High-Speed Autofocusing of Cells Using Radial Intensity Profiles Based on Depth From Diffraction (DFDi) Method

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Abstract—In this paper we propose new image features named radial intensity profiles (RIP) for high-speed autofocusing of cells based on our Depth From Diffraction (DFDi) method. DFDi is a method of extracting depth information of target cells from a single defocused image containing their diffraction patterns. High-speed focusing of separated yeast cells with 20 ms response time and continuous autofocusing with a scanning microscope were demonstrated. The successful continuous autofocusing shows that image-based high-throughput measurement of cells could be realized using the proposed algorithm.

Index Terms—Microscopy, High Speed Autofocusing, Cell, Depth From Diffraction

I. INTRODUCTION

With the rapid development being seen in life sciences and biotechnology, automated observation of cells and tissue using microscopes is becoming increasingly important. A critical step in such automated observation is autofocusing. Since the depth of focus of microscopes is usually very shallow, typically on the order of several micrometers, small shifts in the depth direction cause the image of the specimen to easily become out of focus. Thus, autofocusing is essential to keep the object in focus for precise observation. Major applications of such automated measurement require high throughput because the number of target specimens tends to be enormous. Therefore, high-speed operation is also important.

High-speed autofocusing technology is particularly desired in scanning microscopy. Scanning microscopes are commonly used in the field of image cytometry [1] which is the measurement of cell morphology such as dimension, volume, shape etc. It is impossible to observe all specimens in a static field of view of a microscope due to the limited resolution of the microscope or camera. A scanning microscope solves this problem by moving its field of view (FOV) to observe the specimen as a sequence of images. It is not possible to maintain focus simply by determining the best focus depth at two points on a microscope slide and scanning along the line between them in three-dimensional space [2]. There may be many reasons for this, including mechanical instability of the microscope and irregularity of the glass slide surface. Thus, a high-speed adaptive autofocusing is required to track the specimen in focus while the lateral scanning of the slide. Since the depth variance during the scan is continuous, a distance of defocus is not large if the specimen is in focus at the beginning of scan. Therefore, a wide working range is not important for the autofocusing method for a scanning microscope, considering the initial autofocus can be done using a conventional autofocusing method previously.

Many microscope focusing methods based on the spatial frequency of the acquired image have been proposed, including a method for scanning microscopy [2], a method which works with various illuminations [3], and a method for fast autofocusing [4]. The best focal position providing the highest amount of detail can be estimated from a so-called focus curve formed by sampling the image to obtain a focus score and plotting it against focal position in the depth direction. The best focal position is then found by searching for the peak in the focus curve. However, this sampling takes a considerable amount of time because many images at many focal positions must be individually acquired and processed. Assuming that acquisition and processing of each image takes 40 ms [3] and that 20 samples of the focus score are necessary to estimate the focus curve, the entire autofocus process takes at least 0.8 s.

To solve this problem, Oku et al. proposed the “depth from diffraction” (DFDi) method that estimates the depth of a target cell based on its diffraction pattern [5]. Because this method uses only one defocused image for the estimation, the time-consuming focal position shifting process can be eliminated from the depth estimation process, enabling high-speed focusing. The image processing algorithm described in that study, however, could handle only a single cell. It was also developed for specialized vision systems with parallel processing elements [6], [7]. Thus, conventional

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computers with single processors cannot run this algorithm.

In this paper, we propose a radial intensity profile (RIP) of a cell as an image feature for high-speed autofocusing based on the DFDi method. This image feature can be extracted by a simple serial image processing algorithm, and it also includes sufficient information for the DFDi method. We developed two kinds of image processing algorithms for high-speed depth estimation, for separated cells and clumped cells, respectively. The performance of the proposed algorithm was demonstrated by quick autofocusing of yeast cells. The effectiveness of the algorithm for high-speed measurement of cells was confirmed by demonstrating dynamic autofocusing of a field-scanning microscope that shifts its field of view automatically to measure a large number of cells.

II. DEPTH FROM DIFFRACTION METHOD

Depth estimation of target cells is essential for autofocusing. The “depth from diffraction” (DFDi) method estimates their depth from just one defocused image by using optical characteristics of the cells.

![Fig.1. Bright-field images of yeast and paramecium cells at three focal positions. The yeast cell has a spherical body about 5 μm in diameter. Paramecium is a motile cell with an ellipsoidal body whose longitudinal length is from 100 to 200 μm. The paramecium cell was held in a micro-capillary to keep it in the field of view. In the case of the yeast cell, an intensity variance was observed in the interior of the cell. For z < z_f, no clear inner fringe was observed in the yeast cell, but a bright inner area was observed.](image)

Consider a cell observed with a microscope under Köhler illumination [8]. When the target cell is in focus, a clear image of the cell is observed. If we adjust the focus slightly to defocus the image, bright and dark diffraction fringes can be observed near the periphery of the cell. For example, Fig. 1 shows these fringes in images of paramecium and yeast cells. The depth of the target cell could be estimated from a single defocused image of the cell containing such diffraction fringes. Based on this idea, the “depth-from-diffraction” (DFDi) method, which estimates the depth of cells using their diffraction fringes, was previously proposed [5]. Since this method can estimate the depth from only a single defocused image, the depth estimation can be performed extremely rapidly. Once the depth is estimated, focusing can be easily realized by moving the focal plane to the estimated depth, or by moving the specimen to the focal plane.

Although we have confirmed the validity of the DFDi method only for paramecium (~ 100 μm) [5] and yeast (~ 5 μm) cells [9], we expect this method could be applied to many spherical and ellipsoidal cells. Note that this method depends on refractive indices of medium and cell membrane, cell shape, and cell posture. These factors in a certain condition may limit the validity of the method.

III. RADIAL INTENSITY PROFILE

Depth estimation based on the DFDi method requires an image feature containing information about the fringe intensity and interval. In this paper, we propose a radial intensity profile (RIP) as such a feature. Assume a spherical cell, like a yeast cell, shown in the upper row of Fig. 1. The RIP is an intensity profile along a half-line originating at the center of the cell. Fig. 2 schematically illustrates the RIP. Considering that the brightest and darkest fringes appears near the cell boundary as described above, a part of the intensity profile crossing the cell boundary is assumed to include information of the fringe interval and the order of bright and dark fringes, which is needed for depth estimation.

![Fig.2. Schematic diagram of the extraction process of the radial intensity profiles and their average.](image)

![Fig.3. Average radial intensity profiles measured at -4, 0, and +4 μm in depth.](image)

Fig. 3 shows three of the measured average RIPS of a yeast cell at -4, 0, and +4 μm in depth. The range of distance was decided from a defocus image series of the yeast cell. The following characteristics were obtained from this figure.

- When the cell was in focus, the intensities of pixels outside the cell and those of pixels inside the cell were almost identical.
When the cell depth was +4 μm, the outside of the cell was brighter than the inside.

When the cell depth was -4 μm, the inside of the cell was brighter than the outside.

The results confirmed that the profile depended on the depth of the cell.

When the target cells were spatially separated, RIPs could be measured easily. However, when the cells were clumped together, it was difficult to obtain the profile outside of the cell since the fringes appearing outside of one cell overlapped those of an adjacent cell. In this case, only the partial profile inside of a cell was used for the depth estimation.

IV. IMAGE PROCESSING ALGORITHMS

Two kinds of image processing algorithms were developed, for separated cells and clumped cells, respectively. The target cells are assumed to be in the same plane and not piled up in the depth direction. If some cells are piled up in depth, it is impossible to focus all cells in the viewing field. Thus, this assumption is reasonable from a practical viewpoint.

A. Algorithm for Separated Cells

First, the algorithm for separated cells is explained. The separated-cells assumption is satisfied when the specimen is an isolated floating cell, such as a blood or yeast cell. Even when the target cells are in the form of tissue, some of them can be dissociated into isolated cells using well-known techniques, for example, by using enzymes or surfactants.

1) Cell boundary recognition: To extract the RIP, the algorithm needs to recognize a single cell and its boundary. Since all cells are assumed to be separated, cell boundaries can be extracted by a well-known boundary tracing algorithm and by using a Freeman chain code [10], [11].

A captured microscope image A has a bright background and dark cell boundaries, as shown in Fig. 4 (A). By binarizing A with a suitable threshold, donut-shaped cell boundaries B are obtained, as shown in Fig. 4 (B).

A pixel on a cell boundary is found by searching for a pixel whose intensity is bright and whose left neighbor pixel is dark, by raster scanning. The found pixel is called the first boundary pixel $a_1$.

The cell boundary on which pixel $a_1$ is located is traced using a well-known boundary tracing algorithm. Let $a_1, a_2, \ldots, a_n$ be boundary pixels. The next boundary pixel $a_{n+1}$ is searched for from all 8 neighboring pixels of $a_n$. The search path is in a counterclockwise direction and starts at the next pixel for the previous pixel $a_{n-1}$, as shown in Fig. 5 (a). When a bright pixel is found, that pixel is recognized as the next boundary pixel $a_{m+1}$. Then, treating $a_{n+1}$ as the new current pixel, the algorithm proceeds in the same manner. The tracing stops when the next boundary pixel $a_m$ satisfies $a_m = a_1$. The boundary is represented by a Freeman chain code [10].

Each yeast cell becomes a donut-shaped pattern in the binarized image B, as shown in Fig. 4 (B). This donut-shaped pattern has two boundary curves forming the outer and inner circular edges of the pattern. Although only the outer boundary of the pattern is of interest, the inner boundary might be detected by the boundary tracing process. To prevent such incorrect detection, all of the pixels inside the detected boundaries are marked as cell interior whenever the boundary tracing ends so that only the outer boundaries of cells are recognized. This also prevents redetection of another pixel on the already detected cell boundary. Fig. 4 (M-1) and (M-2) show the images of cell interior marks.

After marking the cell interiors, another pixel without the cell interior mark is searched for by raster scanning. If another boundary pixel is found, the same boundary tracing process is performed in the same manner. The boundary searching and tracing are repeated until the raster scanning reaches the bottom-right pixel.
2) Extraction of the radial intensity profile: The RIPs of each cell are extracted after the boundary recognition.

Each RIP is represented as a set of intensity values of thirteen pixels. These pixels are selected in order of their distance from the cell boundary, namely, -8, -6, -4, 2, 0, 2, 4, 6, 8, 10, 12, 14, and 16. The range of pixels was decided so that the profile covers most region where fringes appear. A negative distance means that the pixel is outside the cell. The distances are calculated from the cell center position and a given position on the cell boundary. The center position of each cell is calculated as the image centroid of its boundary pixels.

For each cell, four profiles are extracted and averaged so that an average RIP can be obtained to reduce noise included in the intensity values, as shown in Fig. 2. The average RIP is also represented as a set of intensity values.

3) Depth estimation by linear regression: According to Fig. 3, partial RIP near cell boundary varies their intensities depending on the cell depth. A linear relationship between the RIP and the cell depth was expected if the defocus distance is small. A result of a preliminary experiment supported this expectation. Thus, the depth of a cell is estimated by multiple linear regression of its average RIP. We assume that the relationship between a cell’s depth and its average RIP is linear and capable of being represented by the following equation:

\[ Z = c_0 + c_1 t_1 + \cdots + c_n t_n \]  

where \( n \) is a number of pixels to represent each profile, \( Z \) is the depth of the target cell, \( t_1, t_2, \ldots, t_n \) are the intensity values of the average RIP, and \( c_0, c_1, \ldots, c_n \) are coefficients of the linear regression. These coefficients are calibrated using cell images captured at various depths.

B. Algorithm for Clumped Cells

Although the outline of the algorithm for clumped cells is almost the same as the one described for separated cells, the cell boundary recognition process and the profile range used for depth estimation are different.

1) Cell boundary recognition using a Hough transform: The previously adopted boundary tracing algorithm cannot be applied to the clumped cells, since it recognizes two or more clumped cells as one cell. Assuming that the cell shape is spherical, circle detection using a Hough transform \cite{12} is adopted to recognize cell boundaries. In our algorithm, a radius gives the average radius of the target cell, and only the center positions of circle are detected so that the image processing can be performed rapidly.

Fig. 6 shows a flow chart of the boundary recognition. A captured microscope image (Fig. 6 (A)) is inverted and binarized (Fig. 6 (B)). Circular boundaries in the binarized image are detected by the Hough transform (Fig. 6 (C)). Since the cells are not always sufficiently circular, some parts of the detected circles are not on the cell boundaries. To avoid recognizing a position that is on the detected circle but not on the cell boundary as a cell boundary, an AND operation is applied to the image of the detected circles (Fig. 6 (C)) and the binarized image (Fig. 6 (B)) to obtain the detected boundaries (Fig. 6 (D)).

![Flow chart of cell boundary recognition algorithm. Note that the original image (A) is shown in the background of the detected circles (C) and detected boundaries (D) for the reader’s comprehension.](image)

2) Extraction of the inner radial intensity profile and depth estimation: Once the cell boundaries are detected, the subsequent processing is almost the same as the case of the separated cells. However, the each detected boundary is not always a whole boundary. Thus, the simple averaging of four RIPs as shown in Fig. 2 can not be used in this case. An average of inner RIPs originated on all the detected boundary pixels of a certain cell is used as an average inner RIP. The inner RIPs that are partial RIPs inside of cells are extracted and represented as a set of intensity values of 15 pixels, since the average radius of the yeast cell was about 15 pixels.

V. EXPERIMENTS

A. Experimental set-up

A block diagram of the developed experimental set-up is shown in Fig. 7. The set-up consisted of an optical microscope (BX50WI, Olympus), a computer vision system (Profile Imager), an XYZ automated stage, a personal computer (PC) for stage control, and another PC for image processing. Both PCs were connected via a shared memory, allowing them to communicate with each other.

A bright-field image of target cells with Köhler illumination was magnified with a 100-times objective lens (NA 0.95, UMPIfl, Olympus) and projected onto the vision system. Note that the diffraction pattern.
depends on the diameter of the aperture stop of the illumination optical system, because the range of incident angles of the illumination light is determined by this diameter. In the experiments, the stop diameter was adjusted so that the diffraction pattern of the cells was clearly visible to the vision system. In the following experiments, the stop diameter was adjusted to be less than half of the whole diameter, and NA of illumination was set to be smaller than that of the objective lens.

To realize high-speed focusing, the vision system must be capable of high-speed image acquisition and processing. Therefore, the vision system we adopted was the so-called Profile Imager system developed by Hamamatsu Photonics K. K. [13], a high-speed CMOS imager with adaptive readout of a region-of-interest (ROI). In our experiment, the ROI was set to 232×232 pixels at 1 kHz frame rate. The effective view field of the Profile Imager was 46.4 μm × 46.4 μm.

The image processing PC included two dual-core Intel Xeon 3.0-GHz processors running Microsoft Windows XP. An image processing program was developed using the C++ language with an Intel C++ compiler and Microsoft Visual Studio.

In the case of separated cells, the PC could execute all image processing algorithms, including binarization, boundary detection, extraction of radial intensity profiles, and depth estimation, within 1 ms when the 232×232 grayscale image included one yeast cell. In the case of clumped cells, the execution time was up to 10 ms due to the relatively complex Hough transform processing.

The estimated depth information was transmitted to the other stage control PC having an Intel Pentium4 3.2-GHz processor running a real-time OS (ART-Linux, Moving Eye). This PC controlled the position of the XYZ stage to the desired position. The control period was 1 ms.

A slide including the yeast cells was fixed on the XYZ stage so that its position could be controlled in three dimensions. The stroke is 25 mm (X,Y) and 10 mm (Z) with sensor resolution of 0.25 μm and repeatability of ±1 μm. The step response time of all axes was around 50 ms. All axes were actuated by AC servomotors (HF-KP053(B), Mitsubishi Electric) driven by AC servo amplifiers (MR-J3-10A, Mitsubishi Electric).

Some experiments described below required high positional resolution in the depth direction. A piezoelectric stage (P-731.10, PI GmbH & Co. KG.) that had a high positional resolution of < 1.0 nm was used instead of the XYZ stage for the experiments.

The yeast cells were cultured in sugar solution. The culture was diluted with pure water, placed on a slide glass, and covered with a cover glass. The slide was put still for about ten minutes so that most cells sink down to the surface of the slide glass.

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**Fig. 7.** Block diagram of the experimental set-up (a), and a photograph of the microscope with the XYZ stage (b). A slide glass including specimens was fixed on the XYZ stage using a metal frame.

**B. Depth estimation of separated cells using the average RIPs**

Experimental results obtained with separated cells are described first.

1) **Depth estimation:** The average RIPs of a yeast cell were measured at various depths to confirm that the profiles actually depended on the depth of the cell. The piezoelectric stage was used for the experiments described in V-B1 and V-B2.

A yeast cell was held on a slide glass with its medium covered by a cover glass. The cell was placed at the center of the field of view of the microscope. The RIPs were measured by scanning the depth of the cell from −6 μm to +6 μm in 0.1 μm steps. The depth origin was defined as the depth when an observer recognized that the cell was in focus. Positive depth means that the cell was on the objective lens side of the focal plane of the microscope.

The linear regression coefficients were estimated from the measured RIPs (4 RIPs per one cell) using the least squares method. Fig. 8 shows the estimated depth profile versus the actual depth of the target cell. Root mean square error (RMSE) of the estimation was 0.70 μm, almost the same as the typical depth of field of the microscope. Thus, the assumed model was sufficiently valid for microscope autofocusing. The maximum error of 3.1 μm was observed at the depth of 1.3 μm.

When the cell depth was more than 6 μm or less than -6 μm, the cell image much blurred and the estimation included large error due to unstable detection of cell boundary. Thus, the applicable range of estimation was from -6 μm to 6 μm with the setup.

2) **High-speed autofocusing:** An autofocusing experiment involving yeast cells was conducted to confirm the high-speed focusing ability of the proposed algorithm. Considering the application to scanning
microscopy, a feedback control of focus was adopted to track the focal plane on the object plane continuously.

In this experiment, the image processing PC captured an image of the cells and estimated their depth. The estimated depth was transmitted to the control PC, which then controlled the Z position of the specimens so that they were in focus. This process was executed for every image captured by the high-speed vision at 1000 fps. Namely, the feedback rate was 1 kHz.

To evaluate the autofocusing speed, a step response of the depth was measured. First, average RIPs of yeast cells at various depths were measured to calibrate the linear regression coefficients. Next, the cells were defocused by -5 μm initially. Then, autofocus control was started at time \( t = 0 \).

![Fig. 8](image)

**Fig. 8.** Estimated depth of the yeast cell versus its actual depth using the average RIP for separated cells.

**Fig. 9 shows the step response of the depth of the target.** The initial rise time was about 7 ms and the settling time was about 20 ms. The settling time was much shorter than the typical response time of 0.8 s of conventional autofocus systems.

![Fig. 9](image)

**Fig. 9.** Step response of the cell depth using the average RIP for separated cells.

3) **Continuous autofocusing for a scanning microscope:** The developed autofocusing algorithm was applied to a scanning microscope to show its effectiveness in a realistic application. The purpose of this experiment was to keep the cells in focus while scanning the field. A slide glass holding many yeast cells was fixed on the XYZ-stage described in V-A.

When one or more cells was included in the image captured by the profile sensor, the depth of a cell located at the upper-leftmost position in the image was estimated. The Z axis of the stage was controlled to bring the estimated cell into focus according to the estimated depth. When no cell was in the image, the Z-axis was controlled to keep the previous position. The X- and Y-axes were controlled to scan a given rectangular outline at a constant linear velocity of 250 μm/s. This velocity corresponded to 5.4 widths of the field per second in our experimental set-up. The linear regression coefficients were estimated in advance for a typical yeast cell with the same illumination conditions adopted in this experiment.

![Fig. 10](image)

**Fig. 10.** Images captured by the profile imager while focusing cells entering the field of view.

![Fig. 11](image)

**Fig. 11.** Trajectory of the Z-axis position. Points denoted by letters correspond to the images shown in Fig. 10. The chattering of Z position between -20 to 0 ms and the sudden change at 80-90 ms are due to the position sensor resolution of 0.25 μm.

**Fig. 10 shows the images captured while focusing cells entering the field of view.** When two cells entered the field of view shown in Fig. 10 (a), they were not focused. The autofocusing was started when the right cell had entered the field completely in (b). Upon completion of autofocusing, the two cells were in focus (f). Fig. 11 shows the trajectory of the Z-axis during...
this focusing. The stage was kept at its depth position after the cells were in focus ($t > 40\text{ms}$). The instability of Z position when $-20 < t < 0$ and $40 < t$ was due to the positional resolution of the stage (0.25 $\mu$m).

By using our autofocusing algorithm, a scanning microscope could scan the field at high speed while keeping the specimen in focus. This suggested that image-based high-throughput measurement or screening of cells could be realized using a scanning microscope.

Current high-throughput cytometers, such as flow cytometers, cannot recognize spatial information, such as the protein distribution in a cell. This drawback could be overcome by using the proposed algorithm.

C. Depth estimation of clumped cells using inner radial intensity profiles

Next, experimental results with clumped cells are described.

1) Depth estimation: The estimated depth profile versus the actual depth of a cell was measured with the piezoelectric stage using the average inner RIP. In this case, a number of inner RIPs for the averaging varies depending on a number of detected boundary pixels.

Fig. 12 shows the result. RMS-E of 0.52 $\mu$m was observed. Thus, sufficient estimation precision was also confirmed with the average inner RIP. The maximum error of 5.6 $\mu$m was observed at the depth of -5.15 $\mu$m. This was caused by a failure of the cell boundary recognition due to a very blurred cell image. Due to the image blur, estimation did not work properly when the depth was out of the range from -6 to 6 $\mu$m.

2) High-speed autofocusing: An autofocusing experiment was conducted using the average inner RIP. To evaluate the autofocusing speed, the step response of the depth was measured. In this case, a feedback control rate of autofocusing was 100 Hz since the depth estimation of one image took 10 ms.

The step response using the piezoelectric stage was measured for comparison with the result obtained with the separated cells. As a target, single yeast cell was adopted. The initial rise time was about 20 ms as shown in Fig. 13. Although this response time is faster than the conventional autofocusing method, it is slower than the initial rise time obtained with the separated cells, which was only 7 ms. This is due to the slow control frequency of 100 Hz, which is limited by the image processing time.

Next, the step response using the XYZ stage with clumped yeast cells was measured. Fig. 14 shows the step response of the depth of the target using the XYZ automated stage. The settling time was about 200 ms. This slower response time is mainly due to the relatively slow response of the XYZ stage. The image sequence captured during the step response is shown in Fig. 15.
D. Calculation time

Calculation time for each of the algorithms is summarized in Fig. 16. Times for the binarization, boundary detection, circle detection and depth estimation were measured by reading processor performance counter data. The margin was necessary to run every image processing periodically.

(a) Separated cells

Exposure (1 ms) | Binarization (~0.3 ms) | Mechanical response (>20 ms) | Boundary detection and depth estimation (~0.3 ms) | Image Transfer (1 ms)
---|---|---|---|---
0 | 1 ms | 1 ms | 1 ms | 1 ms

(b) Clumped cells

Exposure (1 ms) | Binarization (~0.3 ms) | Mechanical response (>20 ms) | Circle detection and depth estimation (0.7-5.0 ms) | Margin (9.0-5.0 ms)
---|---|---|---|---
0 | 10 ms | 5 ms | 5 ms | 5 ms

Fig. 16. Calculation time of every transactions of each of the algorithms, for separated cells (a) and clumped cells (b).

Fig. 17. Typical focused and defocused image around the depth of 1 μm. The arrow indicates the discontinuity of the binarized cell boundary.

VI. DISCUSSION

Both results of depth estimation, shown in Fig. 8 and Fig. 12, includes a large error around the depth of around 1 μm. Around the depth, the contrast of the cell image decreased as shown in Fig. 17, and the both boundary detect algorithms using boundary trace and Hough transform tended to extract less precise boundaries. Actually, the average RIP at the error depth was different by 24% at a maximum in intensity from the average RIPS observed around the depth. This resulted in the large depth estimation error. The low contrast can be explained if we consider the cell as a transparent phase object. No contrast is observed when the phase object is in focus, but a contrast appears when it is defocused slightly. Considering the low contrast was observed at the depth of about 1.0 μm in the experiment, this depth could be optically in focus. Because the in focus depth was determined by an observer who should find a slightly defocused position due to its higher contrast.

The described algorithms need the recognition of cell boundary. Thus, they cannot be applied to the case when the boundary recognition is difficult, such as the case of tissues. The proposed algorithm using the Hough transform cannot be applied to the cells that transform their shape, since it’s based on the assumption that the cell shape is constant.

Although only spherical cells are supposed in this paper, the algorithm for separated cells can be applied to non-spherical cells by extracting an intensity profile along a normal to the cell boundary. In fact, three dimensional tracking of a freely swimming paramecium with an ellipsoidal shape was reported using the modified intensity profile [14]. In the case of clumped cells, boundary extraction of the non-spherical cells tends to be difficult. If boundaries of clumped cells of a certain kind are able to be extracted with some method, the depth could be estimated using an inner intensity profile along the normal.

The diffraction patterns depend on refractive indices of cell formations and a medium. If some of the indices change, for example, due to the change of optical properties of materials included changes in the cells, the re-calibration is required for a precise estimation.

The Hough transform processing for the circle detection is computationally expensive as described above. The calculation cost is roughly proportional to the number of pixels detected as an object in the binarized image (Fig. 6 (B)). Thus, the cost is proportional to the number of cells in a captured image, assuming that every cell has a same appearance.

The fixed radius assumption was adopted for the Hough transform. Thus the proposed algorithm couldn’t recognize yeast cells with an irregular size or with a deformed shape. The recognition rate of yeast cells was around 54–74%. However, this imperfect recognition rate is not a critical problem for autofocusing since a depth recognition of just one cell is sufficient for autofocusing assuming that every cells are coplanar.

When the cells pile up and they are not co-planar, the developed algorithms fail to detect the cell boundaries in most cases. In this case, no depth estimation is executed. Thus, the cells are observed with the previous focus. This is not a serious failure because it is impossible to observe all pile-up cells in focus.

Although the depth estimation of the algorithms is high-speed, it has a limited depth range where the algorithms work properly (-6 to 6 μm for separated cells and -5 to 5 μm for clumped cells). If the cell is defocused beyond the range, another slower conventional autofocus algorithm with wide working depth range should be adopted. The limited range of autofocusing is, however, not a problem for continuous focusing of the cells whose depth shifts randomly, like the cells observed with a scanning microscope, since the depth shift between two frames is small. Thus, the developed algorithms are particularly suited for the continuous focus tracking.

VII. CONCLUSION

In this paper we have proposed a radial intensity profile (RIP) of a cell as an image feature for high-speed autofocusing based on the Depth From Diffraction (DFDi) method. Two kinds of image processing algorithms were developed using the RIP for
high-speed depth estimation: for separated cells and clumped cells, respectively. A microscope system with a high-speed vision system and an XYZ automated stage was developed for experiments. High-speed focusing of separated yeast cells with 7 ms rise time and 20 ms response time, and continuous autofocusing of separated yeast cells with a scanning microscope were demonstrated. The successful continuous autofocusing shows that image-based high-throughput measurement of cells could be realized using the proposed algorithm. High-speed focusing algorithm for clumped yeast cells with 20 ms rise time was also demonstrated. This response time was slower than that of separated yeast cells, mainly due to the period required for cell recognition. Thus, a high-speed cell recognition algorithm is essential for high-speed autofocusing using the DFDi method.

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