Image Analysis Technology for Label-free Cells

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Biological cells are essentially clear and colorless. Since their images lack contrast, it is difficult to observe them, which is why cells are usually labeled with fluorescence before analysis. However, the label-free state is a fundamental requirement for regenerative medicine. This paper introduces two technologies that help observe label-free cells more effectively. One is a digital phase contrast image for enhancing contrast, and the other is machine learning for improving recognition. We have implemented these two technologies in our CellPathfinder high content analysis software. With the assistance of a machine learning function, users can analyze images of label-free cells more precisely.

INTRODUCTION

Label-free cell image analysis can observe and evaluate biological cells under microscopes without using fluorescent proteins or other fluorescent dyes. Since there is no need to fuse cells with a dye that does not inherently exist in the cells, this method eliminates the influence and toxicity of these dyes and can evaluate the characteristics of cells as they are.

In addition, extensive research has been carried out on the practical use of regenerative medicine using iPS cells, cellular immunotherapy, and high-efficiency cell production. In such research, it is necessary to handle cells in the label-free state. However, the imaging and recognition of label-free cells is extremely difficult because cells are essentially clear and colorless. Therefore, technologies for visualizing, recognizing, and evaluating label-free cells in a condition as close to their living state as possible are indispensable for the above research areas, and their practical use is eagerly awaited.

Yokogawa has released high content analysis systems that screen drug compounds, based on its specialty technologies of confocal fluorescence imaging[(1)]. To respond to the increasing need for label-free cells, Yokogawa has developed its original image recognition technologies based on the digital phase contrast (DPC) technology[(2)]. Yokogawa has also developed a method for recognizing the images of label-free cells using machine learning, which was traditionally difficult. These technologies have been implemented in the CellPathfinder analysis software, which is introduced in another paper in this special issue. This paper describes these two core technologies and introduces imaging examples.

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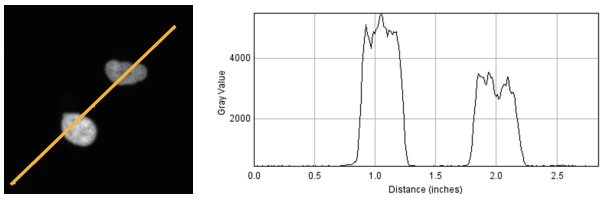
VISUALIZING BIOLOGICAL CELLS

Biological cells are essentially clear and colorless. Since their microscope images have weak contrast, it is difficult to recognize the cells. To solve this problem, technologies have been developed for labelling cells with fluorescent proteins or other fluorescent dyes. This means that cells are observed by means of the intensity and location of fluorescence.

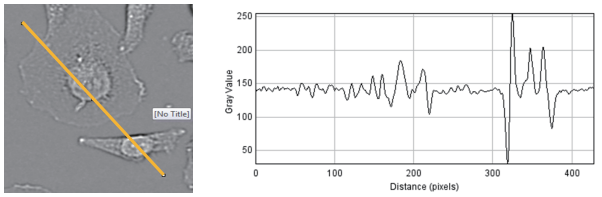
Meanwhile, DPC technology has been applied to the observation of label-free cells. This technology gives sharp contrast to the image of clear cells, enabling their observation. Refractive index differs in cells and the background, which causes the difference in optical phases. Based on this fact and the light propagation equation, the DPC technology calculates the morphology and visualizes cells.

Multiple images are captured by a microscope by moving the objective lens along the axis normal to the cell plane. Then, these image data are substituted into the light propagation equation. By solving it, the difference in optical phases can be quantified. In this way, the DPC technology can recognize cells without labeling.

Figure 1 shows examples of cell images. Figures 1(a) and 1(b) show a fluorescence image and a DPC image, respectively. Cross-section profiles, or brightness profiles, along the yellow line in each image, are shown to the right of each image. In the fluorescence image shown in Figure 1(a), the cell brightness profile shows sharp peaks, and thus the



(a) Fluorescence image and its cross-section profile along the yellow line



(b) DPC image and its cross-section profile along the yellow line

Figure 1 Examples of cell images

cells can be easily separated from the background through the binarization process with a simple threshold value.

In the label-free DPC image shown in Figure 1(b), the cells can be recognized visually. However, different from Figure 1(a), the cross-section profile repeats intermittent swings with no tall peaks. This means that it is extremely difficult to identify cell boundaries and to separate cells from the background through the binarization process. Label-free cells are easier to recognize visually but harder to recognize by mathematical processing than labeled cells.

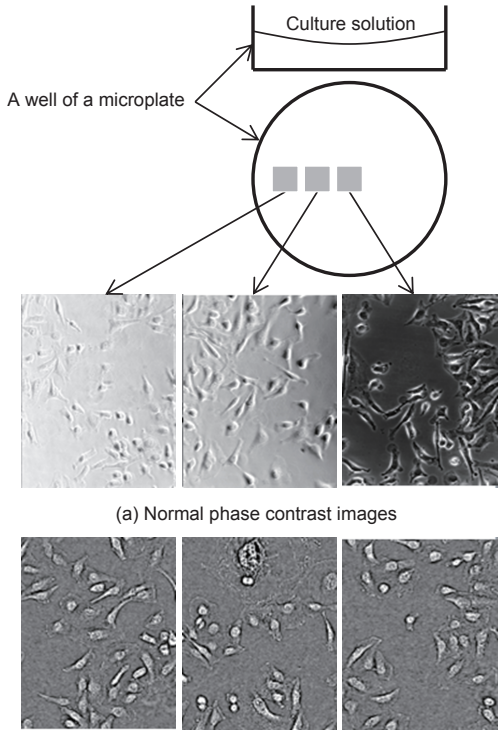
Due to this difficulty, no practical image analysis system for label-free cells is available yet. Recognition of label-free cells still depends largely on visual checking.

Nevertheless, this technology is highly efficient for analyzing gene expressions and detailed functions of intracellular organelles as long as the analysis targets are limited to morphological information such as cell count, area, and length of cells. In this case, there is no need to label cells with various dyes depending on the objects of observation, such as cell nuclei, cytoplasm, and neurites. This saves much labor and expenses. This technology is also useful for analyzing cells that are difficult to label uniformly.

DPC TECHNOLOGY

Label-free cell images are usually obtained by using phase contrast microscopes that emphasize image contrast. Phase contrast microscopy is an excellent method for visualizing transparent cells, and the images obtained, which are called phase contrast images, are suitable for visual checking. However, the phase contrast images contain intense light bands along the boundaries of cells, called halos. Therefore, these images are not suitable for recognizing the fine structure of cells.

Conventional phase contrast images are not available in some wells. Figure 2 shows this limitation. The surface of the culture solution is not planar; surface tension causes the solution to climb the wall of the well. When the well diameter is too small, the culture solution becomes like a concave lens,



(a) Normal phase contrast images

(b) DPC images

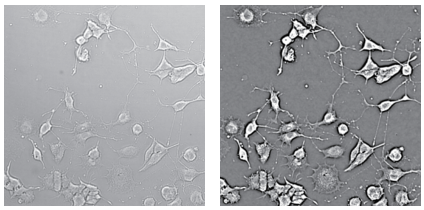
Figure 2 Image quality depending on locations in a well

which disturbs the phase contrast optical system. Figure 2(a) shows this effect. The lens effect is negligible at the center of the well, allowing acquisition of normal phase contrast images with halos. As the imaging point comes closer to the wall of the well, the effect becomes larger and the images become unclear with no halos. Images shown in Figure 2(a) were acquired using a 96-well microplate. Phase contrast images cannot be acquired at all with a microplate of 384 or more wells.

Figure 2(b) shows the images taken as in Figure 2(a), using Yokogawa's DPC technology. These three images show the equal image quality independent of the imaging point.

This DPC technology was applied to imaging of cells with complex shapes, such as neurites, fibroblasts, and iPS cells. Figure 3 shows an example of a neurite image. A bright field image of the same field is shown for comparison. Clearly, the contrast is higher and hence the cell recognition is easier in the DPC image than in the bright field image. Yokogawa's DPC technology is also effective for thick samples, whose whole image cannot be obtained in a single shot. Examples are neurites extending in various directions, and cell colonies containing cells of various sizes. Yokogawa's DPC technology helps to obtain easy-to-analyze images even in such cases.

The DPC technology is expected to be used widely as a standard method for label-free screening applications in drug discovery, because it does not require dedicated optical systems that are necessary for acquiring conventional phase contrast images, and is free from the limitation on the size of wells.



(a) Bright field image (b) DPC image

Figure 3 Example of neurite image

MACHINE LEARNING

Machine learning is a branch of artificial intelligence and has been used for image processing and image recognition. Along with the advancement of computer hardware and software, machine learning has made revolutionary progress and its application to the field of life science is promising.

Machine learning plays a powerful role in various processes of image analysis. Although the DPC technology can visualize label-free cells as shown in Figure 1, it is still difficult to recognize cells and other targets in original images. Yokogawa has applied machine learning to the recognition of label-free cell images and has confirmed its usefulness.

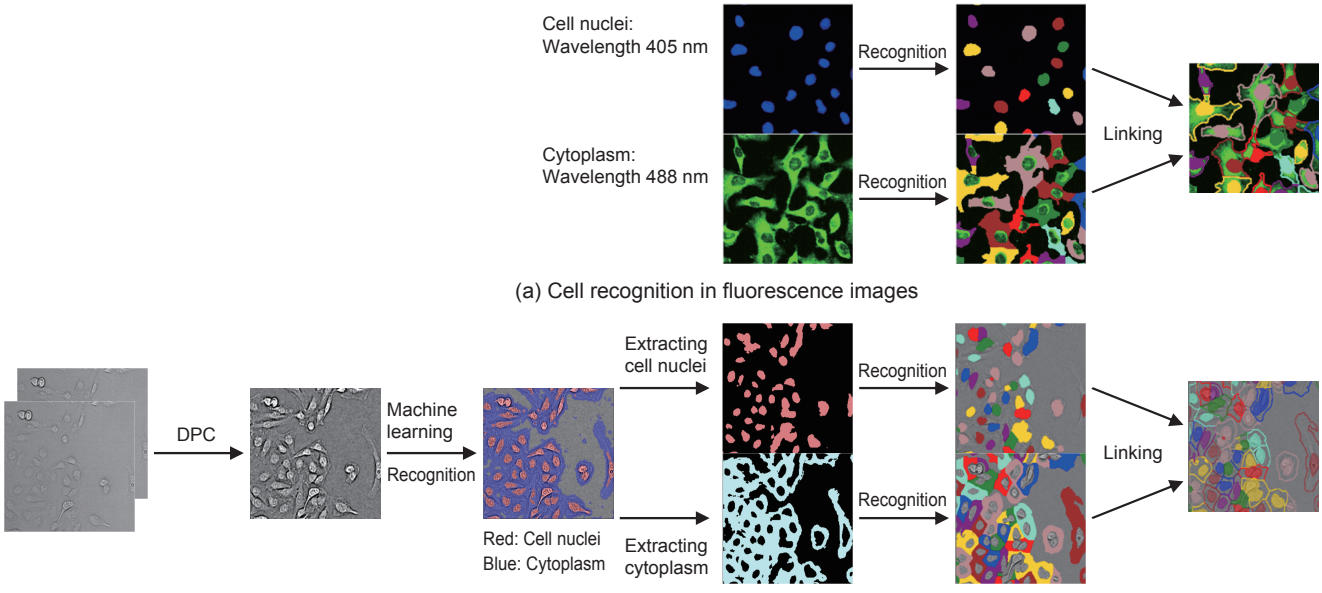
Yokogawa's CellPathfinder analysis software has two machine learning functions. One is the function to recognize cells in native images, and the other is the function to classify the conditions of the recognized cells. The former function works as a filter. It recognizes cell nuclei, cytoplasm, and the background for each pixel and classifies them. The latter is a machine learning gate function. It classifies cell conditions into living cells, dead cells, interphases, foreign matter in the culture solution, and other categories.

Applications of machine learning to label-free cells cover a broad range from simple tasks such as cell counting to difficult ones such as judging the differentiation of iPS cells. The machine learning functions of CellPathfinder were implemented so that they can be easily used in a wide range of applications. For example, users can create supervised data with a small amount of data, simply by clicking parts of an image intuitively and specifying respective categories.

Machine Learning Filter

In the case of fluorescent cells, cell nuclei and cytoplasm can be imaged separately by selecting a proper combination of fluorescent dyes, lasers, and fluorescence filters. As shown in Figure 1, an object is recognized to be a cell nucleus when its brightness is higher than a certain threshold value. Similarly, cytoplasm can be recognized by switching channels. In contrast, recognition with a threshold value cannot be applied to label-free cells. Furthermore, since images are taken at a single channel of transmission illumination, it is even more difficult to distinguish between cell nuclei and cytoplasm. Yokogawa has applied machine learning to this difficult application.

Figure 4 shows the flow of cell recognition for fluorescence images and label-free cell images. In the case of fluorescence images, cell nuclei and cytoplasm are imaged by separate channels as shown in Figure 4(a). After recognizing the targets, the feature values such as area, brightness, and length are calculated in each channel. Based on the positional information, each nucleus is linked to corresponding cytoplasm. In this way, the feature values of the cell nuclei and cytoplasm recognized in separate images are output in the same table and can be plotted in the same scatter diagram.



(a) Cell recognition in fluorescence images

(b) Cell recognition in DPC images

Figure 4 Flow of cell recognition by machine learning

In the case of label-free cells, as shown in Figure 4(b), a cell image is acquired at a single channel and visualized with its contrast emphasized by DPC. Since cell nuclei and cytoplasm are imaged at the same channel, they cannot be distinguished with a threshold value as in the case of fluorescence images. Therefore, machine learning is used to discriminate cell nuclei from cytoplasm. First, each area of cell nuclei, cytoplasm, and the background is selected from DPC images and used as the supervised data. The learning process is repeated with the data. Then, the machine learning function can extract and recognize cell nuclei and cytoplasm in a given image. After that, feature values are calculated and cell nuclei and cytoplasm are linked in the same manner as in fluorescence images. In this way, label-free cell images can be analyzed similarly.

As described above, machine learning enables analyses of label-free cells as well as fluorescent cells. Furthermore, the CellPathfinder analysis software can analyze cell nuclei in a fluorescence image and cytoplasm and neurites in a DPC image simultaneously, according to the user's setting.

Machine Learning Gate

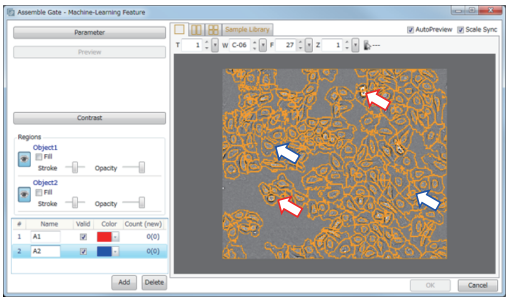
Gating means classifying cell groups for analyses. In scatter diagrams and histograms, cells are classified (gated) with the feature values specified by users. This analysis method is frequently used in fluorescence-based applications such as cell cycle applications.

With the machine learning gate function of CellPathfinder, users create supervised data by classifying cells in images into specified groups. After training with the supervised data, CellPathfinder sorts all cells in new images.

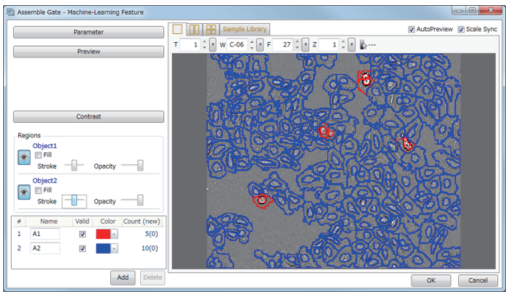
In Figure 5, bright cells are classified into the "red" group, and normal cells into the "blue" group. In Figure 5(a), users click multiple bright cells and normal cells to form the "red" and the "blue" supervised data, respectively. Figure 5(b) shows that after the training, the software precisely classified cells in an image into the "red" and "blue" groups.

In the conventional gate function, the maximum allowable number of feature values is 2 because the classification is carried out in two-dimensional graphs. In contrast, users can use any number of feature values with the machine learning gate of CellPathfinder because it is based on images. Users can specify complex gating.

After cell recognition, CellPathfinder can calculate various quantities ranging from basic feature values such as brightness, area, and perimeter of cells to the feature value of texture representing the pattern of cells. CellPathfinder can also rank the feature values according to their effectiveness



(a) Specifying cells by user for supervised data
(Red: Bright cells, Blue: Normal cells)



(b) Result of grouping by machine learning

Figure 5 Machine learning gate function

for grouping cells. Users select a ranked feature value, define an efficient gate, and execute the desired analysis.

Label-free cell images are often classified by users based on their appearance. Therefore, the surface texture of cells is a critical factor. For such purposes, the machine learning gate of CellPathfinder can automatically classify images based on the texture feature value.

CONCLUSION

This paper described how the DPC technology, which enhances the contrast of label-free cell images, and machine learning, which is an effective tool for image recognition, are powerful means for visualizing and recognizing clear and colorless cells. These technologies still have much room for improvement. Yokogawa will continue to improve its algorithms, establish more practical analysis methods, and implement them in CellPathfinder.

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