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3-D Color Video Microscopy of Intact Plants: A New Method for Measuring Shape and Growth

Kenji OMASA and Masaki KOUDA*

National Institute for Environmental Studies, Tsukuba 305-0053, Japan
**College of Industrial Technology, Nihon University, Narashino 275-0006, Japan*

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We developed a new computerized CCD video light microscopy system for obtaining three-dimensional (3-D) RGB measurements of shape (with color texture) and growth of intact plants under various conditions and over a wide magnification range. We used a modified shape-from-focus algorithm that incorporated a linear regression operator for 3-D reconstruction of images of petunia seedlings, which have a coarse texture. This algorithm was unsuitable for processing images of specimens with glossy texture (e.g., plant cells), but this drawback was mitigated by illuminating the cells in a checked pattern. The algorithm also reduced incorrect range estimates, which are due to defocused areas around the object's edge in original images. Within only a few minutes, the presented system can yield a series of original color images and reconstruct 3-D images incorporating color texture. All operations of our new system are carried out automatically, and the results are displayed by using in-house developed software that allows observation of the reconstructed 3-D image from any direction on the upper side. At angles from 10 to 80° to the objective's face, the surface area can be estimated to within an error of 5%.

INTRODUCTION

Recent advances in computerized light microscopy systems have enabled three-dimensional (3-D) analysis of an object's structure (Erhardt *et al.*, 1985; Hiraoka *et al.*, 1987, 1989; Pawley, 1990; Russ, 1994; Gu, 1996). To obtain information on the 3-D architecture of cells and tissues at high magnification, the confocal laser scanning microscope (CLSM) has been effectively used (Pawley, 1990; Knebel and Schnepf, 1991; Rigaut *et al.*, 1992; Gu, 1996). In this system, the 3-D image is typically constructed by stacking numerous two-dimensional (2-D) images, which are obtained at consecutive confocal planes. The CLSM has fluorescence imaging capability, and it can provide monochromatic or artificial color images. However, this system cannot be used to obtain suitable 3-D full (natural)-color RGB images. In addition, using the CLSM for *in situ* observation of cells and tissues over a wide magnification range under natural growing conditions is difficult. This problem results because in this situation, the laser is operated at a narrow working distance and must be adjusted, thereby affecting the physiological reactions of the target cells.

Traditionally, conventional stereomicroscopes have been used for 3-D color observation in biological applications. However, only low-magnification observation has been possible due to the need for a deep depth-of-focus and a wide working distance. To obtain the stereo, paired images necessary for three-dimensionality, a single camera and a shifting microscope stage or duel video cameras (instead of duel eye lenses) have been used (Hiraoka *et al.*, 1990; Rigaut *et al.*, 1992; Russ, 1994). Many algorithms have been developed for determining a

range image (i.e., depth image) from stereo, paired images. However, these algorithms possess a drawback in automatically matching corresponding points between stereo pair images, while also lacking practicality due to the high computational expense required for calculating a precise range image (Inokuchi and Sato, 1990; Faugeras, 1993; Chellappa and Rosenfeld, 1993).

Range images can even be reconstructed from monocular images through "shape-from-x" algorithms, in which x is focus, shading, texture, or contour (Kanatani, 1990; Nayar and Nakagawa, 1990; Jähne, 1993; Omasa *et al.*, 1997). These methods can be applied to monocular light microscopy. The shape-from-focus (SF) method evolved through studies on automatic focusing techniques and for cases in which limited measurements of an object's depth are available (Krotkov, 1987; Grossmann, 1987; Darrell and Wohn, 1988). The SF method is one of the most practical means for automatically reconstructing range images from fine-texture images. Nayar and Nakagawa (1990) first proposed an SF method, applying it to calculate the range image of a fine-texture steel ball from a series of 2-D monochromatic images that were obtained at consecutive focused planes. However, this algorithm cannot precisely determine the range image of objects with coarse or glossy texture (e.g., plant cells, tissues, and seedlings).

In an attempt to identify an algorithm that would be effective for reconstructing range images from monocular light microscope images of plant cells, tissues, or seedlings, we recently compared the previously described SF algorithm, which is based on a sum-modified Laplacian (SML) operator (Nayar and Nakagawa, 1990) to modified shape-from-focus (MSF) algorithms that were based on either a linear regression (LR) or max-min (MM) operator. We found that the LR operator-based method was the most effective (Omasa *et al.*, 1997). The microscope system that we used in our previous study provided good-quality, 3-D, full-color RGB images. However, it was unsuitable for imaging glossy textures over a wide range of magnification, and it was not applicable to observing seedlings grown under natural conditions. Combining active illumination (e.g., in a checked pattern) with the SF algorithm yielded more exact depth maps of a solder joint on a circuit board (Noguchi and Nayar, 1994), but this type of lighting had not been applied to measuring plant cells with color and glossy textures. These difficulties led to our present study. Here we extend our work by incorporating the enhanced MSF algorithm into a new computerized 3-D light microscopy system. We designed the combined system for imaging the color, texture, and shading of intact plants and seedlings under natural growing conditions and over a wide range of magnification.

MATERIALS AND METHODS

Computerized light microscopy system. The computerized light microscope system that we used (Fig. 1) comprised a modified light microscope (FS-60F, Mitsutoyo) (Fig. 2), a stepping motor control system for auto-focusing and controlling each axis of the microscope stage and lens tube, a CCD color video camera (XC-999, SONY), and a personal computer system (8500/180, Macintosh) for analyzing the resulting 3-D images. Because of the inherent wide working distances (13.0 mm with a 100× objective, 20.5 mm with a 50× objective, and 34.0 mm with a 2× objective), intact plants can be viewed under growing conditions similar to those in their natural environment. The microscope's focal depths (0.6 mm with 100× objective, 0.9 mm with 50× objective, and 91 mm with 2× objective) were adjusted to obtain a series of 2-D images at consecutive focused planes; these images were used to reconstruct the 3-D images. Signals from the stepping motor control system automatically translate the microscope stage along the x, y, and z axes at 0.02 μm per pulse. Movement along the z-axis of the lens tube was also controlled for auto-focusing and measurement of large microscopic

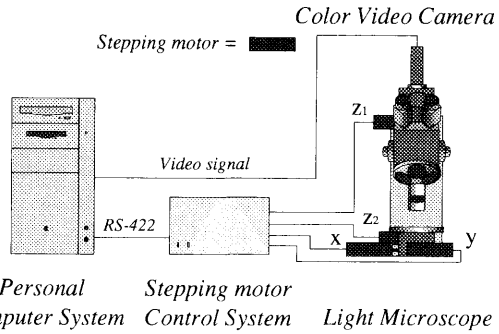


Fig. 1 Computerized light microscopy system for 3-D measurement of shape with color texture and growth of intact plants under natural growth conditions over a wide range of magnification. Movement along the x-axis of the microscope stage is controlled by the stepping motor x, that along the y-axis by the stepping motor y, and that along the z-axis by stepping motor z_2. Movement along the z-axis of the lens tube is also controlled by the stepping motor z_1, being a rapid albeit inexact adjustment.

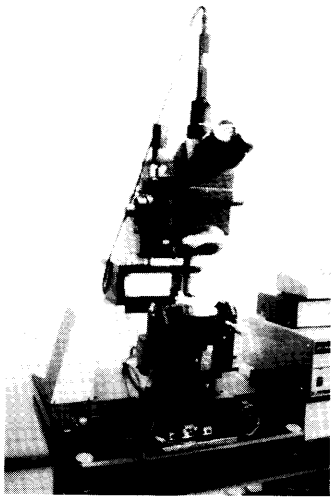


Fig. 2 Photograph of the light microscopy system that we developed and used in the present series of experiments.

objects, being a rapid though rough adjustment. The image was measured by the CCD camera and digitized by a built-in video A/D converter. Each series of RGB digitized images (640×480 pixels; 8-bit color) was stored until subjected to 3-D analysis.

3-D microscopy of growing plants/cells. Petunia (*Petunia hybrida* Vilm cv. Mitchell) plant seedlings (which have a coarse texture) were selected for low-magnification observations and measurements. After germination, seedlings were grown at 25°C in a petri dish covered with moistened filter paper; the dish was then placed on the microscope stage. Illumination during growth was automatically controlled at 60/0 μmol photons $m^{-2} s^{-1}$ (PPFD) in a 12 h : 12 h light : dark cycle. We used a 2× objective [numerical aperture (NA)=0.055; depth-of-focus (DF)=91 μm] and camera relay lens of 0.5×. These conditions allowed visualization of organ growth and of shape (with color texture) under light from the surroundings.

High-magnification microscopy was carried out on cells of an intact pothos (*Epipremnum*

aureum) plant by using a 50× objective (NA=0.55, DF=0.9 μm) and 1× relay lens. To facilitate reconstruction of the 3-D shape of these glossy-textured cells, a fine texture was illuminated on the glossy-texture cells through a checked glass filter and polarizing filter connected to the microscope via an optical cable adapter in addition to light from the surroundings. Pothos plants were grown under typical indoor conditions.

Algorithm sequence for 3-D microscopic measurement and texture mapping. Using the LR operator-based MSF algorithm, we reconstructed focused 3-D RGB images from a series of limited-focus planes, which were obtained by z-axis traversal of the microscope stage and lens tube; we also measured organ growth (Fig. 3). Basically, the algorithm interpolates the depth data such that 3-D shapes with color texture can be visualized in any direction on the upper side using a wire frame-outline filled in with a focused color image. For 3-D analysis of organ growth (e.g., leaf area), the organ image is extracted from the texture mapping images.

Focus measure operator: The LR operator was used to obtain focus measure images from a series of original color images (Fig. 3 (a)), which were collected at limited-focus planes. Data were calculated on each axis of four square-line masks ($M_k(i, j)$, $k=1,2,3$, and 4; see Fig. 3 (a)); i, j indicates the center of the $M_S \times M_S$ line masks, a methodology that reduces computation time. If the intensity at each point (x, y) of the original RGB image is denoted as $I_R(x, y)$, $I_G(x, y)$, and $I_B(x, y)$, then $I_{max}(x, y)$ is

$$I_{max}(x, y)=\max\{I_R(x, y), I_G(x, y), I_B(x, y)\}, \quad (1)$$

The focus measure $f(i, j)$ can subsequently be determined by using the LR operator, in which the sum of the absolute error between $I_{max}(x, y)$ and the regression line of $I_{max}(x, y)$ is calculated by using the least-squares method on the axes (p_k, $k=1,2,3$, and 4) of each line mask. That is,

$$f(i, j)=\sum_{k=1,2,3,4}\sum_{x,y\in M_k(i,j)}\sum |I_{max}(x, y)-\{a_k(i, j)+b_k(i, j), p_k(x, y)\}|, \quad (2)$$

where $a_k(i, j)$ and $b_k(i, j)$ represent the regression coefficients on line mask $M_k(i, j)$ in which $x, y\in M_k(i, j)$ indicates that (x, y) exists in $M_k(i, j)$. The LR operator is better suited for processing images with coarse texture than are sum-modified Laplacian (SML) or max-min (MM) operators (Omasa *et al.*, 1997).

Gaussian interpolation and median filter: The number of focused planes, N, was limited to ten or less in order to reduce computation requirements. Although a discrete value of range of depth could be estimated from the focus measure, adding Gaussian interpolation provided smoother, more accurate range estimates (Fig. 3 (a)). The focus measure that is calculated by assuming the distribution of image data at each image point (i, j) of continuous focused planes can be approximated as a Gaussian distribution in which the optimum focus

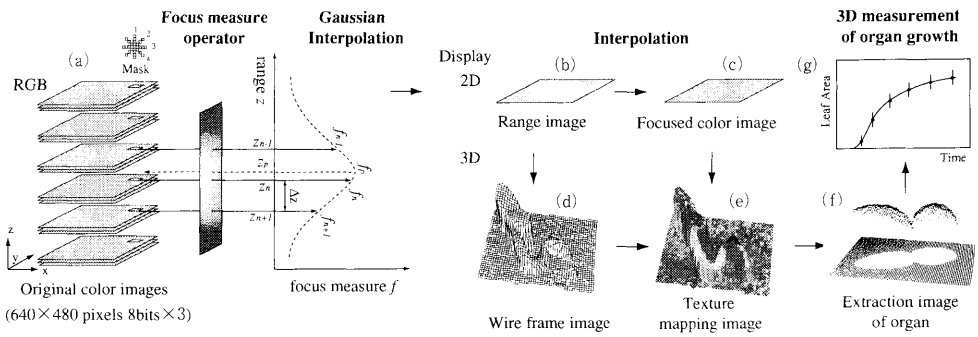


Fig. 3 Algorithm sequence for 3-D measurement and texture mapping.

point, the focused range $z_p(i, j)$, is the peak of the Gaussian distribution. This assumption allows $z_p(i, j)$ to be determined from only three focus measures, and its distance can be determined by using a single reference point on the z-axis, z_n (Nayer and Nakagawa, 1990). Thus,

$$z_p = \frac{(\ln f_n - \ln f_{n+1})(z_n^2 - z_{n-1}^2) - (\ln f_n - \ln f_{n-1})(z_n^2 - z_{n+1}^2)}{2\Delta z\{(\ln f_n - \ln f_{n-1}) + (\ln f_n - \ln f_{n+1})\}}, \quad (3)$$

where coordinates (i, j) are omitted for simplicity, f_n is the largest value in a series of focus measures f_h ($h=1, \cdots, n-1, n, n+1, \cdots, N$) estimated by Eq. (2) at limited-focused planes, f_{n-1}, f_{n+1}, z_{n-1}, and z_{n+1} are the neighboring values of f_n and z_n, and Δz is the distance between neighboring focused planes. The range image estimated by Eq. (3) included strong, spike-like noise, which we removed by using a median filter (i.e., the z_p of each pixel was replaced by the median of z_p's in the line mask). This filter is particularly effective when the noise pattern consists of strong, spike-like components and the characteristic to be preserved is edge-sharpness (Gonzalez and Woods, 1992).

Composition of focused color image and texture mapping: A focused color image was generated after respectively interpolating the original RGB intensities by using the information on the focused range at each image point. That is, the intensity $I_{R,p}(i, j)$ of the R image focused at an image point (i, j) was estimated by

$$I_{R,p} = I_{R,n-1}\left\{\frac{(z_n - z_p)}{\Delta z}\right\} + I_{R,n}\left\{\frac{z_p - z_{n-1}}{\Delta z}\right\}, \quad (4)$$

where coordinates (i, j) are omitted for simplicity, and $I_{R,n}$ and $I_{R,n-1}$ are the intensities of the R image measured at z_n and z_{n-1}. Equation (4) is correspondingly applied to obtain $I_{G,p}(i, j)$ and $I_{B,p}(i, j)$, thereby enabling composition of the focused color image. By using newly developed software that generates a 3-D wire frame-display incorporating color information and a texture mapping display in any direction on the upper side, 3-D shape and color tones can be easily recognized from the computer display. This software incorporated a "rapid preview" function so that the view position could be easily verified.

Measurement of organ growth: Changes in the area of cotyledons, an indicator of seedling growth, were automatically measured from 3-D images. Because the texture-mapping images of cotyledons were predominantly green in color, they could easily be extracted from those of other organs and the background. That is, the cotyledon image was extracted by a set of image points satisfying

$$I_{G,p}(i, j) / \{I_{R,p}(i, j) + I_{G,p}(i, j) + I_{B,p}(i, j)\} \geqq I_{GTH}, \quad (5)$$

and

$$I_{B,p}(i, j) / \{I_{R,p}(i, j) + I_{G,p}(i, j) + I_{B,p}(i, j)\} \geqq I_{BTH}, \quad (6)$$

where I_{GTH} and I_{BTH} are pre-determined, fixed, threshold values. Small holes or uneven edges in the extracted image were filled and smoothed by expanding or shrinking the image as necessary, achieved by dividing the 3-D image into a large number of triangles then applying Heron's formula to compute leaf area after smoothing uneven surface. The measurements of a metallic plate with an uneven surface confirmed the suitability of this method. Automatically extracting an image of a target organ is difficult when its tone and brightness are similar to those of other organs and the background. However, as long as there is even a slight difference in tone and brightness, applying the unsharp masking processing before the thresholding is effective (Omasa and Onoe, 1984). The difference in texture is also used for segmentation and thresholding (Russ, 1994).

RESULTS AND DISCUSSION

A series of original color images of a petunia seedling (Fig. 4) reflects nine focused planes, with the interval $\Delta z = 0.3$ mm. The plane of focus in the first image (1) is the wet filter paper, that for the last (9) is just above the seed leaf. From these color images, the focus measure was estimated by using Eq. (2) and the focused range by using Eq. (3) ; the mask size (including the median filter) for these operations was $M_s = 13$. Despite the small number of discrete original images, the resulting wire-frame 3-D range image (Fig. 5) is smooth and exact.

The ultimate accuracy of the reconstructed images depends on the surface texture (which is related to the focused color image) of petunia seedlings ; however a more precise range image can be obtained by optimizing the mask size. For example, if spike noise in the range image leads to choosing incorrect focused planes, the focused color image becomes unclear at the image points. Too large a mask size also leads to unclear image points (Gonzalez and Woods, 1992 ; Russ, 1994), especially at seedling edges. Applying the LR operator-based algorithm to the focused color image obtained by using Eq. (4) (Fig. 6) reduced image degrading effects produced by spike noise and edges. In fact, with a small mask size, this operator yielded clearer images than did the SML or MM operators (Omasa *et al.*, 1997). This operator also was more effective when using RGB images in place of a monochromatic image (Omasa *et al.*, 1997).

A texture mapping image (Fig. 7), which was generated through combining the 3-D range image and the focused color image, clearly shows the 3-D tone, texture, and shape of the seedling. By manipulating the computer mouse, the observation of reconstructed texture mapping image is allowed from any direction on the upper side. The underside of the seedling cannot be similarly evaluated because the 3-D image is reconstructed from color images (collected through the lenses of the light microscope) of the upper side, but slanting the microscope stage facilitates imaging of this region to some extent.

Determining the range focus in the glossy parts of cells of pothos plants was difficult with the previously described method, and illuminating the checked pattern (Fig. 8A) on the cells had some detrimental effects on imaging. However, at a suitable mask size ($M_s = 15$), which reflects the width of the checking, using the median filter tended to erase the checked pattern (Fig. 8B). In particular, intensity and saturation along red lines were averaged after filtering, whereas hue was only slightly affected (Fig. 9). The reconstructed texture mapping image of pothos cells obtained from a series of eight original color images ($\Delta z = 3$ μm) incorporates checked illumination and median filtering (Fig. 10). The well-rounded cells are smooth ; the color tone changed slightly.

We used the mean area of cotyledons (correlated with growth), as determined from 2- and 3-D images acquired from the upper side of leaves, to document the growth history of an *in situ* petunia seedling over 9 d after providing a water-supply (Fig. 11). Following germination after 4 d, favorable growth is apparent. Note that values from 2-D images are markedly (from 26 to 38%) smaller than those from 3-D images ; the accuracy of leaf area as determined from the 3-D shape was dependent on the angle between the objective and the leaf surface and the texture (unevenness) of the leaf surface. In experiments incorporating a metal disk that had an uneven texture (similar to that of the leaf surface), decreasing the angle to the objective's face increased error ; in Fig. 12, error is about 18% from 10 to 30°. However, this error was reduced to ~5% by using a smoothing filter, which determined the average value for an area of 5 pixels $\times$ 5 pixels. In tests that imaged a metallic hemisphere with 2 mm radius, the error in surface area was within 3% (data not shown).

Our results show that the presented computerized light microscopy system can perform 3-D

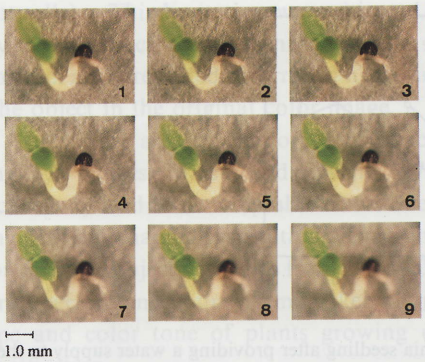


Fig. 4 A series of original color images of a petunia seedling obtained by changing the focus planes from wet filter paper (1) to a plane just above the cotyledon (9) at consecutive intervals of 0.3 mm.

Fig. 6 Focused color image composed by repetitive interpolation [through use of Eq.(4)] at all image points of RGB images.

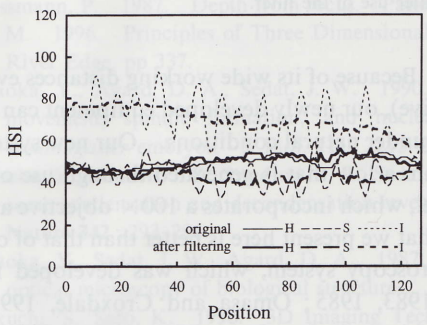


Fig. 9 Hue (H), intensity (I), and saturation (S) values of image points along red lines in Fig. 8 before and after filtering.

Fig. 10 Texture mapping image of pothos cells obtained by using a series of eight original color images that were illuminated in a checked pattern.

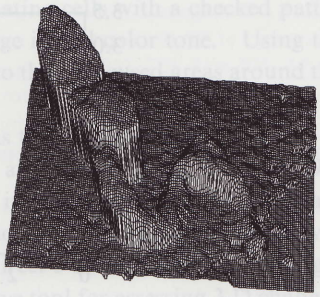


Fig. 5 Wire-frame range image reconstructed by using the original color images in Fig. 4.

Fig. 7 Texture mapping image generated by combining the 3-D range image and the focused color image.

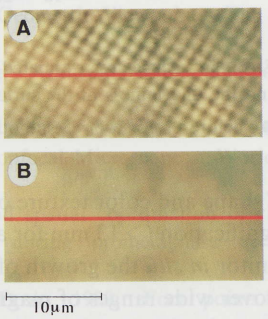
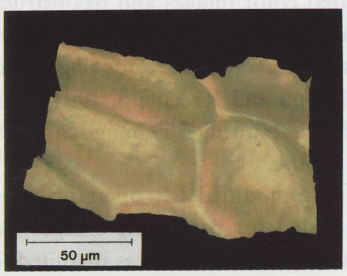


Fig. 8 Effect of the median filter on erasing the superimposed checked pattern.

A: Original image, obtained after illuminating cells in a checked patern. B: Image after filtering.



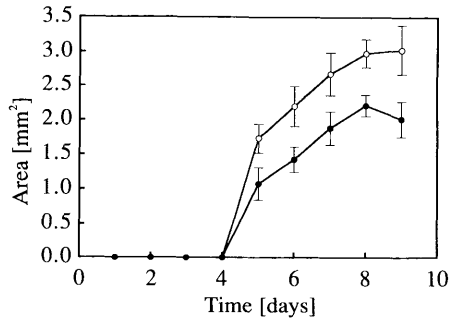


Fig. 11 Changes in area of cotyledons in an intact petunia seedling after providing a water supply to the seed.

●, mean value of leaf area estimated by using a 2-D image measured from the upper side; ○, mean value of leaf area estimated by using the 3-D shape. Vertical bars indicate ±1 standard deviation.

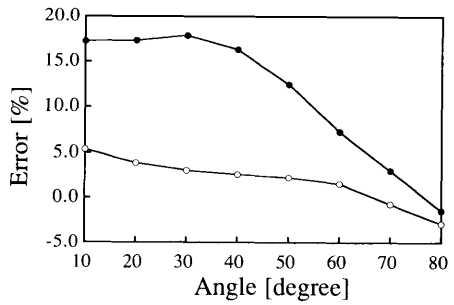


Fig. 12 Errors in surface area estimated by using the 3-D shape depend on the angle to the objective's face and the unevenness of the surface.

●, before use of a 5×5 smoothing filter; ○, after use of the filter.

imaging of shape and color texture of plant cells. Because of its wide working distances even at high magnification (>13 mm for a 100× objective), our newly developed equipment can be used to monitor *in situ* the growth of intact plants under natural conditions. Our new system is effective over wide ranges of magnification—from a low that is generated through use of a 2× objective and a 0.5× relay lens to the high level, which incorporates a 100× objective and a 1× relay lens. The performance of the system that we present here is better than that of our previously described remote-controlled light microscopy system, which was developed for observing stomatal movements (Omasa *et al.*, 1983, 1985; Omasa and Croxdale, 1992). Another advantage of the present system is its rapid processing time. Measuring a series of original color images and reconstructing the corresponding 3-D image with color texture can be accomplished automatically within several minutes, which is probably less time than required for other methods [e.g., analysis of stereo, paired images (Inokuchi and Sato, 1990; Chellappa and Rosenfeld, 1993)]. Moreover, by incorporating the software that we developed, the resulting 3-D image can be observed from any direction on the upper side by the click of a mouse.

The modified LR operator-based shape-from-focus (MSF) algorithm that we used was well-suited for processing images from plants with a coarse texture (Omasa *et al.*, 1997). However, this method was ill-suited for processing images of objects with a glossy texture (e.g.,

plant cells). This limitation was mitigated by illuminating cells with a checked pattern ; the only the drawback of this technique was a slight change in cell color tone. Using the MSF algorithm also prevented incorrect range estimates due to the unfocused areas around the edges of the object in the original color image.

The surface area of each organ of intact plants has traditionally been estimated through using 2-D images (Matsui and Eguchi, 1978 ; Omasa and Onoe, 1984 ; Omasa, 1990) ; this practice may lead to unacceptable errors due to lack of information about the 3-D shape. By using the new imaging system that we present, the surface area can be determined to within an error of 5% at objective angles from 10 to 80° though direct 3-D measurement. In light of its overall performance, our system is likely to be an effective tool for assessing 3-D changes in the shape and color tone of plants growing under natural conditions over a wide range of magnification.

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〈和文抄録〉

生育状態の植物の3次元カラービデオ顕微計測法：形状と生長の新しい計測法

大政謙次・国府田正樹*

国立環境研究所・* 日本大学生産工学部

生育状態の植物の形状や生長を，低倍率から高倍率にわたって，非破壊で計測するための新しい3次元カラービデオ顕微計測法を開発した．この計測法は，線形回帰演算を用いた modified shape-from-focus (MSF) 法によって，カラービデオ画像から，3次元のカラー形状画像を再構築し，器官生長を測る方法である．この MSF 法は，原画像のエッジ周辺のボケにより生じる誤差を改善でき，また，ステレオ画像から3次元形状を再構築する方法に比べて，短時間に，再構成画像を得ることができる．具体的に，低倍率での器官生長の3次元計測の例として，粗いテクスチャをもつペチュニアの実生を，シャーレで生育させながら，受動的な方法で3次元の形状の変化を計測し，発芽後の子葉の生長を求めた．また，高倍率で，表面光沢がある細胞の計測の例として，ポトス細胞を例に，表面の色調や生理状態に影響が出ない程度の格子状のテクスチャ照射し，細胞の膨らみを3次元的に計測した．演算は自動的に行われ，結果の3次元画像を上方の任意の方向から，マウス操作で観察できる．また，この方法を用いた表面積の計測精度は，対物レンズの面に対して，10° から 80° までの角度で 5%以内の精度であった．