EVALUATION OF PLANT SUSPENSION CULTURES BY TEXTURE ANALYSIS

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1. Introduction

Plant cell suspension culture has been widely used as a way for cell proliferation in research and is extending to commercial use. To make the best use of this technique, it is essential to maintain cell quality. Selection of cell suspensions having desirable properties is a routine work in plant cell suspension culture [1]. Image analysis techniques appear to be one of the promising methods for evaluation of cell suspension cultures because it can offer non-destructive monitoring of culture giving an objective index for visual information [1,2]. The macroscopic visual appearance of cell suspensions may vary with colour and size distribution of cell aggregates in the cell suspensions, depending on culture conditions, culture periods, or cell lines. Hence, the visual texture of a macroscopic image of a cell suspension may be used for evaluation of cultured cell quality [1,3].

In this chapter, the feasibility and problems of methods for the non-destructive evaluation of cell suspension cultures will be discussed, focusing on texture analysis of macroscopic images of cell suspensions. First, macroscopic images will be compared with microscopic images from the viewpoint of their use for non-destructive evaluation of cell suspension cultures, and basics of texture analysis for biological objects will be explicated. Next, as an example of application of texture analysis for macroscopic images, a research on evaluation of somatic embryogenic potential of carrot cell suspension culture will be introduced.

2. Microscopic and macroscopic image uses in plant cell suspension culture

Normally, objects in cell suspension culture are single cells or cell aggregates. Therefore, to identify cells or cell aggregates, images of cell suspensions acquired using microscopy, are necessary. As plant cells are normally several micrometers to several tens of micrometers in size, a spatial resolution of at least several micrometers per pixel is needed in microscopic images to analyze single cells or small cell aggregates. Use of microscopic images has the advantage of allowing direct observation of individual cells,

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cell aggregates and differentiated cell masses. However, this microscopic image analysis has difficulties in image acquisition [1]. Generally, to acquire microscopic images, sampling of the culture is necessary. Sampling may be destructive with risks of contamination, and is labour-intensive. In addition, sampling raises questions of whether the sample population is truly representative of the cell suspension, and it may be necessary to increase the number of samples or use effective statistical methods [1]. By using an inverted microscope attached with a camera or a long working distance microscopic CCD camera, image can be acquired without sampling. However, it is difficult to obtain microscopic images of suspended cells suitable for direct observation of individual cells and cell aggregates because of cell overlapping by sedimentation or limitation in working distance. In addition, whether the populations recorded in sampled images are truly representative remains a problem.

Several microscopic imaging system in which an image of suspended cells is acquired in an imaging cell connected to a bioreactor, have been proposed. Grand d'Esnon *et al.* [4] first reported this type of system for acquiring cell microscopic images. Suspended *Ipomoea batatas* Poir. cells were passed into the imaging cell by a peristaltic pump from the bioreactor. This system was used to monitor the population dynamics of embryogenic and non-embryogenic cell aggregates in cell suspension cultures used for somatic embryo production. Smith *et al.* [5] have developed a similar system that evaluated pigment production of *Ajuga reptans* cells. Ibaraki *et al.* [6] also developed a system to acquire images of carrot somatic embryos (*Daucus carota* L.) for sorting. Harrell *et al.* [7] developed an improved system and measured cell aggregate distribution and growth rate in embryogenic cell suspension cultures of *Ipomoea batatas* Lam. In this system, to avoid cell damage the cell aggregates could not be allowed to go through the pumping unit, and a method to calculate total reactor population from the number of observed aggregates was proposed. These methods are effective for serial quality evaluation in cell suspension cultures. However, it should be noted that the population density of single cells and cell aggregates is crucial if image analysis is used to measure the properties of individual cells and cell aggregates. Low cell population density is needed to prevent cells from overlapping, and this may not be optimal for cell growth or metabolite production [1].

In contrast, macroscopic images have an advantage in imaging and have been used for quality evaluation of cell suspensions although applications are limited to a few studies. A macroscopic image of a cell culture is defined as an image viewed with normal or macro lens whose field of view contains almost a whole culture [1]. Macroscopic images can be acquired from the outside of a culture vessel without special devices if the culture vessel has transparent walls, i.e., it is perfectly non-destructive imaging. Depending on the imaging devices, these images have spatial resolutions of several hundreds of micrometers per pixel and do not allow us to identify a small cell aggregate. However, macroscopic images have often been used for quantification of cell masses on solid media [8,9,10] and in cell suspensions [11] because they included one whole culture in their fields of view. Moreover, colour /grey level analysis and/or texture analysis of macroscopic images of suspension cultures can provide us with information related to status of suspended cells and tissues. Texture analysis has the potential of characterizing individual objects in a macroscopic image, in which the individual objects were not clearly identified [12]. Experimental evaluations in plant

cell culture very frequently include visual examinations [2]. Image analysis of a macroscopic culture image may substitute for the visual examination, supporting objective decision and contributing to improvement in reproducibility in plant cell culture.

3. Texture analysis for macroscopic images of cell suspensions

3.1. TEXTURE FEATURES

As simple texture features, mean grey level, variance, range (i.e., the difference between maximum and minimum values of grey level), and other statistical features derived from grey level histogram such as skewness and kurtosis, are used for classification and segmentation of images based on texture although these texture features can not involve information on spatial distribution.

Texture analysis methods considering spatial distribution include two-dimensional frequency transformation, grey level run lengths method, spatial grey level dependence method, etc. Two-dimensional frequency transformation method has been widely used for image analysis. It can derive the power spectrum image (frequency-domain image), which expresses periodic features in the image texture. From power spectrum images, wedge-shaped features related to texture direction and ring-shaped features expressing periodic characteristics can be extracted.

In grey level run lengths method [13], features are extracted from the matrix which is a set of probabilities that a particular-length line consisting of pixels with the same grey level will occur at a distinct orientation. It is valid for analysis of band pattern texture.

Texture features extracted using spatial grey level dependence method (SGDM) developed by Haralick *et al.* [14] have been often used for texture analysis for biological objects. In SGDM, a co-occurrence matrix is determined and 14 texture features are calculated from the matrix. The co-occurrence matrix is a set of the probabilities $P(i,j)$ that a combination of a pixel at one particular grey level (i) and another pixel at a second particular grey level (j) will occur at a distinct distance (d) and orientation (θ) from each other. Of the 14 features, major features are as follows:

$$\textit{Angular Second Moment} = \sum_{i=0}^{N-1}\sum_{j=0}^{N-1} P(i,j)^2 \tag{1}$$

$$\textit{Contrast} = \sum_{n=0}^{N-1} n^2 \sum_{|i-j|=n} P(i,j) \tag{2}$$

$$Correlation = \frac{\sum_{i=0}^{N-1}\sum_{j=0}^{N-1} ijp(i,j) - \mu_x \mu_y}{\sigma_x \sigma_y} \tag{3}$$

$$Entropy = -\sum_{i=0}^{N-1}\sum_{j=0}^{N-1} p(i,j)\log(p(i,j)) \tag{4}$$

Where, N is the number of grey levels, and μ_x, μ_y, σ_x, σ_y denote the mean and standard deviation of the row and column sums of the co-occurrence matrix, respectively. Briefly, "Angular Second Moment" is a measure of homogeneity, "Contrast" is a measure of local contrast, "Entropy" is a measure of the complexity or randomness of the image, and "Correlation" is a measure of grey-tone liner-dependencies. The number of grey levels, N, is often lessened for reducing calculation time and for suppressing noise effect. If the image is assumed to be isotropic, only one orientation (θ) is often tested. Moreover, recently, texture analysis using the colour co-occurrence matrix has been used [15].

A wide variety of new texture analysis methods have been proposed extensively in various research fields. Tuceryan and Jain [16] divided texture analysis methods into four categories: statistical, geometrical, model-based, and signal processing. Of these categories, histogram-derived features, grey level run lengths method, and SGDM are classified into statistical methods, and two-dimensional frequency transformation is classified into signal processing methods. Geometrical methods consider texture to be composed of texture primitives, attempting to describe the primitives and the rules governing their spatial organization [17]. Model-based methods hypothesize the underlying texture process, constructing a parametric generative model, which could have created the observed intensity distribution [17].

3.2. TEXTURE ANALYSIS FOR BIOLOGICAL OBJECTS

In remote sensing, texture analysis has been used for classification of land use or plant species identification extensively. In proximal remote-sensing for plant canopies, applications of texture analysis have been also reported. Shearer and Holmes [15] identified plant species using colour co-occurrence matrices, which were derived from image matrices for each colour attribute: intensity, hue, and saturation. Shono *et al.* [12] compared the effectiveness of several methods for texture analysis, including grey level run lengths method, SGDM, and power spectrum method, on estimation of the species composition in the pasture filed.

In addition, in the filed of quality evaluation in agriculture, machine vision systems based on texture features have been used. Sayeed *et al.* [18] evaluated snack quality by neural network using textural and morphological features. Maturity in shell-stocked peanuts was detected by the histogram characteristics or the texture descriptor derived from the analysis of gradient images [19]. Texture analysis which is based on the frequency of co-occurrence of a random event and is named as Frequency Histogram of

Connected Elements was used for detection and recognition of cracks in wood boards [20]. Shono [21] analyzed leaf orientation by texture features extracted by power spectrum method. Murase *et al.* [22] quantified plant growth by analyzing texture features using neural network.

Texture analysis has been used for biological objects besides plants extensively. The applications include assessment of chromatin organization in the nucleus of the living cell [23], and medical applications for brain MR images [24], for bone radiographs [25], and for pulmonary disease images [26].

3.3. TEXTURE ANALYSIS FOR CELL SUSPENSION CULTURE

Although applications of texture analysis for plant cell suspension culture are still limited to a few studies, texture analysis has the potential of evaluating and/or selecting cell suspension cultures. The macroscopic visual appearance of cell suspensions reflects on colour and size distribution of cell aggregates, which may be indicators of cell suspension culture status. Cell aggregate size distribution patterns in cell suspension culture vary significantly between cell lines and also a consequence of culture age and culture conditions [27,28]. It has been reported that the visual appearance of suspension cultures changes with the number of subcultures [29] or with variations in embryogenic potential [3,29]. In fact, statistical texture features were effective for describing the difference in macroscopic appearances between carrot embryogenic and non-embryogenic suspensions [3]. The study will be introduced in 4.2. Texture analysis is expected to contribute to maintenance of cell quality in plant suspension culture, offering objective index for macroscopic appearance of suspension culture.

3.4. CONSIDERATIONS FOR APPLICATION OF TEXTURE ANALYSIS

It should be noted that as texture features are not the direct measures of biological properties in many cases, it is required to determine the relationships between texture features and the targeted biological properties by modelling methods such as regression analysis [3] and artificial neural network [18,20,22] to use the features for evaluation of biological properties. In addition, dependency of texture features on the experimental set-up including image acquisition, sampling, and pre-processing, should be considered [17]. All experimental results should be considered to be applicable only to the reported set-up [17]. For routine use of texture analysis of macroscopic images, simple indices for describing cell suspension culture properties without the complicated model are required. In addition, more efforts for developing the robust way to acquire a macroscopic image of a cell suspension should be made in view of dependency of texture features on image acquisition set-up.

4. Evaluation of embryogenic potential of cultures by texture analysis

4.1. EVALUATION OF EMBRYOGENIC POTENTIAL OF CULTURES

The productivity of somatic embryos depends on the quality of embryogenic cultures [3]. The embryogenic potential of cultures must be sustained in maintenance phase for

the stable production of somatic embryos. The embryogenic potential depends on genotypes. Moreover, it can change with culture period and is affected by medium composition and environmental conditions. To monitor embryogenic potential of culture would be useful to stably produce somatic embryos [30].

Using microscopic observation, a pro-embryogenic mass (PEM), which is a cell cluster to become somatic embryos under certain conditions, could be identified. In a number of systems studied to date, PEMs shared similar structural features. They consist of small and highly cytoplasmic cells which often have an accumulation of starch within the plastids [31]. On the other hand, non-embryogenic cells are large and vacuolated. Therefore, a PEM could be selected with regard to its transparency and shape under microscopy. The amount of PEMs in cell suspensions may be one direct index for determining the embryogenic potential of the culture. In a similar way, the amount in cultures of other embryogenic tissues as materials for embryo production such as embryo suspensor masses and early globular embryos can be used for evaluation of cultures.

Microscopic image analysis for suspension culture could be used to select PEMs. Grand d'Esnon *et al.* [4] monitored population dynamics of PEMs in suspension cultures of *Ipomoea batatas* for somatic embryo production using image analysis. PEMs and non-embryogenic cell aggregates were divided by using a correlation between the size and the mean transparency of the object.

Culture growth rate may be one of indices for evaluation of the embryogenic potential [1]. Differences in growth characteristics between embryogenic and non-embryogenic cultures have been reported in maize suspension culture [28], in carrot suspension culture [11,32], and in *Ipomoea batatas* callus culture [33]. Growth rates can be calculated through non-destructive cell quantification by image analysis. There have been several reports on image-analysis-based quantification of cells on gelled media [8,9,10,34]. In addition, Ibaraki and Kurata [11] quantified embryogenic suspension cultures by image analysis of macroscopic images of the suspensions. They showed the relationship between growth rate estimated by image analysis and embyrogenic potential of carrot embryogenic culture.

4.2. TEXTURE ANALYSIS BASED EVALUATION OF EMBRYOGENIC POTENTIAL

Other indices to be potentially used for evaluation of suspension culture are colour, cell aggregate distribution, and consequent macroscopic texture [1]. Ibaraki *et al.* [3] reported the system for evaluation of embryogenic potential of cell suspension cultures based on texture analysis. They acquired macroscopic images of carrot cell suspensions from the bottom of a culture vessel (Erlenmeyer flask) with a video camera (GR-S95, JVC) using transmitted light. The video signal was digitized as a 24-bit RGB colour image whose size was 640 by 480 pixels. As the B component of the RGB was more sensitive to yellow carrot cells than the other two components, each image was converted into an 8-bit monochrome image based on the B value. A part of the flask bottom in the image was extracted as an elliptic region and transformed into a circle with 400-pixel diameter (Figure 1). In this condition, the spatial resolution in the image was about 0.23 mm/pixel. Texture features were extracted using SGDM. Of 14 features in SGDM, 3 features, Angular Second Moment, Contrast, and Entropy were calculated

from co-occurrence matrix and tested. Actual embryogenic potential of a cell suspension was determined by the number of PEMs in the unit volume suspension (hereafter, PEM density) or total number of embryos induced using each cell suspension.

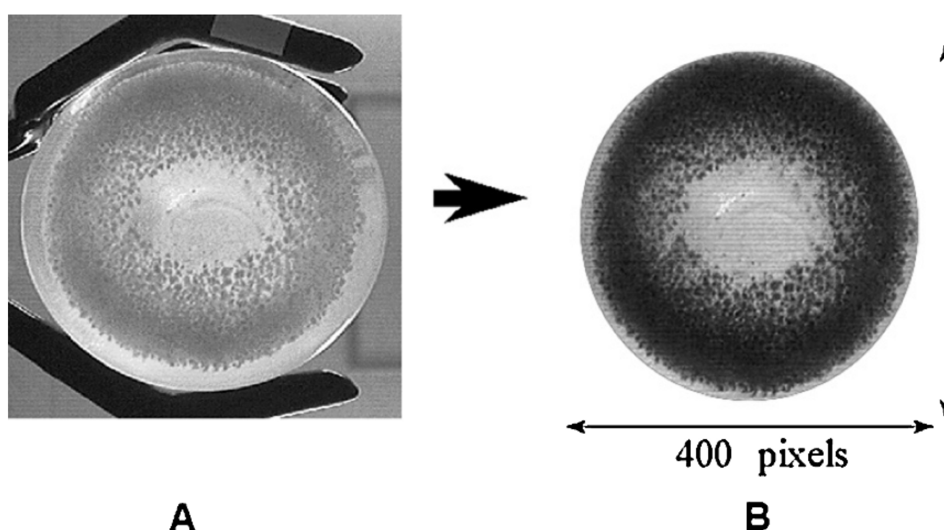

Figure 1. Macroscopic images of carrot cell suspension viewed form the bottom of culture vessel. A part of the flask bottom in the original colour image (A) was extracted after conversion into 8-bit monochrome image based on the B component value as an elliptic region and transformed into a circle with 400-pixel diameter (B).

Different carrot cell suspensions had various embryogenic potentials expressed by the PEM density. Differences in visual appearance due to differences in cell aggregate size distribution pattern between embryogenic and non-embryogenic suspensions were observed (Figure 2). Images of cell suspensions possessing high embryogenic potential had course texture, while those of non-embryogenic suspension had fine texture. In embryogenic cell suspensions, many large cell aggregates could be observed. In contrast to this, non-embryogenic suspensions had few large cell aggregates and consisted mainly of small cell aggregates. Several reports have been shown difference in cell aggregate size distribution patterns between embryogenic and non-embryogenic cultures [28,35]. The difference in textural appearance due to cell aggregate distribution patterns could be detected by texture analysis. The most useful texture feature for evaluating the embryogenic potential was Entropy, which is a measure of complexity of an image. Images of cell suspensions with higher PEM density had higher values of texture feature Entropy (Figure 3). In addition, suspensions with higher values of texture feature Entropy have the potential to produce more somatic embryos (Figure 4). These results suggested that texture analysis of a macroscopic image of a cell suspension could be used to evaluate the embryogenic potential of the suspension.

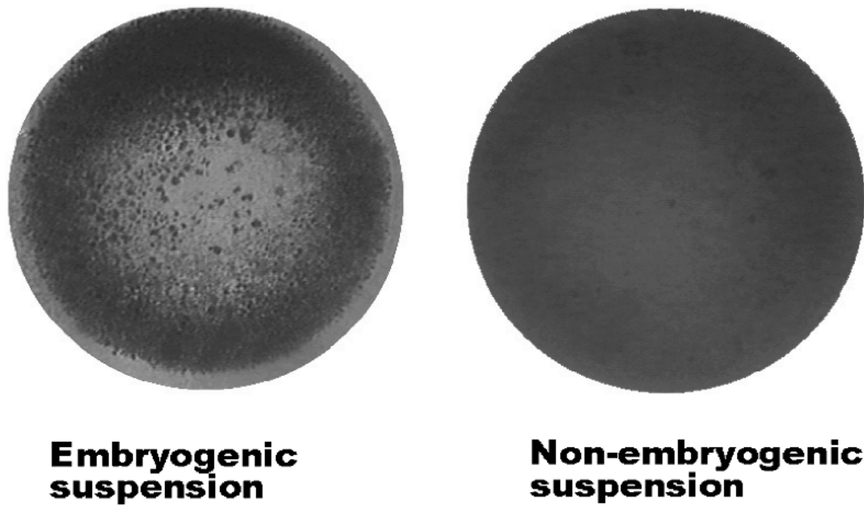

Figure 2. Images of embryogenic and non-embryogenic suspensions.

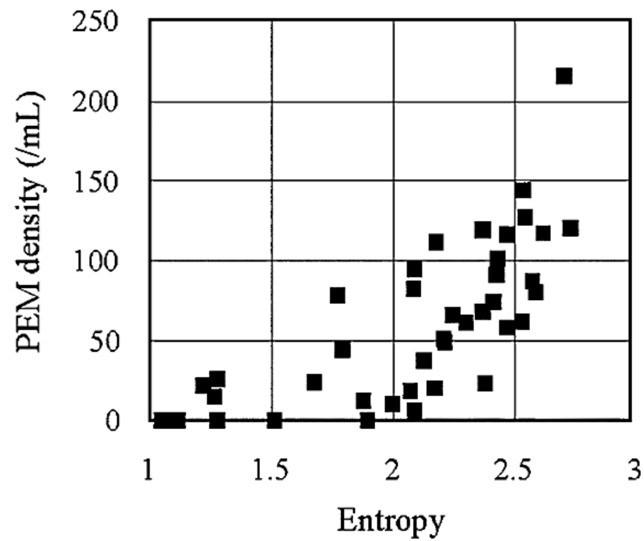

Figure 3. Relationship between texture feature entropy and PEM density when the number of grey level =8 (n=43). Reprinted from Ibaraki et al. (1998) [3].

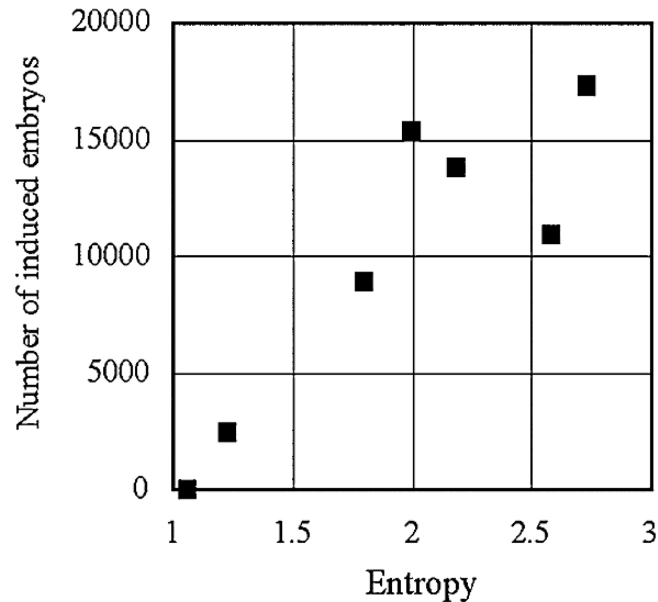

Figure 4. Relationship between texture feature entropy when the number of grey level =8 and number of induced somatic embryos. Reprinted from Ibaraki et al. (1998) [3].

5. Concluding remarks

Image analysis has potential to provide simple, non-destructive, and objective quality evaluation of cultured cells for plant cell suspension culture. As compared with microscopic images, macroscopic images are more easily acquired without sampling, showing the potential for non-destructive evaluation. The visual texture of a macroscopic image of a cell suspension can be an indicator of cultured cell quality. The texture analysis of the macroscopic image was used for evaluation of embryogenic potential in cell suspension cultures. Texture analysis techniques are expected to contribute to maintenance of cell quality in plant cell suspension culture. Texture analysis is now used extensively for biological objects in various areas and novel methods have been reported. These technologies are expected to be transferred to plant tissue culture area.

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