluation accuracies in each class for the above-mentioned methods are not stabilized.

Categorization of crystallization droplets

The purpose of the present study is to determine whether the crystallization droplet contains the subject for the X-ray diffraction experiment. In the score decision criteria (Figure 2), scores between 6 and 9 indicate the condition in which the sample contains the subject, and scores between 0 and 5 indicate the condition in which the sample does not contain the subject. We realized a highly accurate determination of the scores 0, 1, 2, 3, and 4–9 in a previous study Saitoh *et al.* (2004, 2005). Thus, in the present study, we discuss only scores between 4 and 9, and images of crystallization droplets evaluated scores between 4 and 9 are classified into either category A, in which a sample containing the subject for the X-ray diffraction experiment is indicated by scores between 6 and 9, or category B, in which a sample that does not contain the subject for the X-ray diffraction experiment is indicated by scores between 4 and 5 (Figure 3).

Scores between 5 and 9 indicate that the sample contains protein crystals. A score of 5 (microcrystal) is not considered to be an experimental subject for the X-ray diffraction experiment due to its insufficient size. The relationship between score and crystal size is shown in Table I. A score of 4 (amorphous grain) denotes that the sample is not crystal and is not an experimental subject for the X-ray diffraction experiment.

Figure 4 shows some typical examples of images captured by the TERA system. The proposed method was evaluated using such images, which depict several crystalline objects in

Figure 2 Score decision criteria

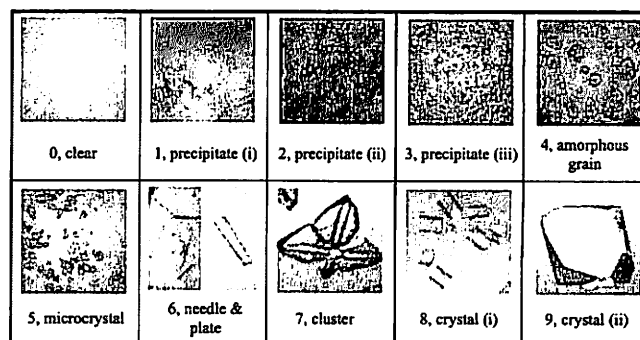


Figure 3 Decision category

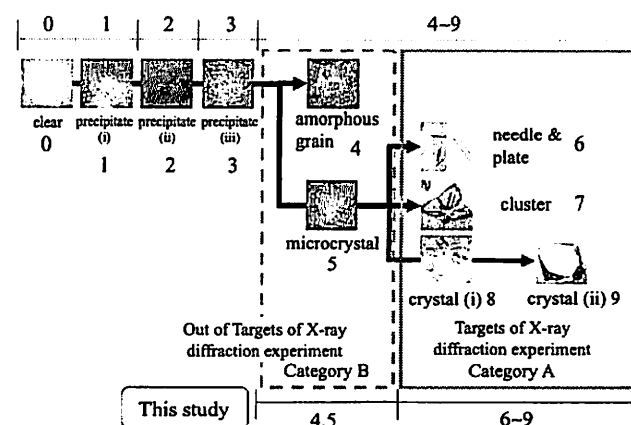
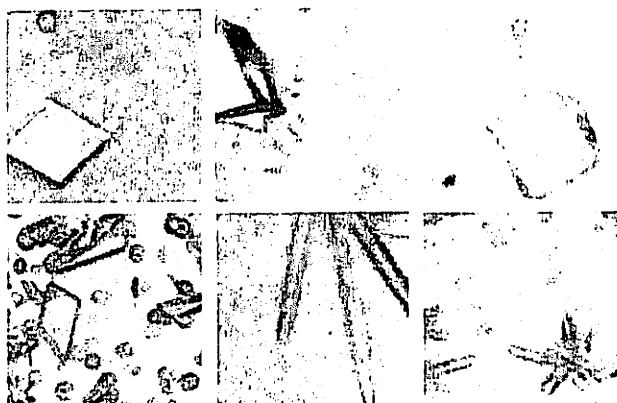


Table 1 Categorization criteria

	Score 5 (Category B)	Scores 6 ~ 9 (Category A)
Size		
Length (long side)	Less than 0.05 mm	0.05 mm or more
Thickness	Less than 0.01 mm	0.01 mm or more
Width	Less than 0.01 mm	0.01 mm or more

Figure 4 Examples of images obtained by TERA. Several growth states are present in the same droplet



various stages of growth (amorphous grain, microcrystal, and crystal) in the same droplet. For example, some of the droplets contain amorphous grains and microcrystals, and others contain microcrystals and crystals.

In the present paper, we discuss a method by which to determine whether a detected crystal is actually a subject for the X-ray diffraction experiment based on image processing.

Proposed method

The determination process, in which it is determined whether the crystallization droplet contains an object for the X-ray diffraction experiment, is a sort of pattern recognition process. The determination process is used to categorize images of crystallization droplets as images that contain the subject for the X-ray diffraction experiment or images that do not contain the subject for the X-ray diffraction experiment. The pattern recognition process consists of a sequence of preprocessing, feature extraction, and determination. For accurate determination, it is important that proper features are selected for the problem. In order to evaluate the second half of the growth period (scores between 4 and 9), the experts observe objects in the crystallization droplet (such as crystal amorphous grain). The experts commented that they determined whether the subjects were appropriate for the X-ray diffraction experiment based on shape or size. Thus, the characteristic shapes of objects are regarded as characteristic contours, and these contours are employed as feature values. Figure 5 shows the workflow of the proposed method.

Preprocessing

For the extraction of feature values, images are preprocessed, and the contours of the objects are extracted as binary edge images (Figure 6). For this purpose, original color images are

Figure 5 Workflow of the proposed method

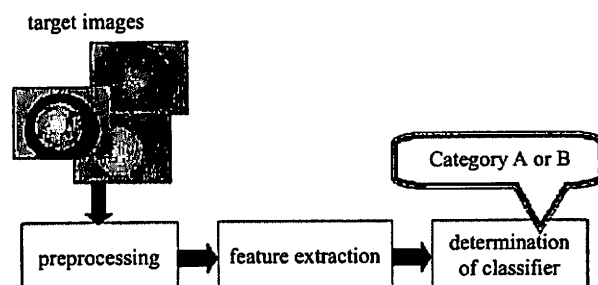
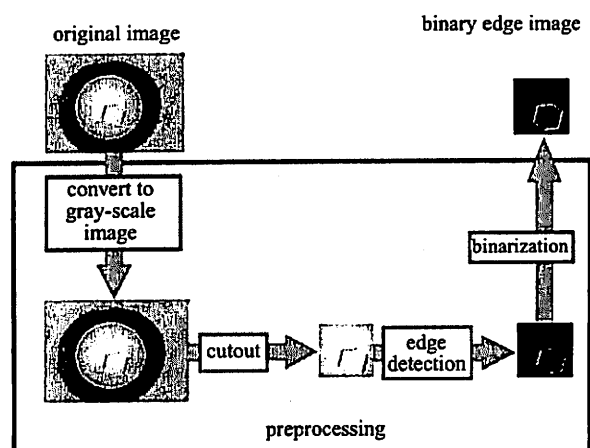


Figure 6 Workflow of preprocessing



converted into 256-level grayscale images. Since, the original images depict not only the crystallization droplet but also the lateral side of the droplet plate and near the base of the plate, only part of the image containing the crystallization droplet need be cut from the grayscale images. The size of the cut-out images was set at 450×450 (pixel) such that most of the droplet at the base could be included. In the present paper, we focus on crystal growth evaluation. Therefore, in this stage, the images are cut out manually and an automated system is a subject of future research.

Next, the edge detection process is applied to the cut-out images in order to emphasize the contours of objects in the crystallization droplet. Among the various edge detection methods, the Sobel transformation, which is a general method, was employed in the present study. Following the Sobel transformation, binarization processing is conducted on the edge-detected images to extract the contours of objects as the binary edge images. In the usual binarization, a certain threshold value is fixed, and only the pixels with grayscales that are equal or greater than the threshold value are considered as objects (edge pixels). However, the threshold configuration remains a key problem. When crystallization droplet images of the objects were binarized using the threshold value that was configured by the discriminant analysis, several problems were encountered. For example, a subtle concentration difference in the crystallization droplet was extracted as a contour line, and the contours of the objects could not be completely extracted.

More concretely, the edge magnitude distribution of 100 images that did not contain any objects from among the

images with a score of 0 (clear) was investigated (Figure 7). A threshold value of 29 that binarized 99.9 percent pixels of this distribution was employed as a background to extract the contour lines. As a result, TERA maintained its illumination condition for capturing the image, and so the threshold value can be used as a fixed value in this experiment.

Here, we discuss the determination of the threshold value for the images captured by TERA. The determination sequence can be applied to the images of another system when such a system can maintain the same condition for capturing the image of the droplet.

Feature extraction

Using the binary edge images of the contours of the extracted object that were obtained by preprocessing, the effective feature values for the determination were extracted based on the relationship between the size of the crystals and the scores, as shown in Table I. Comparison of the contour lines belonging to category A and category B shows that most of the contour lines of crystals belonging to category A are longer than the contour lines of microcrystal (score 5) belonging to category B (Figure 8).

In addition, if the curved contours lines of non-crystalline grain (score 4) consist of a collection of short line segments, the contour lines of objects in category B have short line segments. In other words, in categories A and B, the lengths of the line segments that comprise the contour lines of the objects differ from each other (Figure 9).

Consequently, the longest line segments within the images (maximum length, L_{max}) were utilized as the feature value. Furthermore, the number of objects in images belonging category B tends to be greater than that in the images belonging category A. As a result, the number of line segments is also proportionally greater (Figure 10). Therefore, the number of line segments within the object images (N_{all}) was employed as a feature value.

In order to extract the maximum length, L_{max} , a number of line segments, N_{all} , from among the feature values, linear features are scanned within the binary edge images. The edge pixels that exist sequentially in-line are considered to be the line segments, and their lengths and numbers are determined. However, since it is impossible to predict either the location at which objects will be formed in the crystallization droplet or the location of contour lines that will be obtained by preprocessing,

Figure 7 Edge magnitude distribution of clear images

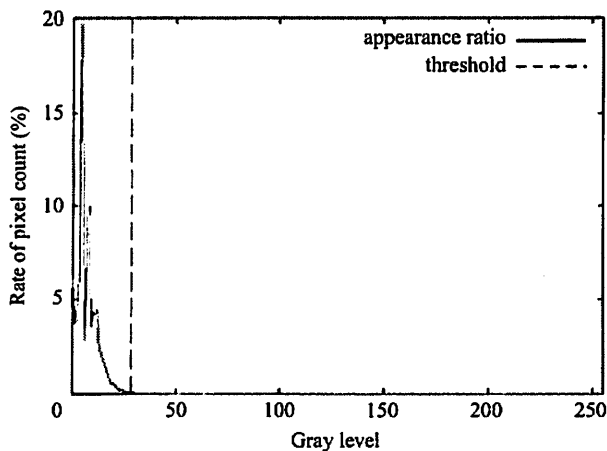


Figure 8 Examples of categories A and B

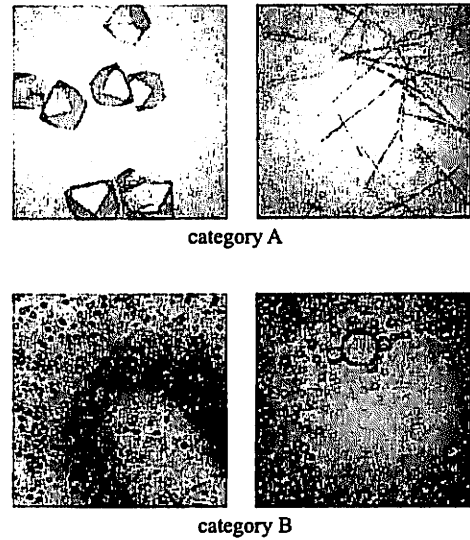


Figure 9 Contour lines comprising the objects

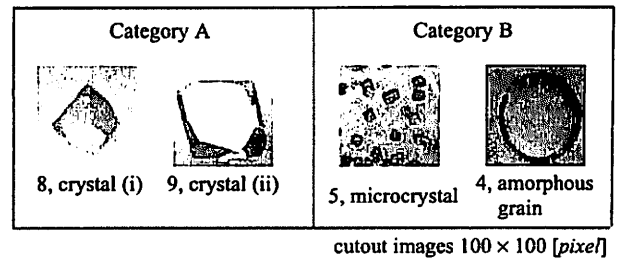
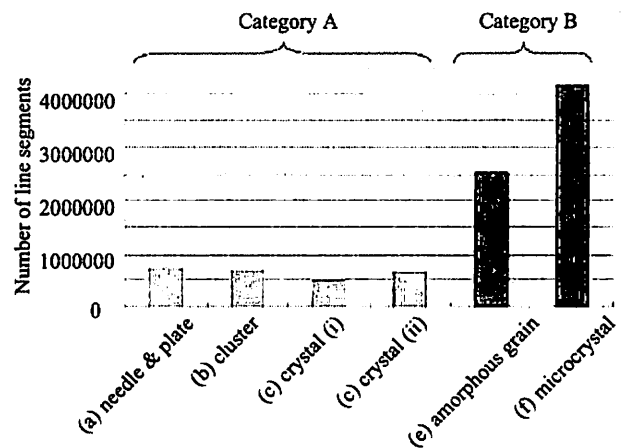
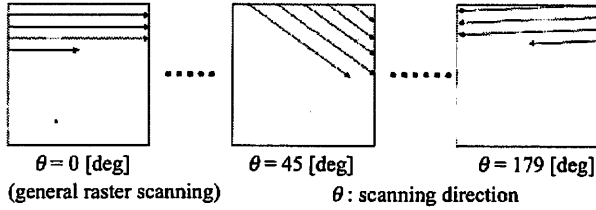


Figure 10 Number of line segments in categories A and B



the object images must be scanned from every possible direction. Thus, raster scanning was applied by altering the scanning direction, θ , ($0 \leq \theta < 180$ (deg), resolution: 1 (deg)) (Figure 11).

An edge pixel that was detected on a scanning line, when $n-1$ line segments had already been detected, was counted as the n th line segment, and the pixel was considered to be the origin of the n th line segment $S_n(x_{ms}, y_m)$, and the scanning

Figure 11 Raster scanning in the θ direction

process was continued. Next, when the edge pixel was discontinued on the scanning line or a scanning point was reached at the margin of an image, the last edge pixel was considered to be the terminal $E(x_{en}, y_{en})$. In this case, the length of the n th line segment (L_n) was defined as:

$$L_n = \sqrt{(x_{en} - x_{sn})^2 + (y_{en} - y_{sn})^2} \quad (1)$$

and is added to the total number of line segments. If a total of m line segments were detected at the completion of scanning, then the maximum length L_{\max} and the number of line segments N_{all} can be evaluated as:

$$L_{\max} = \max(L_n) \quad (1 \leq n \leq m) \quad (2)$$

$$N_{\text{all}} = m, \quad (3)$$

respectively.

Classifier for categorization

The maximum length of the extracted feature values, L_{\max} , and the number of line segments, N_{all} , are input to the classifier to determine whether the object images belong to either category A or B. Various classifiers, for example, self-organizing neural nets (Spraggon *et al.*, 2002), C5.0 (Bern *et al.*, 2004), and Bayes theorem (Wilson, 2002), have been used in previous studies for crystal image analysis. Although various identifiers are available, the proposed method employs the support vector machine (SVM) (Vapnik, 1995), (Duda *et al.*, 2001), which provide efficient for two-class identification.

The SVM performs pattern recognition for two-class problems by determining the separating hyper-plane with the maximum distance to the closest points of the training set. These points are called support vectors. If the data is not linearly separable in the input space, a nonlinear transformation $\Phi(\cdot)$, which maps the data points $\mathbf{x} \in \mathbf{R}^n$ into a high-dimensional space H called a feature space, can be applied. The data in the feature space is then separated by the optimal hyper-plane, as described above.

The mapping $\Phi(\cdot)$ is represented in the SVM classifier by a kernel function $K(\cdot, \cdot)$ that defines an inner product in H , i.e. $K(\mathbf{x}, \mathbf{x}') = \Phi(\mathbf{x})^T \Phi(\mathbf{x}')$. The decision function of the SVM has the form:

$$f(\Phi(\mathbf{x})) = \sum_{i=1}^r \alpha_i y_i K(\mathbf{x}, \mathbf{x}_i) \quad (4)$$

where r is the number of data points, and $y_i \in \{-1, 1\}$ is the class label of training points \mathbf{x}_i . Coefficients α_i in (5) can be determined by solving a quadratic programming problem with linear constraints. The support vectors are the nearest points to the separating boundary and are the only points for which α_i in (5) can be nonzero.

Examples of admissible kernel functions are the polynomial kernels:

$$K(\mathbf{x}, \mathbf{x}') = (\mathbf{x}^T \mathbf{x}' + 1)^d \quad (5)$$

where d is the degree of the polynomial, and the Gaussian kernels:

$$K(\mathbf{x}, \mathbf{x}') = \exp(-\|\mathbf{x} - \mathbf{x}'\|^2 / 2\sigma^2) \quad (6)$$

where σ is the variance of the Gaussian.

The SVM is used for classifying categories A and B based on line features extracted from the droplet image.

Experiment and results

In order to verify the effectiveness of the proposed method, the experiment is conducted using the SVM as classifiers. In this experiment, 866 images (600 images of category A and 266 images of category B), the correct categorization of which was performed ahead of time by experts, were prepared. Then, 300 of these images (200 images of category A and 100 images of category B) were used as training data to construct the classifier, and the remaining 566 images (400 images of category A and 166 images of category B) were used as test data to estimate the identification ability of the classifier. These images were autographed by several TERA systems at different times, and the possibility exists that these images may be slightly different based on the illumination condition. Figure 12 shows some of the actual images used in the experiment.

The images of training data were preprocessed, and the line features were extracted. Next, using the extracted features, the classifier was constructed using each classifier to decide the discrimination function. The classification performance was then evaluated using test data. Using the extracted features, the discrimination function is constructed using the SVM algorithm ($\sigma = 0.01$). The classification performance of the SVM was then evaluated using the test data.

As a result, using the SVM, the correct answer ratio for the learning data were 86.3 percent (Table II (a)) and the correct

Figure 12 Examples of experimental images

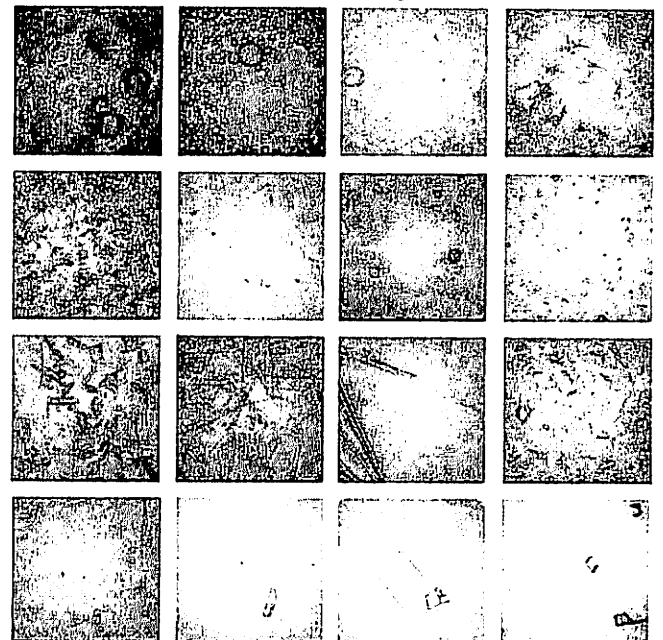


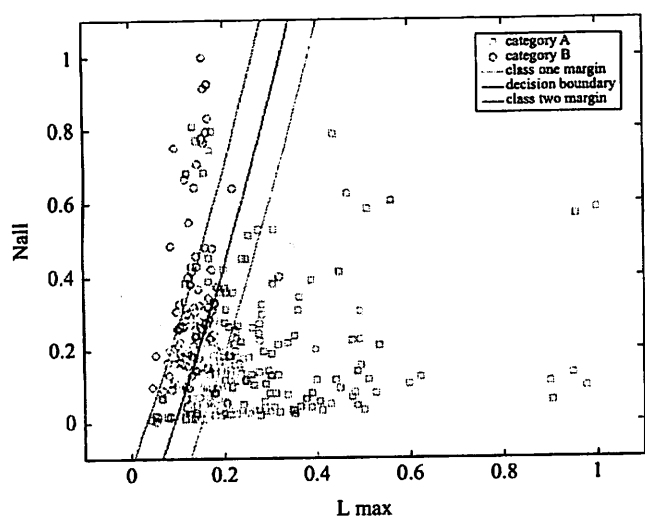
Table II Classification results of SVM

		Classification result	
		A	B
(a) Results using training data			
Human evaluation	A	180	20
	B	21	79
(b) Results using test data			
Human evaluation	A	334	66
	B	14	152

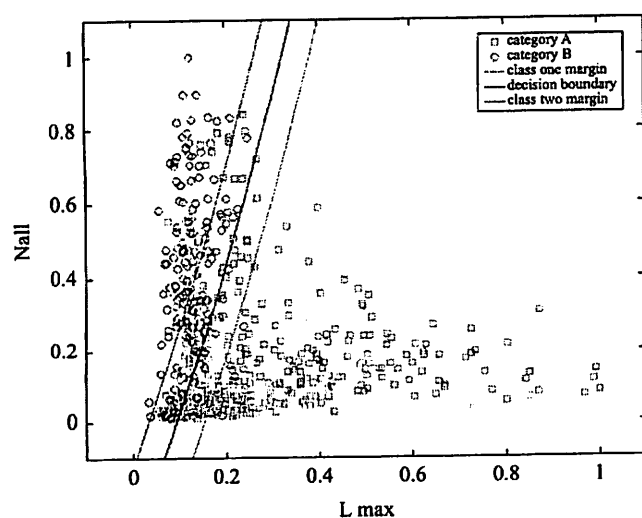
answer ratio for the test data were 85.9 percent (Table II (b)). The correct answer ratios for both the training data and the test data exceeded 80 percent.

Figure 13(a), (b) show the results by using derived discrimination function with training data and experimental data.

Figure 13 Discrimination by SVM: (a) training data with discrimination function (b) experimental result with discrimination function



(a) training data with discrimination function



(b) experimental result with discrimination function

Discussion

As mentioned above, TERA maintains the illumination condition for taking pictures. Therefore, in this experiment, the threshold for binarization was a fixed value (gray level: 29). Such a threshold value can be determined for each machine. However, this would require the introduction of an automated threshold decision method for such problems.

In the feature space, the distribution of data sets belonging to categories A and B used at this time had a distributed complex shape in the region of the boundary.

In addition, since the feature values, the maximum length, and the number of line segments were selected by investigating the entire image, in the case of the images containing both categories A and B, it is ascertained that these features caused erroneous decisions by extracting the features of both categories A and B. For example, the images in Figure 14 belong to category A. However, the effect of extracting feature values from the objects belonging category B, which occupy most of the image, caused an error. Thus, if only the feature of a crystal belonging category A can be extracted from the images, the error for images that include both category A and category B objects may decrease. Consequently, extraction from partial images, i.e. the extraction of feature values from an object as a unit, will be considered in the future.

Conclusion

In the present paper, we discussed the improvement of the efficiency of the crystallization experiment for protein X-ray structural analysis and proposed a classification method to decide whether a protein crystallization droplet contains the experimental subject for X-ray diffraction. The processing flow of the proposed method is as follows. First, the contour lines of objects in the images were extracted as binary edge images by preprocessing. Next, the feature values, i.e. the maximum length and the number of line segments, are considered for classification based on the expert's comments.

Figure 14 Erroneous decision images



Furthermore, these extracted feature values are utilized for discriminant analysis via SVM. Using the classifier, the correct answer ratio with respect to the test data were over 80.0 percent, verifying the applicability of the proposed method.

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