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Non-invasive quality evaluation of confluent cells by image-based orientation heterogeneity analysis

Kei Sasaki,[1] Hiroto Sasaki,[2] Atsuki Takahashi,[3] Siu Kang,[1] Tetsuya Yuasa,[1] and Ryuji Kato[3,*]

Graduate School of Science and Engineering, Yamagata University, Jonan, Yonezawa, Yamagata 992-8510, Japan,[1] Graduate School of Engineering, Nagoya University, Furocho, Chikusa-ku, Aichi 466-8602, Japan,[2] and Graduate School of Pharmaceutical Sciences, Nagoya University, Furocho, Chikusa-ku, Aichi 466-8601, Japan[3]

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In recent years, cell and tissue therapy in regenerative medicine have advanced rapidly towards commercialization. However, conventional invasive cell quality assessment is incompatible with direct evaluation of the cells produced for such therapies, especially in the case of regenerative medicine products. Our group has demonstrated the potential of quantitative assessment of cell quality, using information obtained from cell images, for non-invasive real-time evaluation of regenerative medicine products. However, image of cells in the confluent state are often difficult to evaluate, because accurate recognition of cells is technically difficult and the morphological features of confluent cells are non-characteristic. To overcome these challenges, we developed a new image-processing algorithm, heterogeneity of orientation (H-Orient) processing, to describe the heterogeneous density of cells in the confluent state. In this algorithm, we introduced a Hessian calculation that converts pixel intensity data to orientation data and a statistical profiling calculation that evaluates the heterogeneity of orientations within an image, generating novel parameters that yield a quantitative profile of an image. Using such parameters, we tested the algorithm's performance in discriminating different qualities of cellular images with three types of clinically important cell quality check (QC) models: remaining lifespan check (QC1), manipulation error check (QC2), and differentiation potential check (QC3). Our results show that our orientation analysis algorithm could predict with high accuracy the outcomes of all types of cellular quality checks (>84% average accuracy with cross-validation).

[**Key words:** Confluent cells; Cellular quality; Orientation complexity; Microscopic image; Image-based analysis]

In order to guarantee maximum safety and efficacy of cell-based therapies for the development of regenerative medicine, for which patient-derived cells are cultured and expanded *in vitro* for long periods, it is essential to evaluate the quality of cultured cells. Although several molecular-biological methods are available for evaluating cellular status, their invasiveness is incompatible with production of cells for regenerative medicine, which requires intact cells for use in treatments (1–3). In current cell-culture techniques, daily maintenance of cultured cells is commonly accomplished through manual microscopic observation and handling, based on human experience (4). However, because such manual techniques are neither quantitative nor descriptive, improvement of the safety and efficiency of cellular production has been the greatest challenge in the industrialization of cell-based therapies. To overcome such difficulties, novel image-based analysis technologies (5–11) have been introduced into these production processes, with the goal of monitoring and quantitating the cellular state in a non-invasive and high-throughput manner. We have described the successful application of image processing and machine learning techniques to quantitatively evaluate, or even predict, the ultimate cellular status based solely on phase-contrast microscopic images collected during the early stages of culture (8–11).

Several protocols for production of therapeutic cells require maintenance of the cells at confluent or near-confluent status (i.e., the maximum cell density achievable in culture). Similarly, when cell therapy requires certain yields for the purpose of clinical treatment, cells tend to be cultured to a nearly confluent state (70–90% confluence) in order to maximize cell number. In some cases, cells are shifted from a proliferation mode into a period of differentiation induction after they reach the fully confluent state. However, even though confluence frequently occurs during practical cell production for regenerative medicine, the daily and continuous evaluation of this stage without the use of staining has been extremely uncommon. Although several exclusively image-based analysis methods have been applied to cellular evaluation, the quantitation of later stages of the culture process, near the confluent state, has not succeeded in quantitatively predicting cellular status. One of the main obstacles to such image-based evaluation of confluent cells is the difficulty of cellular segmentation within images of dense cultures. In non-stained cellular images consisting only of gray-scale pixels, the morphological features of intact cells are rare. In addition, when cells are confluent, their borders become ambiguous. Above all, the performances of cell-segmentation image-processing technology can only be evaluated by reference to the ground truth, which is commonly assessed from

* Corresponding author. Tel.: +81 52 747 6811; fax: +81 52 747 6813.
E-mail address: kato-r@ps.nagoya-u.ac.jp (R. Kato).

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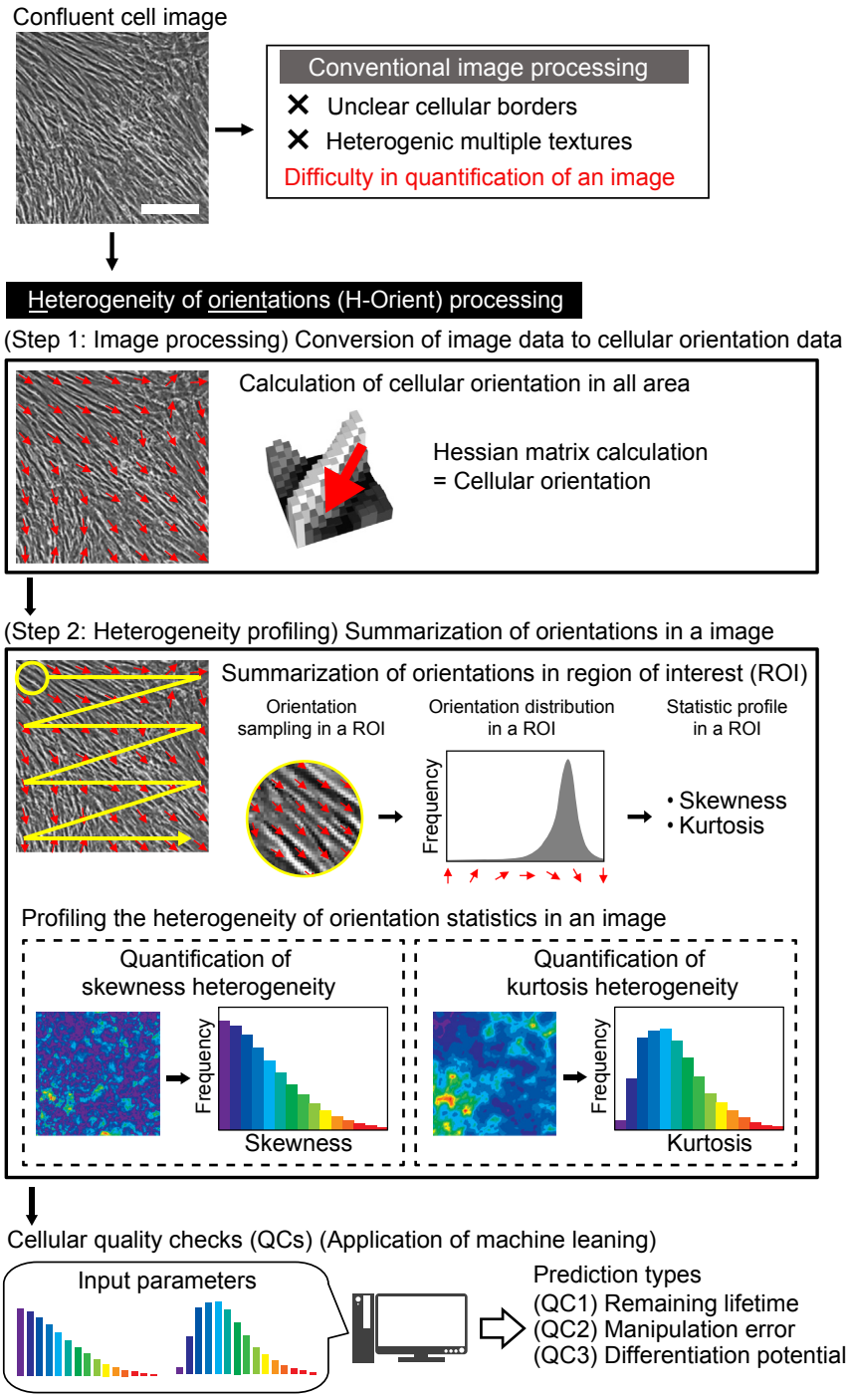

FIG. 1. Schematic illustration of heterogeneity of orientation (H-Orient) processing in confluent cells. Scale bar in the confluent cell image: 100 μm.

staining results; consequently, it is difficult to apply these methods to images without an established ground truth. For example, if nuclei or cytosol is stained with fluorescent labels, the high signal-to-noise ratio pixels in such fluorescent images provide a biologically relevant clue that is useful for estimating the border of neighboring cells. However, in the case of patient-derived cells intended for use in therapy, such staining cannot be performed. In fact, in CellProfiler (1), ImageJ (12), and ICY (13), the software most frequently used for cellular image processing, it is difficult to achieve cellular segmentation of confluent cells in non-stained phase-contrast images.

We developed a novel concept for analyzing images of confluent cells for the purpose of non-invasive and quantitative evaluation in the context of regenerative medicine. In this work, we abandoned the effort to segment cells individually, which commonly fails to achieve accuracy and robustness, and instead designed an algorithm for evaluating confluent cellular images as patterns of pixels reflecting cellular heterogeneity. Our concept for converting images of confluent cells is designated as heterogeneity of orientation (H-Orient) processing. In this concept, we effectively combined two algorithms to describe the heterogeneous density of cells in the confluent state (i.e., to distinguish between more highly occupied