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Image-based focused counting of dividing cells for non-invasive monitoring of regenerative medicine products

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Despite the growing numbers of successful applications in regenerative medicine, biotechnologies for evaluating the quality of cells remain limited. To evaluate the cultured cells non-invasively, image-based cellular assessment method holds great promise. However, although there are various image-processing algorithms, very few studies have focused to prove the effectiveness of phase contrast images with risk assessment example that reflects actual difficulties in regenerative medicine products. In this study, we developed a simple image-processing method to recognize the number of dividing cells in time-course phase-contrast microscopic images, and applied this method to assess the irregular proliferation behavior in normal cells. Practically, as a model, rapid proliferating human fibrosarcoma cells were mixed in normal human fibroblasts in the same culture dish, and their sarcoma existence was evaluated. As a result, the existence of sarcoma population in normal cell sample could be feasibly detected within earliest period of cell culture by their irregular rise of accumulated counts of dividing cells. Our image-processing technique also illustrates the technical effectiveness of combining intra-frame and inter-frame image processing to accurately count only the dividing cells. Our concept of focused counting of dividing cells shows a successful example of image-based analysis to quickly and non-invasively monitor the regular state of regenerative medicine products.

[**Key words:** Image processing; Cell proliferation; Dividing cells; Sarcoma; Fibroblast]

With the recent momentous rise in cell-culture technologies, the growing field of regenerative medicine has expanded its potential to provide cellular therapies for defects that are not treatable by conventional medical strategies (1). This growth in cellular therapies has resulted in new requirements for producing cells as regenerative medicine products; because these therapies require intact cells, techniques for assessing the state of cultured cells during the cultivation and maintenance process have become critical for the production of stable and safe cells for therapeutic applications. Currently, several methods exist for evaluating cellular status using conventional techniques in molecular biology; these methods include next-generation sequencing, quantitative PCR, immunohistochemical staining, flow-cytometry analysis, and one of the most recently developed techniques, high-content image analysis (2–4). Although these conventional techniques have proven to be quantitative and reliable for evaluation of cellular status, they are all based on invasive methods. Such invasive methods only allow partial quality assessment of regenerative medicine products, and not able to be expanded to whole cellular quality check to assure maximum safety of cells. To practically check suspicious risks in cultured cells, microscopic observation has been an essential method for monitoring daily changes in cells (5). However, because the results of manual microscopic observation are difficult to quantify (i.e., convert into numerical data), there is a rapidly growing needs for new technologies (6–12) that can quantify microscopic images to measure daily cellular status.

Previously, our group has reported on the effectiveness of image-based cellular evaluation, focusing especially on images of non-stained cells, in production of regenerative medicine products (9–12). In particular, we reported that cellular morphological information extracted from a time-course of phase-contrast microscopic images could provide quantitative and predictive information that enables non-invasive real-time daily evaluation of cellular quality such as proliferation and differentiation potentials. These studies demonstrated that it is possible to use computational image analysis to convert traditional microscopic observations of cellular properties into quantitative data. In this study, we expanded our concept of cellular evaluation using non-labeled phase-contrast images to monitor the proliferation state of regenerative medicine products. As suggested by regulatory agencies, the irregular cellular proliferation rate is one of the most recent assessment criteria required to check the quality of cellular products, because such growth kinetics (proliferation rate or number of population doublings) of cells is suggested as informative criteria to detect the risk of tumor formation (13). In this work, we here propose the risk assessment concept of detecting irregularly increasing population of dividing cells in cultured cells by simply counting the numbers of dividing cells in non-stained images.

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Daily monitoring of cellular doubling time is one of the most practical and feasible tests for detecting risk of tumorigenicity in regenerative medicine products (13). Commonly, doubling times are important indicators of monitoring cellular quality (14–16). Although doubling time can provide an important indicator of the "irregularity" in daily cell maintenance, the protocols for measuring this parameter has remained primitive. One practical method for evaluating growth irregularity is measuring the doubling time by simply counting cells at the time of passage. In practice, a small partial sample of harvested cells are counted (either manually or automatically), and the result is used to estimate total cell number (in general, 1000 to 10,000-fold greater than number of counted cells). However, because these sampling-based cell counting can only be performed at the time of passage, this method is not capable of everyday monitoring. Moreover, sampling of such a small subset of cells is associated with a large estimation error rate.

To develop the method of image-based assessment for monitoring the regular proliferation state of regenerative medicine products, we designed our work based on two major concepts that distinguishes our approach from those of others. Concept 1: Development of an original image-processing algorithm focused on counting only cells in the dividing state, customized to phase-contrast microscopic images of human cells actually applied in clinical use. Concept 2: Evaluation of the performance of focused counting, in which only the dividing cells in each image are counted, for detection of irregular cellular population growth in normal cell population.

First, regarding concept 1, we primarily used phase-contrast microscopic images, i.e., images of non-stained cells. Based on recent progress in technologies for high-content analysis, there are numerous commercially available systems and software capable of quantifying cellular images. However, it should be noted that most high-content analysis systems are limited to acquisition of bright-field images and fluorescently labeled images. Cell-recognition image processing using bright-field images uses a basic algorithm very different from that used for phase-contrast images. CellProfiler (2), which is one of the best-performing software for cellular image analysis, also provides various algorithmic possibilities for cellular image processing; however, it has not yet been applied to focused counting of dividing cells in non-stained phase-contrast images. Several previous studies aimed at developing image-processing algorithms for detection of cellular events (cell division, cell death, or cellular mobility) (17–23). However, few reports have evaluated the performance of these algorithms in recognizing cell division with clinically-used cell types. It has been also rare to aim their application for solving practical problems related to cell therapy, especially for risk assessment of regenerative medicine products. Therefore, although the concept of image-based cell evaluation holds great promise for a wide range of applications, practical methods that address bottleneck problems in real-world cell therapy remain rare. To monitor the proliferation status of cellular products, we designed a cell model containing human fibrosarcoma cells (HT-1080) mixed in human normal fibroblasts (normal human dermal fibroblast: NHDF), which can be used to produce an increase of the irregular subpopulation in normal cells. Because fibroblasts are widely obtained and used from human tissues, they are clinically used as a rich source of cells for skin-defect therapies (24–27), and they have also been used recently for the establishment of induced pluripotent stem cells for clinical applications (28). To date, however, there are no significant biological markers that can define normal fibroblasts; therefore, it is difficult to evaluate the purity of samples of this important cell type. The irregular transformation of fibroblasts leads not only to tumorigenic risk, but also to form scar contracture in injected areas when cells express the myofibroblastic response. Therefore, the detection of irregular characteristics, especially increasing dividing frequency compared to the normal daily data, should contribute to more delicate quality maintenance. Previous attempts at cellular image analysis have not evaluated such practical events. Technically, in order to implement the idea of detecting irregular event in cells lacking useful markers, we developed the morphology filter to count only the dividing cells. In practical, we constructed the image processing algorithm that recognizes the halo-like feature of dividing cells (reflecting a spherical morphology), combining the workflow of intra- and inter-frame image processing on time-course images. We assumed that such focused counting of dividing cells should reflect the increase in the irregularly proliferating cell subpopulation within a heterogenous population of cells. Our data also shows technical solution of focused cell-counting by evaluating the effect of inter-image processing on the accuracy of cell recognition.

Second, we evaluated the performance of our algorithm in discriminating different proportions of sarcoma cells intentionally seeded in normal fibroblasts. Focused counting of dividing cells could detect the HT-1080 sub-population within a population of NHDF as their irregularly-higher frequency, within the first few days of cell culture. This result demonstrates the practicality of our image-based risk assessment method to monitor the quality state of adhesive-type normal cells produced for cell therapies.

MATERIALS AND METHODS

Cells and cell culture Human normal dermal fibroblasts (NHDF, Kurabo, Osaka, Japan) and a fibrosarcoma cell line (HT-1080, ATCC, Manassas, VA, USA), a model of tumorigenic cells derived from mesenchymal cells such as fibroblasts, were cultured and maintained in Dulbecco's modified Eagle medium (DMEM) containing 10% fetal bovine serum. Cells were grown in 3.5-cm dishes at 37°C in 5% CO_2. To model contamination by tumorigenic cells, various proportions of HT-1080 cells were mixed with NHDF cells at the time of seeding: (i) HT-1080:NHDF = 0:100, (ii) HT-1080:NHDF = 20:80, (iii) HT-1080:NHDF = 50:50, and (iv) HT-1080:NHDF = 100:0. Cells were seeded at the density of 2000 cells/cm^2.

Image acquisition Time-course images were taken using CellWatcher (Corefront, Tokyo, Japan), starting at the time of seeding and adhesion, thereafter at 15-minute intervals (From 200 to 400 frames obtained in each cell conditions as total data, common 125 frames that express similar cellular conditions were extracted for further analysis). From three replicates of the image-acquisition experiment, the sample with the smallest cell-seeding bias was chosen for image analysis. Each image was a 24-bit BMP file of 1024 × 768 pixels. Example images acquired from three samples are shown in Fig. 1. Several periods were used to evaluate the algorithm's performance. Term 1: (i) 171–181 frames, (ii) 70–80 frames, (iii) 50–60 frames, (iv) 100–110 frames; term 2: (i) 211–221 frames, (ii) 100–110 frames, (iii) 100–110 frames, (iv) 150–160 frames; term 3: (i) 351–361 frames, (ii) 170–180 frames, (iii) 150–160 frames, (iv) 200–210 frames. For representative image sampling for Fig. 2, frame 368 (Fig. 2A), frame 288 (Fig. 2B), frame 194 (Fig. 2C), and frame 288 (Fig. 2D) was selected.

Image-processing algorithm Analysis of all the images acquired (1138 images, including the images that were not further analyzed for plotting dividing cells) covering all cell conditions and time-courses taken prior to the actual data analysis revealed that characteristic features of cell-like objects in phase-contrast images could be categorized into three types (Fig. 1): type I, expanding cell-like objects with low-intensity pixels in the majority of the object area, surrounded by halos at the edges of their contours, considered to represent cells engaged in growth; type II, dividing cell-like objects which their major area consists of halo, and a very round peripheral morphology, considered to represent cells engaged in division; and type III (not indicated in Fig. 1), objects smaller than nuclei (<20 pixels) with low-intensity pixels throughout the majority of the object area, considered to represent debris.

In practice, the intra-frame image-processing method consisted of major four steps of workflow (Fig. 3): Step 1, image processing for recognition of both type I and type III objects. From the raw image (Fig. 3A), all pixels in the frame were searched using the bottom-hat filter (circle of 20 pixels in radius was used as the structural element). The binalized result image contains both type I and type III objects (Fig. 3B). Step 2, image processing for recognizing objects with halo, containing type II objects and false-positive objects (halo area near type I/III objects that are not cells) (Fig. 3C). In this step, two kinds of morphological filters were applied to the raw image: filter 1 (top-hat filter, radii of 20 pixels as structural elements), and filter 2 (bottom-hat filter, radii of 200 pixels as structural elements). Next, the binalized image resulting from filter 2 was subtracted from that of filter 1 to reduce the false-positive objects. Step 3, subtraction binalized images of step 2