

BRIEF REPORT



Label-free cell detection of acute leukemia using ghost cytometry

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Abstract

Early diagnosis and prompt initiation of appropriate treatment are critical for improving the prognosis of acute leukemia. Acute leukemia is diagnosed by microscopic morphological examination of bone marrow smears and flow cytometric immunophenotyping of bone marrow cells stained with fluorophore-conjugated antibodies. However, these diagnostic processes require trained professionals and are time and resource-intensive. Here, we present a novel diagnostic approach using ghost cytometry, a recently developed high-content flow cytometric approach, which enables machine vision-based, stain-free, high-speed analysis of cells, leveraging their detailed morphological information. We demonstrate that ghost cytometry can detect leukemic cells from the bone marrow cells of patients diagnosed with acute lymphoblastic leukemia and acute myeloid leukemia without relying on biological staining. The approach presented here holds promise as a precise, simple, swift, and cost-effective diagnostic method for acute leukemia in clinical practice.

KEYWORDS

acute leukemia, acute lymphocytic leukemia, acute myeloid leukemia, flow cytometry, ghost cytometry, imaging flow cytometry, machine learning, support vector machine

1 | INTRODUCTION

Acute leukemia is one of the most common types of cancer and remains a leading cause of death worldwide [1–3]. Early diagnosis is critically important to enhance survival chances and improve prognosis. Acute leukemia is diagnosed through morphological examination of bone marrow (BM) smears using microscopy [4, 5]. In addition, immunophenotyping of BM cells using flow cytometry (FCM) is widely utilized as a diagnostic complement to the microscopic examination

[5–10]. However, manual reading under microscopy necessitates skilled specialists and is often resource-intensive and time-consuming. While FCM allows a quantitative and high-speed analysis of cells, it relies on cell staining with fluorophore-conjugated antibodies, which may lead to errors due to a lack of cell morphological correlation, reduced antigen expression, and nonspecific binding. In addition, an FCM test involving antibodies is expensive, and its procedures and interpretations are often complex, limiting facilities capable of performing it. Thus, a simple, accurate, and cost-effective method for diagnosing acute leukemia is desired. Recent efforts have focused on label-free techniques for the cell detection of acute leukemia [11, 12];

Yoko Kawamura and Kayoko Nakanishi contributed equally to the work.

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however, their practical implementation has yet to be achieved possibly due to the difficulty of effectively balancing accuracy and simplicity.

Ghost cytometry (GC) is a recently developed high-content flow cytometric approach, which enables a machine vision-based, high throughput analysis of cells based on their detailed structural information [13, 14]. In a previous study, we have demonstrated that its label-free mode (label-free GC: LF-GC) can distinguish different cell states, cell types derived from human-induced pluripotent stem cells, and subtypes of peripheral white blood cells without the need for biological cell staining [15]. However, its application to BM cells and its utility for diagnosis in clinical practice have yet to be explored. This study demonstrates that LF-GC can detect leukemic cells in BM cells from patients diagnosed with acute lymphoblastic leukemia (ALL) and acute myeloid leukemia (AML), presenting that this approach is a promising candidate as a novel diagnostic method for acute leukemia.

2 | MATERIALS AND METHODS

2.1 | Cell line

The NB-4 cell line (human, B-cell leukemia cell line) was purchased from the German Collection of Microorganisms and Cell Cultures (DSMZ, Braunschweig, Germany). The cell line was cultured in the Roswell Park Memorial Institute (FUJIFILM Wako Pure Chemical, 189-02025, Japan) 1640 medium with 10% fetal bovine serum (Merck, F7524, Germany) and 1% penicillin/streptomycin (FUJIFILM Wako Pure Chemical, 168-23191, Japan) at 5% CO₂ and 37°C.

2.2 | Bone marrow specimens

BM aspirates of a healthy donor and two patients diagnosed with acute leukemia were collected in accordance with the Declaration of Helsinki and approved by the Kyoto University Graduate School and Faculty of Medicine, Ethics Committee. Patient 1 was a 22-year-old male diagnosed with B-cell ALL. The May-Grunwald-Giemsa (MGG) stained-BM smear showed 60.0% leukemic blasts. The blast cells were small to medium-sized and had scanty basophilic cytoplasm with homogeneous fine chromatin. Patient 2 was a 52-year-old female diagnosed with overt AML. The MGG stained-BM smear showed 31.2% leukemic blasts. These blast cells were medium to slightly large-sized, exhibiting a high nuclear/cytoplasm ratio with basophilic cytoplasm. The blast cells had soft, fine chromatin and nucleolus. Patient 2 had received a BM transplantation for myelodysplastic syndromes, and the BM specimen was obtained during the treatment after relapse.

2.3 | Sample preparations

NB-4 cells were suspended in phosphate-buffered saline (PBS, FUJIFILM Wako Pure Chemical, 045-29795, Japan) containing 0.5%

bovine serum albumin (BSA, Merck, A7906-100G, Germany). NB-4 cells and the BM aspirate of a healthy donor were first fixed with BD FACS™ Lysing Solution (BD bioscience, 349202, USA). The lysing solution was 10-fold diluted with deionized water before use. A total of 2 and 20 mL of the diluted lysing solution were added to 500 µL of the BM aspirate and 2 mL of the NB-4 cell suspension, respectively, and the cells were incubated for 10 min. After fixation, the cells were washed with PBS containing 1% Tween20 (Merck, P1379-25ML, Germany). The NB-4 cells were then labeled with Fixable Far Red Dead Cell Stain (FFR, Thermo Fisher Scientific, L34974, USA). The solid product of FFR was dissolved in 50 µL of dimethyl sulfoxide (DMSO) before use. In total, 4 µL of the FFR DMSO stock solution was added to 200 µL of NB-4 cell suspension, and the cells were incubated for 60 min. In total, 4 µL of a DMSO (FUJIFILM Wako Pure Chemical, 046-21981, Japan) solution was added to 200 µL of the BM cell suspension, and the cells were incubated for 60 min so that the exposure to DMSO became equivalent between the NB-4 and BM cells. After staining, the cells were washed with PBS containing 1% Tween20. All the above staining procedures were performed at room temperature. The NB-4 and BM cells were mixed at approximately equal ratios before LF-GC measurements.

The BM aspirates of patients with ALL and AML were first lysed using VersaLyse Lysing Solution (Beckman Coulter, A09777, USA). A total of 24 and 23.5 mL of the lysing solution were added to 2.4 and 2.35 mL of BM aspirates for ALL and AML, respectively, and the samples were incubated for 15 min. After lysis, the cells were washed with PBS. The cells were then stained with the APC/Cyanine7 anti-human CD45 Antibody (BioLegend, 304014, USA). About 10 µL of the antibody was added to 400 µL of each BM cell suspension, and the cells were incubated for 30 min. After staining, the cells were washed with PBS. The cells were then fixed with BD FACS™ Lysing solution. About 5 mL of the lysing solution 10-fold diluted with deionized water was added to 500 µL of each BM cell suspension, and the cells were incubated for 10 min. After fixation, the cells were washed with PBS containing 0.5% BSA. All the above staining procedures were performed at room temperature.

2.4 | Experimental setup

Cells flowed through a glass flow cell (Hamamatsu Photonics Inc., Japan) with a channel cross-section dimension of 150 × 150 µm² at the measurement position. The sample flow was hydrodynamically focused into a narrow stream using sheath flows. The sheath fluid (IsoFlow, Beckman Coulter, USA) was driven at a pressure of about 305 kPa using a homemade pressure pump, and the sample fluid was driven at a flow rate of approximately 10–20 µL/min using a syringe pump (KD Scientific, USA). The GC setup equips two colors of continuous-wave lasers: a 405-nm-wavelength violet laser and a 637-nm-wavelength red laser. The violet laser passes through a diffractive optical element, forming a structured illumination pattern on the focal plane of an objective lens. When cells flow through the structured light illumination in a microfluidic channel, compressed imaging signals are acquired as temporal waveforms, called ghost

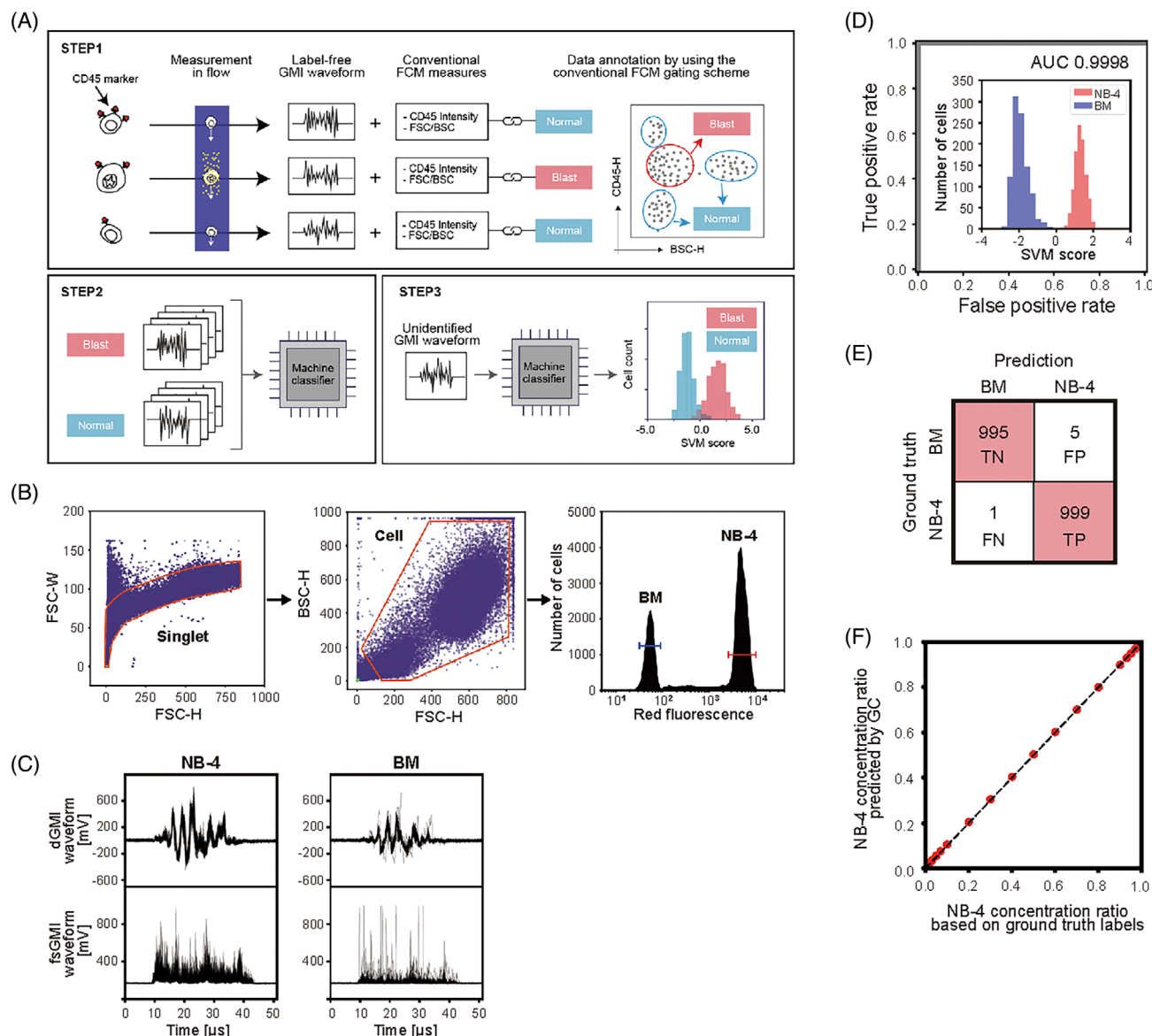


FIGURE 1 Label-free detection of NB-4 cells spiked in BM cells from a healthy donor. (A) A workflow for the label-free detection of blast cells in BM using LF-GC. STEP1, LF-GMI waveforms, as well as the conventional flow cytometric measures such as forward-scattering (FSC), back-scattering (BSC), and fluorescence signals as ground truth labels, are collected using a GC setup. Subsequently, the LF-GMI waveforms of blast cells and normal cells in BM are identified (annotated) using ground truth labels defined by the widely used CD45 gating schemes in the detection of acute leukemia by FCM (upper panel). STEP2, a classifier based on a machine-learning algorithm, the SVM in this study, is trained using the annotated LF-GMI waveforms (lower left panel). STEP3, the classifier predicts the class (blast or normal) of cells based solely on unidentified LF-GMI waveforms, a test dataset that was not used to develop the classifier, without using biological labels. (B–F) Detection of NB-4 cells spiked in the BM cells of a healthy donor. (B) Gating strategies to identify (annotate) NB-4 and BM cells to train a classifier. Coincidence data were removed in a homemade software pre-processing step before the gating process. Singlets were then selected in the FSC-H (height)/FSC-W (width) plots (left panel). Debris was excluded, and cells were selected in the FSC-H/BSC-H plots (center panel). NB-4 and BM cells were identified (annotated) based on the intensities of red fluorescence stained as a ground truth label (right panel). (C) Representative LF-GMI waveforms: pairs of dGMI and fsGMI waveforms, obtained for NB-4 and BM cells after annotation. A total of 50 waveforms were overlapped in each panel. A total of 6,000 pairs of LF-GMI waveforms (3,000 pairs for each class) were used to train a classifier. (D) A ROC curve and SVM score histogram (the inset figure) in classifier evaluation. A total of 2,000 pairs of LF-GMI waveforms (1,000 pairs for each class) were used for validation. The AUC score of 0.9998 was obtained. Red and blue colors at the inset of the figure correspond to NB-4 and BM cells defined by the red fluorescence intensities shown in (B), respectively. (E) A confusion matrix in classifier evaluation when the decision boundary of SVM scores was set at 0. Accuracy, sensitivity, and specificity values of 0.997, 0.999, and 0.995, respectively, were obtained. Accuracy is calculated as the number of cells correctly predicted (TP + TN) divided by the total number of cells (TP + FP + TN + FN), where TP represents true positive, TN denotes true negative, FP indicates false positive, and FN represents false negative. Sensitivity is calculated by dividing the number of cells correctly predicted as blast cells (TP) by the total number of actual blast cells (TP + FN); specificity is calculated by dividing that of cells correctly predicted as normal cells (TN) by that of actual normal cells (TN + FP). (F) Detection of NB-4 cells spiked in BM cells at the concentration ratios of 1–99%. Data from NB-4 cells and BM cells were computationally mixed and analyzed. A total of 5,000 pairs of LF-GMI waveforms were used in each condition. The decision boundary of SVM scores was set to 0. The dashed line shows $x = y$.

motion imaging (GMI) waveforms, encoding detailed morphological information of individual cells [13–15] (Figure 1A). GMI waveforms include two label-free GMI (LF-GMI) modalities called diffractive and forward-scattering GMI (dGMI and fsGMI, respectively) waveforms,

which are analogous to diffractive and forward-scattering darkfield microscopy images, respectively [15]. The red laser beam is focused using an objective lens to create an elliptical spot, similar to a conventional flow cytometer. The spot at 637 nm wavelength was utilized to

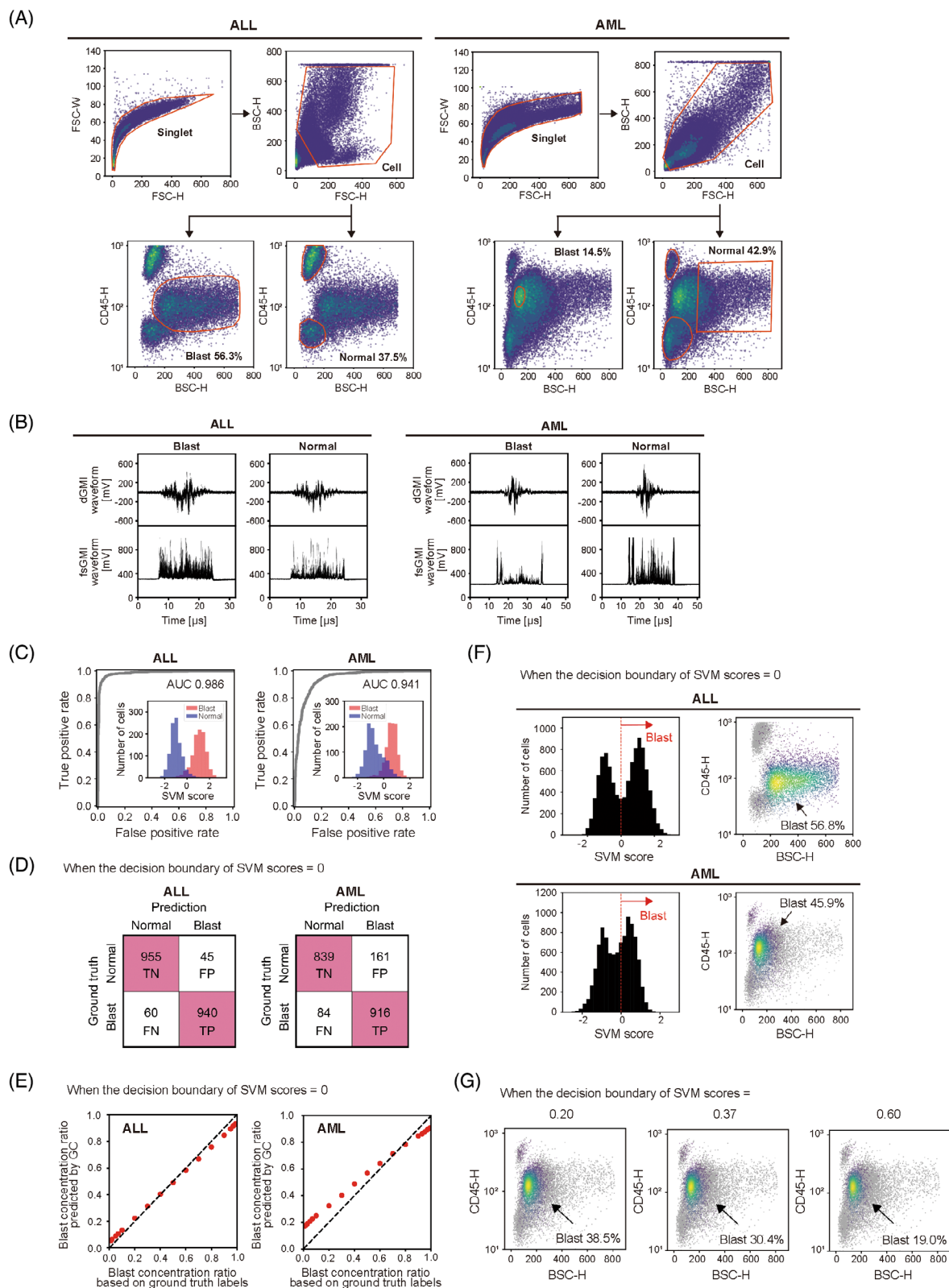


FIGURE 2 Legend on next page.

obtain conventional flow cytometric measures such as red fluorescence, forward scattering (FSC), and backscattering (BSC), which is an analogy of side scattering (SSC) and can be used interchangeably with SSC [15]. The red fluorescence, FSC, and BSC signals provided ground truth labels for annotating training and evaluation datasets (see Section 2.5). The spatial relative positions of these two excitation lights along the microchannel are configured to position the red laser spot approximately at the center of the structured illumination on the same focal plane. dGMI and fsGMI waveforms, as well as FSC, BSC, and red fluorescence signals, were acquired by photomultiplier tubes (PMT, Hamamatsu Photonics Inc., Japan). Homemade software and a field-programmable gate array development board (FPGA, TR4, Terasic, Taiwan) with a homemade analog/digital converter were used to record a fixed length of signal segments simultaneously from each PMT channel with a fixed trigger condition applied to the FSC signal. Prior to each measurement, the PMT gains and the position of the glass flow cell relative to the light sources were calibrated using a micro polystyrene bead-containing solution (Spherotech Inc., Fluorescent Particles, USA) as a quality control of the setup.

2.5 | Data analysis using machine learning

While full image reconstruction was demonstrated in our previous study, fluorescence GC [13], LF-GC employs reduced acquisitions of compressed LF-GMI (dGMI and fsGMI) waveforms and directly analyzes them using a machine-learning algorithm without the need for computationally intensive digital image production, enabling high-speed and accurate data processing [15]. Conventional flow cytometric data modalities such as FSC, BSC, and red fluorescence signals from biological labeling were utilized for annotation in supervised machine-learning analysis. Specifically, the biological labels were used as ground truth labels to identify (annotate) blast and normal cells in BM based on conventional flow cytometric gating schemes. The annotated LF-GMI waveforms were subsequently used to train a classifier based on the support vector machine

(SVM) algorithm. The performance of the trained classifier was evaluated by comparing predicted results with the ground truth labels based on a receiver operating characteristic (ROC) curve, the area under the ROC curve (AUC), and the confusion matrix. Finally, by applying the trained classifier to unidentified (non-annotated) LF-GMI waveforms, a test dataset that was not used to develop the classifier, we demonstrated the detection of blast cells in BM without relying on biological labels. All the data analyses were performed by using homemade software.

3 | RESULTS AND DISCUSSION

3.1 | Detection of NB-4 cells in BM cells from a healthy donor

We first detected NB-4 cells experimentally spiked in the BM cells of a healthy donor, including immature hematopoietic cells that appear morphologically similar to leukemic cells (Figure 1B–F). Before measurements, we stained NB-4 cells with a red fluorescence marker (FFR) as a ground truth label, as described above. After data collection, we identified (annotated) NB-4 and BM cell populations through gating based on the red fluorescence intensities (Figure 1B). Representative LF-GMI waveforms obtained for NB-4 and BM cells after annotation are shown in Figure 1C. Using the annotated LF-GMI waveforms, we trained a classifier and evaluated its performance. Figure 1D shows a ROC curve with an AUC score of 0.9998 in classifier evaluation, exhibiting a high classification ability. Figure 1E illustrates a confusion matrix generated when the decision boundary of SVM scores was set at 0 (SVM score ≥ 0 , predicted as blast; SVM score < 0 , predicted as normal). The classifier demonstrated excellent performance, achieving high accuracy, sensitivity, and specificity scores of 0.997, 0.999, and 0.995, respectively. Furthermore, to test the robustness of the classifier, we detected NB-4 cells computationally spiked in BM cells at various concentration ratios (1–99%) using the classifier. As shown in Figure 1F, the LF-GC-based detection of

FIGURE 2 Label-free detection of blast cells in BM cells from patients diagnosed with ALL and AML. (A) Gating strategies to identify (annotate) blast cells and normal cells in BM for training classifiers. Coincidence data were removed in a homemade software pre-processing step before the gating process. Singlets were then selected in the FSC-H/FSC-W plots (upper left panels). Debris was excluded, and cells were selected in the FSC-H/BSC-H plots (upper right panels). Blast and normal cells were identified (annotated) in the CD45/BSC plots based on the conventional CD45 gating schemes (lower panels). (B) Representative LF-GMI waveforms: pairs of dGMI and fsGMI waveforms, obtained for blast cells and normal cells in the BM samples after annotation. A total of 50 waveforms were overlapped in each panel. In each ALL and AML case, 6,000 pairs of dGMI and fsGMI waveforms (3,000 pairs for each class) were used to train a classifier. (C) ROC curves and SVM score histograms (the inset figures) in classifier evaluations. Red and blue colors in the inset figures correspond to blast and normal cells defined by gating shown in (A), respectively. In each case, 2,000 pairs of LF-GMI waveforms (1,000 pairs for each class) were used for the validation. The AUC scores of 0.986 and 0.941 were obtained for ALL and AML, respectively. (D) Confusion matrices in classifier evaluations when the decision boundary of SVM scores was set at 0. The trained classifiers achieved accuracy, sensitivity, and specificity scores of 0.948, 0.940, and 0.955, respectively, for the ALL case, and 0.878, 0.916, and 0.839 for the AML case. (E) Detection of blast cells spiked in normal cells at the concentration ratios of 1–99%. Data from blast cells and normal cells were computationally mixed and analyzed. A total of 1,000 pairs of LF-GMI waveforms were used in each condition. (F) Histograms and BSC/CD45 back plots of the predicted results obtained by applying the classifiers to unidentified (non-annotated) LF-GMI waveforms. In total, 10,000 pairs of LF-GMI waveforms were classified in each case. Predictions as blast and normal cells are depicted as color density and light gray plots, respectively, in each BSC/CD45 panel. The decision boundary of SVM scores was set at 0 in both cases. (G) BSC/CD45 back plots of the predicted results were obtained in the same manner as in (F) with different decision boundaries of SVM scores: 0.20, 0.37, and 0.60, corresponding to the left, center, and right panels, respectively, exclusively for the AML case.

the acute leukemia cell line consistently showed high performance over a wide range of concentration ratios.

3.2 | Detection of blast cells in BM cells from patients with acute leukemia

Next, we detected blast cells in the BM of patients diagnosed with acute leukemia. As described above, BM aspirates were collected from two patients diagnosed with B-cell ALL and overt AML. Before LF-GC measurements, we stained the BM samples with a CD45 surface marker as a ground truth label. After data collection, we identified (annotated) blast and normal cells in the BM samples according to the commonly used CD45 gating strategies [16, 17] (Figure 2A). Representative LF-GMI waveforms obtained for NB-4 and BM cells after annotation are shown in Figure 2B. Subsequently, we trained classifiers and evaluated their performances using the annotated LF-GMI waveforms. Figure 2C shows ROC curves in classifier evaluations, showing high AUC scores of 0.986 for ALL and 0.941 for AML. Figure 2D depicts confusion matrices generated when the decision boundary of SVM scores was set at 0 (SVM score ≥ 0 , predicted as blast; SVM score < 0 , predicted as normal). The classifiers achieved accuracy, sensitivity, and specificity scores of 0.948, 0.940, and 0.955, respectively, for the ALL case, and 0.878, 0.916, and 0.839 for the AML case. Furthermore, to test the robustness of the trained classifier, we detected blast cells computationally spiked in normal BM cells at various blast concentration ratios (1–99%) using the classifiers. Figure 2E illustrates that, for the ALL case, the LF-GC-based detection of leukemic cells consistently demonstrated high performance across a broad range of concentration ratios. For the AML case, predicted blast ratios were higher than those based on ground truth labels, particularly at low concentration ratios, reflecting a relatively moderate specificity score (0.839) compared to the ALL case (0.955).

Finally, we applied the developed classifiers to test datasets of unidentified (non-annotated) LF-GMI waveforms (Figure 2F,G). The SVM score histograms of the test datasets (Figure 2F, left panels) exhibit bimodal peaks corresponding to blast and normal cells for both cases. Furthermore, there is a good agreement between the blast high-density regions defined by LF-GC-based predictions (the color density plot areas in Figure 2F, right panels, and Figure 2G) and those defined by the CD45 gating methods (the blast gating areas in the BSC/CD45 density plots in Figure 2A). When the decision boundary of SVM scores was 0, the blast ratios were calculated to be 56.8% for ALL and 45.9% for AML (Figure 2F). Based on the MGG staining, morphological examinations of the same cases by medical technologists at diagnosis determined the blast ratios to be 60.0% for ALL and 31.2% for AML. In the case of ALL, the predicted blast cell ratio is thus highly consistent with the result of the manual diagnosis. In the case of AML, on the other hand, the predicted blast cell ratio was higher than the result of the manual examination, as expected from the results (Figure 2E) of the classifier evaluation using annotated datasets. To fill this gap, we optimized the decision boundary of SVM scores. As

depicted in Figure 2F,G, selecting cells with high SVM scores as positive (blast) cells effectively narrows and refines the blast regions in the CD45/BSC plots, suggesting that LF-GC accurately captures the morphological characteristics of AML blast cells with high morphological heterogeneity. Notably, the sample in this AML case was collected from a patient who experienced relapse post-treatment, which presented particular challenges due to their significantly high morphological heterogeneity. The fact that LF-GC showed effectiveness in a complex case further strengthens the value of our research. Furthermore, when the decision boundary of SVM scores was 0.37, we obtained a 30.4% blast cell ratio (Figure 2G, center panel). The results indicate that by refining the decision boundary, LF-GC can be a valuable tool for predicting blast cell percentages in BM cells, even those with a low blast cell ratio. This capability holds particular significance in its application to diagnostics. We believe that selecting appropriate training cell data and their accumulation from various patient cases will lead to developing a robust, accurate, and reliable protocol, including optimizing the classifiers and decision boundary conditions. In conclusion, the machine vision-based method presented here is expected to be applied as an accurate, simple, rapid, and economical diagnostic method of acute leukemia in a clinical setting.

AUTHOR CONTRIBUTIONS

Yoko Kawamura: Data curation; investigation; validation; formal analysis; resources; writing – original draft; writing – review and editing; methodology; conceptualization; project administration; visualization; software. **Kayoko Nakanishi:** Conceptualization; resources; writing – original draft; writing – review and editing; methodology. **Yuri Murata:** Investigation; formal analysis. **Kazuki Teranishi:** Investigation; formal analysis; writing – review and editing. **Ryusuke Miyazaki:** Resources. **Keisuke Toda:** Methodology. **Toru Imai:** Methodology. **Yasuhiro Kajiwara:** Software. **Keiji Nakagawa:** Methodology; software. **Hidemasa Matsuo:** Writing – review and editing; conceptualization; supervision; methodology. **Souichi Adachi:** Conceptualization; supervision; writing – review and editing; methodology; resources. **Sadao Ota:** Writing – review and editing; supervision; writing – original draft; conceptualization; methodology; visualization; project administration. **Hidefumi Hiramatsu:** Writing – review and editing; conceptualization; supervision; writing – original draft; methodology; resources.

ACKNOWLEDGMENTS

We thank all members of ThinkCyte K.K., in particular Keiki Sugimoto, Hiroaki Adachi, Hirofumi Nakayama, Yuichi Yanagihashi, and Hikari Morita, for their discussions. We thank the patients and their families.

CONFLICT OF INTEREST STATEMENT

Sadao Ota is the founder and shareholder of ThinkCyte K.K., a company engaged in developing GC-based cell sorting; Yoko Kawamura, Yuri Murata, Kazuki Teranishi, Ryusuke Miyazaki, Keisuke Toda, Toru Imai, and Keiji Nakagawa have shares of stock options of ThinkCyte K.K.; Sadao Ota, Yoko Kawamura, Keisuke Toda, Toru Imai, and Keiji Nakagawa have filed patent applications related to this article.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

How to cite this article: Kawamura Y, Nakanishi K, Murata Y, Teranishi K, Miyazaki R, Toda K, et al. Label-free cell detection of acute leukemia using ghost cytometry. *Cytometry*. 2024. <https://doi.org/10.1002/cyto.a.24821>