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Size-based Differentiation of Cancer and Normal Cells by a Particle Size Analyzer Assisted by a Cell-recognition PC Software

Babita Shashni, a Shinya Ariyasu, b Reisa Takeda, a Toshihiro Suzuki, c Shota Shiina, a Kazunori Akimoto, a,d Takuto Maeda, e Naoyuki Aikawa, b,e Ryo Abe, c,d Tomohiro Osaki, f Norihiko Itoh, f and Shin Aoki, a,b,d

a Faculty of Pharmaceutical Sciences, Tokyo University of Science; 2641 Yamazaki, Noda, Chiba, Japan; b Center for Technologies against Cancer, Tokyo University of Science; 2641 Yamazaki, Noda, Chiba, Japan; c Research Institute for Biomedical Sciences, Tokyo University of Science; 2641 Yamazaki, Noda, Chiba, Japan; d Division of Medical Science-Engineering Corporation, Research Institute for Science and Technology, Tokyo University of Science; 2641 Yamazaki, Noda, Chiba, Japan; e Faculty of Industrial Science and Technology, Tokyo University of Science; 6-3-1 Niijuku, Katsushika, Tokyo, Japan; and f Laboratory of Veterinary Surgery, Joint Department of Veterinary Medicine, Faculty of Agriculture; Tottori University, Tottori, Japan

* Correspondence e-mail:
shinaoki@rs.noda.tus.ac.jp
Summary

Detection of anomalous cells such as cancer cells from normal blood cells has the potential to contribute greatly to cancer diagnosis and therapy. Conventional methods for the detection of cancer cells are usually tedious and cumbersome. Herein, we report on the use of a particle size analyzer for the convenient size-based differentiation of cancer cells from normal cells. Measurements made using a particle size analyzer revealed that size parameters for cancer cells are significantly greater (e.g., inner diameter and width) than the corresponding values for normal cells (white blood cells (WBC), lymphocytes and splenocytes), with no significant difference in shape parameters (e.g., circularity and convexity). The inner diameter of many cancer cell lines is greater than 10 μm, in contrast to normal cells. For the detection of WBC having similar size to that of cancer cells, we developed a PC software “Cancer Cell Finder” that differentiates them from cancer cells based on brightness inflection points on a cell surface. Furthermore, the aforementioned method was validated for cancer cell/clusters detection in spiked mouse blood samples (a B16 melanoma mouse xenograft model) and circulating tumor cell cluster-like particles in the cat and dog (diagnosed with cancer) blood samples. These results provide insights into the possible applicability of the use of a particle size analyzer in conjunction with PC software for the convenient detection of cancer cells in experimental and clinical samples for thera nostics.

Keywords: cancer cell detection; particle size analyzer; size and shape characterization; PC tool; surface roughness
1. Introduction

The occurrence of anomalous cells deviating from the original cells may be a symptom of a diseased state in a body, hence their detection and classification are very important. One typical example of diseases that are characterized by the uncontrolled division and growth of abnormal cells would be cancer. Some cancer cells, referred as circulating tumor cells (CTCs), spread and reach an arresting state beyond the original tumor sites to secondary organs (metastasis), which is a prime cause of cancer-associated mortality. Therefore, quick and convenient detection of cancer is highly desirable for diagnosis and treatment in personalized (precision) cancer medicine.1-7)

Localized cancers can be detected by a histopathological examination of suspicious tissue, but this technique has constraints due to the fact that the analysis varies from person to person according to their visual acuity and experience.8) On the other hand, it has been reported that non-adherent abnormal CTCs can be detected based on the expression of tumor antigens and biophysical deformities.9,11-18) The positive expression of CTC protein markers such as cytokeratin -8, -18 and -19, CD44 and the epithelial cell adhesion molecule (EpCAM) is often found in a CTC.10) To date, the CellSearch system (Veridex, LLC, USA), which is based on immunostaining for CKs+, CD45− and nucleus+, is the only device that is currently approved by the Food and Drug Administration for CTC detection.19-21) Since the expression of CTC markers has been reported to vary with cancer heterogeneity, dynamic protein turnover, and during epithelial-to-mesenchymal transition (EMT), immunological based detection may be a less reliable approach.22-24)

The capture, isolation and enrichment of non-adherent abnormal CTC are based on their difference from hematologic cells in size and biophysical deformities such as such as shape, stiffness and electrical polarizability.13-18, 25-29) Many studies have reported that tumor cells are larger in size as compared to hematologic cells and this difference accounts for the
basis for size-based isolation using filtration systems and Isolation by Size of Epithelial Tumor cells (ISET, Rarecells) etc.\textsuperscript{30,31} In a recent study, CellSearch confirmed CTC’s (EpCAM\textsuperscript{+}, CK\textsuperscript{+}, DNA\textsuperscript{+}, CD45\textsuperscript{−}) median size was 10.7 μm (prostate), 11 μm (colorectal cancer), 13.1 μm (metastatic breast cancer).\textsuperscript{30} Contrary to this, it has also been reported that the size of leukocytes may also fall in the same range as that of CTCs, hence size based enrichment may have limited efficacy in terms of discriminating target cells.\textsuperscript{32} In contrast to their normal cell counterparts/ hematologic cells, cancer cells have been reported to differ cytomorphologically e.g., cancer cells exhibit a higher nuclear cytoplasmic ratio, variable size, abnormal nuclear DNA content etc.\textsuperscript{33-34} Cytomorphological deformities in CTC has been correlated to a poor clinical outcome in colorectal, prostate and metastatic breast cancers.\textsuperscript{35} To overcome the limitation of size-based enrichment methods, additional approaches should be used to increase the probability/efficacy of CTC detection/capture.

In this study, we combined two approaches to discriminate cancer cells from hematologic/normal cells: 1) the detection of cancer cells based on their size and shape and 2) the recognition of cancer cells based on cytomorphological deformity (surface contour). Namely, a Jasco-Occhio particle size analyzer IF nano-200 was employed for the comprehensive measurement of various size and shape parameters of cells. For the physical characterization of cells, a suspension of cells was introduced into a flow cell equipped with a high definition CCD camera that captures images by using stroboscopic illumination. The captured images are automatically analyzed for shape and size parameters by an in-house PIA-Pro Particle Image Analysis Software. Using the aforementioned Injection Flow Particle Image Analyzer, we first determined the appropriate combination of size and shape parameters to discriminate various cancer cells (established cancer cell lines) from normal cells (red blood cells, white blood cells, splenocytes and lymph node cells). With circularity (shape parameter) and inner diameter (size parameter; maximum inscribed disc within a
particle) as chosen parameters for analysis, we confirmed that a majority of cancer cell lines have greater inner diameters (≥10 μm) than those of normal cells. Furthermore, a PC software program “Cancer Cell Finder” was designed to differentiate cancer cells from white blood cells (inner diameter ≥10 μm) based on surface luminance (brightness/darkness). This software was validated on various established cancer cell lines and mouse derived lymph node cells and splenocytes. The detection of CTC- and cluster-like particles in blood samples from a cat and dog diagnosed with cancer was also carried out. The effect of the measurement using the particle size analyzer on cell viability, growth, and CTC marker expression was also assessed.

2. Results and Discussion

2.1. Detection of cultured cancer cells on particle size analyzer (Jasco-Occhio particle size analyzer, IF-200nano)

A JASCO-Occhio particle size analyzer IF-200nano from JASCO INTERNATIONAL CO., LTD was employed for the comprehensive size and shape characterization of cells. The Jasco-Occhio particle size analyzer, IF nano-200, consists of 1) a sample inlet jet nozzle 2) an injection pump, 3) a flow cell equipped with a high definition CCD camera, and 4) in-built PIA-Pro particle image analysis software for the analysis of the sample images (Figure S1 in Supporting Information). The IF-200nano is a wet-dispersion particle image analyzer that can analyze cell suspensions that are spatially separated. Cells dispersed in a liquid (the recommended concentration of cells is ca. 2000 cells/mL) are introduced into a flow cell via the jet nozzle and a CCD camera captures sample images passing through the flow cell by using stroboscopic illumination. A threshold needs to be set for image analysis for background correction. The captured images are then extracted and morphological parameters of each particle are calculated using the PIA-Pro Particle Image
Analysis Software which are presented in various user-selectable formats. Information related to characteristic shape such as ISO solidity, convexity, luminance, luminance RSD, ISO circularity, O. elongation, ISO aspect ratio, and ISO roundness, and size parameters such as ISO inner diameter, ISO area diameter, mean diameter, width, length and ISO max distance (Table S1 in Supporting Information) of a particle is displayed with the image of the individual particles for visual verification.

In this work, SP2/O myeloma cells were chosen as the first example, because the results of our previous experiments suggested that the average inner diameter of SP2/O cells is 11.5 μm, which is greater than that of mouse red blood cells (4.4 μm on average).13) We first observed SP2/O cells spiked in mouse blood at different dilution ratios on the particle size analyzer (Figure 1a-1f). The images obtained from the particle size analyzer clearly showed an increase in the number of larger cells (SP2/O cells) at increasing ratios of SP2/O cells added to blood, confirming the possible application of the particle size analyzer for the size-based detection of cancer cells in blood. Furthermore, the size distribution of blood cells and SP2/O cells were visualized by number (horizontal axis as area diameter in μm and vertical axis as the number (%)) in Figure 1g and by volume (horizontal axis as area diameter in μm and vertical axis as volume (%)) in Figure 1h, indicated that the size distribution by volume exhibits a better sensitivity in detecting cancer cells than by number due to the fact that the radius of SP2/O cancer cells is larger than that of blood cells.

Various combinations of sizes and shapes were tested in an attempt to detect a mixture of SP2/O cells in blood. Figure 2 displays a representative two-dimensional distribution of SP2/O cells (1x10⁶/mL) in mouse blood (the cell suspension in blood was diluted 51 X with PBS for the analysis to include 5.1 X 10⁹/mL red blood cells (RBC) and ca. 2 x 10⁴ SP2/O cells/mL) as scatterplots based on size and shape parameters (each small dot in the scatterplot corresponds to a cell (particle) as depicted in Figure S1b in Supporting
Information). For example, Figure 2a-2d are scatterplots of SP2/O cells spiked in mouse blood (by volume) with respect to circularity (%) (vertical axis) versus mean diameter (μm) (horizontal axis) (Figure 2a), convexity (%) (Figure 2b), luminance (%) (Figure 2c), and max distance (μm) (Figure 2d). Figure 2e-2g displays distribution of SP2/O cells in blood by volume as scatterplots with respect to inner diameter (μm) (horizontal axis) versus area diameter (μm) (Figure 2e), roundness (%) (Figure 2f) and circularity (%) (vertical axis) (Figure 2g).

In addition, Figure 2h and 2i display the distribution of mouse blood (1000 X dilution in PBS) and SP2/O cells (2 x 10^3/mL of PBS) by volume with respect to inner diameter (μm) (x axis) and circularity (%) (y axis), respectively. Scatterplot of the blood cells confirmed that almost all blood cells are located in the left quadrant of the scatterplot with inner diameters of less than 10 μm, whereas SP2/O cells are mainly located in the right quadrant with inner diameters of greater than 10 μm. These results suggest that inner diameter and circularity are the optimal combination for detecting cancerous SP2/O cells spiked in mouse blood.

Based on the aforementioned results, 11 adherent and non-adherent cancer cell lines, LLC, B16, K562, HeLa, A549, Colon-26, Jurkat, Molt-4, MDA-MB-468, MDA-MB-157 and MC38 were analyzed on the particle analyzer using a combination of inner diameter and circularity, as presented in the form of a scatterplot in Figure 3a-3k (displayed by volume), indicating that all of these cancer cell lines have majorly inner diameters of greater than 10 μm.30,33) The values of these inner diameters are summarized in Table S2 in Supporting Information. In addition, images of the top 5 largest individual cells of these cancer cell lines obtained by the particle size analyzer are shown in Figure S2 in Supporting Information with circularity (circ) and maximum diameter (Dimax) values. Images of the cells captured by
particle size analyzer offer an advantage over other size-based detection system to identify a cell/clusters/debris.

For comparison of normal blood cells and cancer cell lines, the size and shape parameters of normal cells from blood, spleen and lymph nodes of mouse (C57BL/6) were examined. Total blood cells were segregated into platelets, peripheral blood mononuclear cells (PBMCs) and red blood cells (RBC) by density gradient centrifugation and analyzed by the particle size analyzer.\textsuperscript{36,37} Splenocytes deprived of RBC and lymph node cells were also analyzed to confirm the size of white blood cells. Scatterplots (circularity versus inner diameter) of the normal cells such as RBCs, platelets, WBCs, lymph node cells and splenocytes are shown in Figure 3l-3p, respectively. In addition, images of the largest 5 individual cells of the normal cells are shown in Figure S3 in Supporting Information. Size distribution curves for cancer cells and normal cells are also shown in Figure S4 and S5 in Supporting Information, respectively. The scatterplot and size distribution graph obtained from the particle size analyzer clearly shows the size difference between normal cells and cancer cell lines and also confirms that a combination of “inner diameter” and “circularity” are appropriate parameters for further analyses to differentiate blood cells (inner diameter <10 μm) and cancer cells. Images of cancer cell lines and normal cells taken by the particle size analyzer are presented in Figure S6 in Supporting Information.

Shape and size parameters for normal cells were compared with those of cancer cell lines such as adherent (LLC and B16) and non-adherent (Jurkat and Molt-4) type cells, as summarized in Figure 4. The shape and size parameter values of all normal cells and cancer cell lines are listed in Table S2 of Supporting Information. Statistical analyses indicated that the size parameters, inner diameter, area diameter, mean diameter and width were all significantly higher in cancer cell lines compared to those of normal cells (Figure 4a), while no significant difference was found in shape parameters between cancer cells and normal
cells (Figure 4b). For further analyses, we used the inner diameter as a typical parameter, because a combination of inner diameter and circularity was found to be appropriate for discriminating cancer cells from normal cells.

2.2. Development of a new software program “Cancer Cell Finder (CCF)” to distinguish cancer cells from normal cells

It has been reported that the size of some WBCs is similar to that of cancer cells. In our study, we observed one WBC having an inner diameter of 10.3 μm among 777 particles (inner diameters of 4-50 μm) (Figure 3n). This information prompted us to develop a new computer software program to distinguish WBCs (inner diameter of >10.3 μm) from cancer cells using images obtained from the particle size analyzer. Deformity in cancer cells in contrast to normal cell is widely known and some reports have linked the deformity in cancer cells with their metastatic propensity. As shown in Figure 5, the difference in surface deformity (roughness) between cancer cell line (SP2/O cells and LLC cells) and normal cells (WBC and lymph node cell) formed the basis of our new method for distinguishing cancer cells from normal cells. In Figure 5a and 5b, WBC and lymph node cells exhibit bright area in the center and darker area on the edge and smoother surface, while darkness curves of SP2/O and LLC are more complicated (Figure 5c and 5d). This difference could be attributed to the abnormally increased DNA content in cancer cells which might have affected cell luminance during image capture by the particle size analyzer.

Based on this observation, we decided to count the inflection point (IFP) (IFP means change in darkness/brightness at a point) for discriminating cancer cells from normal blood cells based on the following algorithm (Figure 5): 1) Prepared binarization (black and white) images of the microscopic images (or images from the particle size analyzer) of the cells with a given threshold. 2) Based on the obtained binarization images, cell areas were
extracted and their center of gravity determined (Figure 5a-d). 3) The defined two cross sections passing through the center of gravity of each cell (bold plain and bold dashed lines in Figure 5a-d) and respective luminance (darkness) functional curves of sections were obtained (Figure 5e-5h and 5i-5l). 4) The total number of IFP were counted on the aforementioned two luminance curves (red and blue lines in Figure 5a-d), based on which the cells were classified into two categories: i) a cell having IFP number of less than or equal to 10 (≤ 10) is considered as a non-cancer cell, and ii) a cell having IFP number greater than 10 (> 10) is considered as a cancer cell. This PC software is denoted as “Cancer Cell Finder (CCF) ver. 1.0”.

The resolution (pixel density) of particle size analyzer’s camera is 0.185 μm/pixel, based on the maximum outer diameter that was evaluated using the following formula: outer diameter (μm) = maximum horizontal pixel x 0.185. The outer diameters calculated for WBC, lymph node cell, SP2/O and LLC, are 13.1 μm (10.3 μm inner diameter), 9.4 μm (6.6 μm inner diameter), 19.8 μm (14.3 μm inner diameter) and 26.1 μm (23.0 μm inner diameter), respectively, are shown in Figure 5a-5d.

In Figure 5m and 5n, normal cells, WBC and lymph node cells have total inflection points of lower than 10, whereas LLC and SP2/O cancer cells have inflection points of greater than 10 (Figure 5o and 5p). The probabilities of “CCF ver. 1.0” for detecting normal cells such as WBC and lymph node cells was 83% and 100%, respectively, and for cancer cells, such as SP2/O cells and LLC cells, was 95% and 100%, respectively, as summarized in Table 1. Similarly, we analyzed various other cancer cell lines and evaluated their inflection points, and the results are shown in Table 1. Based on these results, it was concluded that this “CCF ver. 1.0” may be more useful in differentiating localized cancer-originated CTCs rather than lymphoma/leukemia cells from WBCs.
2.3. Reculture of particle size analyzer processed cells

To check the effect of particle analysis on the viability of cancer cells, LLC, MDA-MB-157 and MDA-MB-468 cells were recultured (24 h) after analysis using the particle size analyzer. As shown in Figure S7 in Supporting Information, after being processed through the particle analyzer, the cancer cells retained their adherent characteristics. The results of a dye exclusion assay of these cancer cell lines before and after particle analyzer measurements shows that LLC and MDA-MB-157 cells are sensitive to the analysis on the particle analyzer, while other cell lines Jurkat, Molt-4, and MDA-MB-468 cells are not (Figure S8 in Supporting Information).

Further, the effect of this method on the proliferation of B16, LLC, MDA-MB-468, MDA-MB-157 and Jurkat cells was evaluated by means of a WST assay. After the particle size analyzer measurement, the cells were subjected to a proliferation rate evaluation for 24-45 h. The data suggest that there was a significant difference in proliferation between LLC, Jurkat and MDA-MB-468 cells (Figure S9b-d in Supporting Information) with that of control cells but no significant difference was observed in the case of B16 and MDA-MB-157 cells (Figure S9a and S9e in Supporting Information).

The effect of the particle size analyzer processing on the expression of CTC markers (CD44 and EpCAM) in MDA-MB-468 cell lines was also examined. After processing though the particle size analyzer, MDA-MB-468 cells were cultured in vitro for 4 days and the expression of CTC markers in the particle size analyzer processed and control cell samples was evaluated by immuno-blotting with CD44 and EpCAM (Figure S10 in Supporting Information). No significant difference in the expression of CTC markers (CD44, p=0.975 and EpCAM, p=0.871) was observed in MDA-MB-468 cells before and after the particle size analyzer measurements (Figure S10b in Supporting Information), suggesting that the processing of cancer cells on the particle size analyzer negligibly affects the CTC
characteristics of cancer cells, at least, at the expression level. The effect of particle size analyzer measurement on cells is summarized in Table S3 in Supporting Information.

2.4. Detection of cancer cells and cancer clusters spiked in blood

The detection of cancer cells spiked in blood was carried out using LLC cells expressing the enhanced green fluorescent protein (LLC-EGFP) for easy visualization on the microscope. Mixtures of LLC cells (1x10^7/mL) in 0.9% saline (Figure 6a) and in blood (estimated number of RBC: 0.7-1.3 x 10^10/mL) (Figure 6b) were observed on the particle size analyzer (final LLC cell concentration: 8 x 10^4/mL after dilution (126 X) in 0.9% saline). A typical example of a scatterplot of LLC-EGFP cells spiked in blood is presented in Figure 6c (LLC cells are shown in the inset). Individual cells observed with inner diameters greater than 10 μm were subjected to a “CCF ver. 1.0” analysis to classify them to cancer or non-cancer cells based on surface roughness/brightness, as explained in Figure 5 (Figure 6d). The “CCF ver. 1.0” analysis data for the cells confirmed that 8 out of 10 cells have over 11 IFP values and hence can be assumed to be cancer cells (indicated by “*” in Figure 6e). In 166 pictures taken on particle size analyzer (LLC cells spiked in blood sample), ca. 1.1 x10^10 RBC/mL and ca. 1.6 x10^7 LLC cells/mL in blood were detected which were approximately similar to their initial numbers in the spiked samples prepared for these experiments.

Nicola Aceto and coworkers reported that CTC clusters possess a greater metastatic potential and a higher resistance to apoptosis than single CTCs, indicating the clinical relevance of CTC clusters in metastasis. To examine the efficiency of detection of CTC clusters by our method, we prepared cancer cell line tumor spheres (clusters) in vitro. B16 and LLC-EGFP cells were cultured in a mixture of 0.6% methylcellulose and DMEM, for 5-6 days and then harvested carefully for analysis by the particle size analyzer (images of clusters are shown in Figure S11 of Supporting Information). Scatterplots of LLC and B16 cell
clusters (in saline) confirms that the clusters can also be detected by the particle size analyzer and their inner diameters are greater than 10 μm (Figure 6f and 6h). Moreover, the detection of cancer cell clusters in blood was carried out. LLC-EGFP and B16 clusters were carefully spiked in C57BL/6 mouse blood and then diluted with PBS for analysis on the particle size analyzer. As shown in Figure 6h and 6i, the analyzed clusters were located in the right quadrant of the scatterplot with inner diameter greater than 10 μm. The data implies that cancer cell clusters can also be detected on the particle size analyzer.

We next carried out the filtration of LLC-EGFP and B16 clusters through 10 μm nylon mesh and the changes in numbers and size of these clusters were assessed with particle size analyzer before and after filtration to confirm the damage to clusters during particle size analyzer measurement. Figure S12a-1 and S12b-1 in Supporting Information show the scatterplot of LLC and B16 clusters (x-axis: inner diameter and y-axis: circularity) before filtration and Figure S12a-2 and S12b-2 show those after filtration. Tables S12a-3 and S12b-3 in Supporting Information indicate that total number of particles was reduced and negligible clusters (>10 μm) were observed after filtration through 10 μm nylon mesh, implying that clusters and larger particles were filtered off and not broken down into small particles during measurement on the particle size analyzer. The largest 10 distribution particles before and after filtration are shown in Figure S13 of Supporting Information, confirming the results mentioned above.

2.5. Detection of tumor cells in B16 tumor mouse model.

We next verified our approach for differentiating cancer cells in a mouse model, to which B16 cells had been injected because B16 cancer cells possess high metastatic nature and its detection probability by “CCF ver. 1.0” is 98 % (Table 1). Blood and splenocytes harvested from the tumor bearing mouse (13 days post inoculation of B16 cancer cells) were
first analyzed by flow cytometry to check the presence of B16-EGFP cancer cells (Figure S14 in Supporting Information). Because a greater number of tumor cells was detected by flow cytometry in the spleen sample than in the blood of the tumor bearing mouse, we measured splenocytes on the particle size analyzer (Figure 7). Scatterplots of splenocytes derived from a normal mouse and a B16 tumor bearing mouse are shown in Figure 7a and 7b, respectively. Cells 1-8 in Figure 7c (spleenocytes from normal cells) with inner diameters of >10 μm were subjected to “CCF ver. 1.0”, which confirmed that all of them had inflection points of <10 (Figure 7e). In the tumor bearing mouse samples, 5 cells (No. 9-13) among 12 cells (No. 9-20) in Figure 7d were judged as cancer cells and the rest as normal cells (No. 14-20 in Figure 7d), which possibly are splenocytes or activated white blood cells (Figure 7f). The features of the cells No. 9-13 are similar to those of in vitro cultured B16 cells, 21C-24C in Figure 7d and 7f.

As pointed by the reviewers, we do not deny the possibility that the lymphocytes will be enlarged upon stimulation by cytokines, but based on our aforementioned data we believe that it is possible to verify larger lymphocytes as normal cells using our software “CCF”.

2.6. Detection of CTC cluster-like particles in the blood of cat and dog diagnosed with cancer

As a clinical application of the particle size analyzer for detecting CTC clusters in the whole blood of cancer patients, blood samples obtained from normal and cancer diagnosed cats and dogs were analyzed in a similar manner to the aforementioned method. As shown in Figure 8a and 8e, scatterplots (y axis-circularity (%) and x axis-inner diameter (μm)) of normal dog (sample ID: TU-0001-D) and cat blood (sample ID: TU-0003-C) showed negligible particles (cell/clusters) greater than 10 μm, respectively. On the other hand,
particle size analyzer detected 2 larger cancer cluster-like particles in the blood sample of the dog with osteosarcoma (sample ID: TU-0002-D) (Figure 8b) and 1 cluster in the blood sample of a cat diagnosed with a mammary adenocarcinoma (sample ID: TU-0004-C), Figure 8d (debris are marked as 2 in sample TU-0004-C). Top 10 individual cells based on inner diameter were subjected to “CCF ver. 1.0” analysis to categorize them into cancer/non-cancer cells (Figure 8c and 8g). These data confirmed that one cell (>10 inflection points) out of 2 cells (inner diameter >10 µm) in the dog blood sample (sample ID: TU-0002-D) appeared to be a cancer cell and other cells having inner diameter of <10 µm were judged to be normal cells (Figure 8c and 8d). For cat blood samples, one CTC-like cluster particle was similarly confirmed as a cancer cell, whereas cells with inner diameter of <10 µm were judged as normal cells (Figure 8g and 8h). More clinical information is provided in Table S4 of Supporting Information. These data have allowed us to conclude that a combination of particle size analyzer and “CCF ver. 1.0” is also capable of detecting CTC cluster-like particles in blood samples of cancer-bearing animals.

2.7. Detection of cancer cells in normal human blood cells

We conducted measurements of human lung adenocarcinoma A549 cells spiked in human blood. The size of A549 cells in human whole blood (diluted in saline) was measured by the particle size analyzer (Figure 9a), in which only a few large normal cells (inner diameter >10 µm) were observed. A typical scatterplot of a blood sample with large cells (inner diameter = 10.2 µm) is shown in Figure 9a and a “CCF ver. 1.0” analysis of the top 7 largest cells (Figure 9b) indicated that their IFP’s are <10 (Figure 9c), implying that these are normal cells. Next, white blood cells isolated from human blood were measured on the particle size analyzer (n=6) and “CCF ver. 1.0”. As shown in Figure 9e and 9f, all of the 8 cells picked up from Figure 9d were judged as normal cells (IFPs are <10), although some of
them had an inner diameter of >10 µm. Additional information is provided in Figure S15a and S15b in Supporting Information.

In Figure 9g-i, human A549 cancer cells were analyzed and the largest 8 cells were confirmed as cancer cells. We next analyzed a mixture of human white blood cells and A549 cells at the same concentration (Figure 9j-l). In this case, cell number 1-5 were judged as cancer cells among 10 cells having inner diameter of 6.8~25 µm (Figure 9k and l). Pictures of normal white blood cells and A549 cells taken on particle size analyzer in shown in Figure S16.

These results prompted us to attempt to detect A549 cells (1 x 10^5) spiked in human whole blood (6.3 x 10^6 normal cells) (diluted) on the particle size analyzer. As shown in Figure 9m and 9n, cell number 1-5 among 8 cells were judged as cancer cells (Figure 9o). Furthermore, the ability of the particle size analyzer to detect cancer cells in spiked sample was verified by an analysis of larger cells (A549 cells) in the absence and presence of blood cells. Figure 9m presents a scatterplot of A549 cells (in blood) at the same concentration as those analyzed in spiked saline (Figure S15c in Supporting Information). The total number of A549 cells (inner diameter >10 µm) spiked in blood sample (n=11) was 6.5 ± 1.0 x 10^4 large cells/3.2 x 10^6 blood cells. For comparison, the number of A549 cells spiked in saline was determined to be 6.0 ± 2.0 x 10^4 A549 cells/mL, which is almost same number as that of the aforementioned numbers in blood (Figure S15d), suggesting that human cancer cells can be detected in human blood without substantial loss.

Finally, we checked the morphology of human umbilical vein endothelial cell (HUVEC), which are a type of normal cells. As shown in Figure S17a, the inner diameter of HUVEC are 4–50 µm and some large cells possessing peculiar shapes and different circularity values were unexpectedly judged to be cancer cells by “CCF ver. 1.0” (Figure S17b and c). The reason for this finding is unclear but may be attributed to the
harvesting methods (trypsinization) employed to detach the adherent HUVEC. More detailed investigations need to be done to analyze adherent normal cells.

3. Conclusion

In this study, we employed a Jasco-Occhio particle size analyzer IF nano-200 for the size- and size- based detection of cancer cells and clusters. Sample measurement using the particle size analyzer is more convenient than previously reported methods. It permits particles/cells to be comprehensively characterized in a short time (ca. 5 min for 1 mL of sample). The automated analysis data can be displayed in various user-selectable formats such as size and shape distributions curves and numerical values. Debris, single cell or clusters in a scatterplot can be verified visually by images of individual particles, thereby ruling out the possibility of generating any negative data. The test samples do not require any major preprocessing (e.g. cell fixation) and can be operated with any diluent, thereby permitting the viability of the target cells to be maintained for in vitro establishment and for further characterization, contrary to immunological based detection which requires cells to be fixed.

It should be noted that the definition of the detection limit (sensitivity) of CTC in our method is somewhat different from that of previous methods. Namely, the detection limit of our method is dependent on the volume of blood sample analyzed and the number of images acquired by the particle size analyzer, and hence, it is dependent on the amount of memory and the processing speed of the computer (the greater is the memory and processing speed, the higher the sensitivity is). In our current experiments (particle size analyzer used in this work), the detection limit was ca. 2000 CTCs/mL of blood (possibly, containing 1.1 x 10^6 RBCs). Because the cancer cell numbers used in these cell spiking experiments are greater
than those in actual clinical samples, the limit of detection of the CTC will need to be improved.

A convenient software program “CCF ver. 1.0” was developed to distinguish cancer cells from normal cells based on differences in the luminance (darkness) extent of curve of the cell surface. It is suitable for differentiating adherent cancer cells from WBCs and splenocytes, thus making it attractive for use in hospitals, medical facilities and laboratories, thereby overcoming the need for expensive CTC detecting devices. We do not deny the possibility that the size and shape of CTC and normal cells are altered by anticancer agents, cytokines such as TGF-β or other effects. Further studies will need to be done to examine these issues and will be reported elsewhere. Over all, an analysis of blood samples (diagnosed with cancer) with a particle size analyzer or more simple and cheaper instruments assisted by a cell-recognition software such as “CCF” provides insights into possible clinical applications of our combination approach for detection CTCs and CTC clusters for evaluating the progression and theranostics of cancer and related diseases.

4. Experimental Section

4.1. Reagents

Dulbecco’s modified Eagle’s medium (DMEM), RPMI 1640 medium, fetal calf serum (FCS), streptomycin sulphate, benzypenicillin potassium, sodium chloride, potassium chloride, tris base, glycine, sodium phosphate dibasic, sodium dihydrogen phosphate were purchased from Wako Pure Chemical Industries, Osaka, Japan. Trypsin-EDTA was procured from Gibco, Japan.

4.2. Cell culture

The cell lines, LLC-EGFP (mouse; lewis lung carcinoma expressing green fluorescent protein), B16 (mouse; melanoma), Jurkat (human; T cell lymphoma), Molt-4 (human; T cell
lymphoma), MC38 (mouse; colon cancer), K562 (human; chronic myelogenous leukemia), HeLa (human; uterine cancer), A549 (human; lung adenocarcinoma), Colon-26 (mouse; colorectal cancer), and SP2/O (mouse; melanoma) were provided by Prof. Ryo Abe (Tokyo University of Science), MDA-MB-468 (human; breast cancer) and MDA-MB-157 (human; breast cancer) were purchased from ATCC and HUVEC were purchased from RIKEN cell bank (Japan). Carcinoma and normal cells were maintained at 37 °C in a humidified 5% CO2/95% O2 in their respective media supplemented with 10% FCS, L-glutamine and antibiotic mixture (penicillin; 69.9 ng/mL and streptomycin; 139.3 ng/mL). LLC-EGFP, B16, MDA-MB-468, MDA-MB-157, A549 and MC38 were maintained in DMEM, Jurkat, Molt-4 and SP2/O cell lines were maintained in RPMI 1640 medium supplemented with HEPES (2-[4-(2-hydroxyethyl)-1-piperazinyl]ethanesulfonic acid) and mono-thioglycerol (0.5 mM/L). Colon-26 cells and HeLa cells were maintained in RPMI 1640 and MEM medium, respectively. HUVEC were maintained in medium 200 supplemented with low serum growth supplement (Gibco).

4.3. Analysis in particle size analyzer

Adherent and non-adherent cell lines were cultured to 80-90% confluency for analysis. Cells were harvested and pelleted down by centrifugation at 1400 rpm for 7 min at 4 °C. Obtained pellets were suspended in a sterile phosphate buffer solution (PBS) at pH 7.4 for measurement on particle size analyzer. Prior to cell measurement, flow chamber of the particle size analyzer was washed with 5-10 mL of double-distilled water (dd H2O), followed by background and light calibration, and subsequently analysis of cells (1-2 mL aliquot of the cell suspension solution) with the following parameters: light intensity (9), volume sampling (0.5%), volume analyzed (1-2 mL), magnification (4.5 X), camera pixel (0.185) and syringe diameter (6515 μm).
4.4. Standardization of shape and size parameters

For analysis of only blood, the blood was diluted 1000 X with PBS and analyzed. To standardize suitable parameters for the detection of cancer cells in blood cells, SP2/O cells (1 x 10^6/mL) were spiked in C57BL/6 mouse blood. A 100 μL aliquot of this sample was diluted with PBS (50 X) (final cell concentration: ca. 2 x 10^4 SP2/O cells/mL) and analyzed on the particle size analyzer. Various shape and size parameters combination were standardized to identify suitable parameters for the detection of cancer cells in the presence of normal cells.

4.5. Cell cluster preparation

LLC-EGFP and B16 cells were seeded (1.5x10^3 cells/well) in 0.6% sterile methylcellulose/DMEM (500 μL/well) in a 24-well dish (Ultra low attachment plate, Costar) and incubated for 5-6 days. Post 3 days of seeding, 200-300 μL of media was added. After incubation, the clusters were imaged using a microscope (Biorevo, BZ-9000, Keyence) at 40x and 200x magnifications. For particle size analyzer measurements, the clusters were collected (from 3 wells, ~ 250-300 clusters) and pelleted down by centrifugation at 1400 rpm for 6 min at 4 °C and suspended in 4 mL sterile PBS (pH 7.4). The clusters were kept on ice until used.

4.6. Cancer cell and cluster detection in particle size analyzer

LLC-EGFP cells (1 x 10^7) were spiked in 1 mL of mouse blood (C57BL/6) and in saline, and then further diluted (126 X) with 0.9% saline for measurement on the particle size analyzer (final cell concentration: ca. 8 x 10^4 cells/mL). For the analysis of individual cluster analysis, 1 mL of cluster suspension in PBS was analyzed (see cluster preparation section for details). After the analysis, the suspension was filtered through 10 μm nylon mesh and the filtrate was analyzed again on particle size analyzer. The filtered clusters on the mesh were visualized by a microscope (Biorevo BZ-9000, Keyence) at a 200x magnification. For the detection of clusters in blood, clusters were harvested (from 3 wells of a 24-well plate),
centrifuged at 1400 rpm at 4 °C for 6 min and the resulting pellet was carefully suspended in 50 μL of C57BL/6 healthy mouse blood. The spiked mixture was diluted with PBS (126 X for LLC-EGFP clusters and 251 X for B16-EGFP clusters) and analyzed on the particle size analyzer (2 mL).

4.7. Normal cells isolation and detection

Prior to the analysis of cancer cell lines, we examined the shape and size parameters of normal blood cells (red blood cells (RBC), white blood cells (WBC), and platelets), splenocytes and lymph node cells that were harvested from 4-5 months old C57BL/6 mice (SANKYO LABO SERVICE Co. Inc., Shizuoka, Japan). To harvest the RBCs, WBCs and platelets from blood, blood was obtained through cardiac puncture (500-1000 μL) from anesthetized C57BL/6 mice (isoflurane) and collected in a vial containing heparin (1000 U/mL). The blood sample was diluted with 2 mL of wash buffer (PBS supplemented with 0.5% calf serum (CS) and azide) followed by the addition of 1 mL of lymphocyte separate media (Immuno-Biological Laboratories Co., Ltd., Gunma, Japan) to the base of the vial and the resulting suspension was centrifuged at 1400 rpm at RT for 30 min. After centrifugation, the sample solution in the vial was separated into 3 different layers. 1) The top transparent layer (platelets) was diluted in 2-5 mL RPMI (0.5% CS) and analyzed using the particle size analyzer. 2) The middle buff-colored white layer corresponding to peripheral blood mononuclear cells (PBMCs) was collected in new vial with the addition of 2 mL of wash buffer and centrifuged at 1400 rpm at 4 °C for 8 min. 3) The pellet (RBC) was suspended in 400-500 μL of RPMI (10% CS) and filtered through a 70 μm mesh before use. All 3 separated cell suspensions were maintained on ice until used. For analysis using the particle analyzer, the cell suspension was diluted with PBS and around 1 mL was used in the analysis. The lowest RBC layer was diluted with PBS (100 X) and 1 mL was analyzed on particle analyzer.
For the detection of lymph node cells, axillary and inguinal lymph nodes were harvested from C57BL/6 mice and suspended in 10 mL of cold RPMI media (0.5% CS). The cells were squeezed out through the nodes by sterile forceps and filtered through a 70 μm mesh followed by centrifugation at 1400 rpm for 8 min at 4 °C. The pellet was suspended in RPMI (10% CS) and filtered again through 70 μm mesh to remove cell aggregates. The resulting filtrate (1 mL) was analyzed on particle analyzer.

To isolate splenocytes, spleens from C57BL/6 mice were harvested, minced and squeezed with sterile glass slides. The isolated cells were suspended in 25 mL of cold RPMI media (0.5% CS) and centrifuged at 1400 rpm for 8 min at 4 °C. The resulting pellet was treated with 1 mL of ACK (Ammonium-Chloride-Potassium) lysing buffer for 1 min at RT to lyse the RBCs. The reaction was terminated by the addition of 40 mL of RPMI media (0.5% CS) followed by filtration through 70 μm mesh. The suspension was then centrifuged at 1400 rpm at 4 °C for 8 min and the resulting pellet was suspended in 10 mL RPMI media (0.5% CS). The resulting mixture was filtered through 70 μm mesh, 1-2 mL of which was analyzed using the particle analyzer. All animal experiments were conducted in accordance with the guidelines of Tokyo University of Science (approved animal experiment number S15005).

4.8. B16-EGFP tumor mouse model

All animal experiments were conducted in accordance with the guidelines of Tokyo University of Science (approved animal experiment number: S15005). To test the applicability of our method for cancer cell detection, a B16-EGFP tumor mouse model was used. Briefly, 4 x 10⁶ B16-EGFP cells/200 μL of PBS was injected by tail vein in 4-5 months old C57BL/6 mice. Mice were sacrificed 13 days post cell inoculation and spleen and peripheral blood (by cardiac puncture) were harvested. Individual cells were collected from the samples as mentioned in the above section and analyzed in particle size analyzer and flow cytometry.
4.9. Detection of CTC-like clusters in cat and dog blood samples.

Cat and dog blood samples were obtained from Tottori University, Japan, with clinical information. For particle size analysis, the blood samples were diluted (500 X) in PBS (pH 7.4), 5 mL of which was used for the analysis on the particle size analyzer. All animal experiments were conducted in accordance with the guidelines of Tottori University.


Human whole blood serotype A from a single donor was purchased from BioPrendic International (France). For the analysis of human samples, the detection of A549 cells spiked in human blood was carried out. Whole blood was diluted 100X with saline before measurement on the particle size analyzer. White blood cells (lymphocytes, monocytes) from human blood were isolated using lymphocyte separation medium (MP Biomedicals, France) following the manufacturer’s instructions measured on particle size analyzer suspended in saline. A549 cells at a concentration of ~8 x 10⁴/mL in saline was measured on particle size analyzer. A mixture of white blood cells (1 x 10⁶/mL) and A549 cells (1 x 10⁶/mL) in saline was measured on the particle size analyzer. Human blood sample was diluted 50X in saline before spiking with A549 cells. HUVEC, harvested by trypsinization, were suspended in saline and analyzed on the particle size analyzer (7 x 10⁴/mL).

4.11. Statistical analysis

The quantitative data are representative of three or more independent experiments and expressed as the mean ± SEM. Statistical analysis was performed using analysis of variance (one way analysis of variance) followed by Bonferroni’s test to determine significant differences in mean and p < 0.05 was considered as statistically significant.
Acknowledgements

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Conflict of Interest (COI)

The authors declare no conflict of interest.

Supplementary materials

The online version of this article contains supplementary materials.
References


**Figure 1. Detection and analysis of SP2/O cancer cells.** SP2/O cells (indicated by circles in Figure b-f) spiked in diluted C57BL/6 mouse blood (200 X), at various ratios (by volume) of mouse blood to the SP2/O cell suspension (1x10^6 cells/mL) (a) 1:0, (b) 1: 0.125, (c) 1: 0.25, (d) 1:0.5, (e) 1:1 and (f) 1:2 (v/v) were analyzed on the particle size analyzer. The size distribution of blood cells and SP2/O cells were analyzed by number (g) and volume (h). The red curves in (g) and (h) are integration curves corresponding to the number (g) and volume (h) plotted against area diameter (μm) from 0.1 μm (left) to 1000 μm (right). Plot by volume (h) showed better sensitivity than plots by number (g).
Figure 2. Representative two-dimensional distribution of SP2/O cells spiked in mouse blood. SP2/O cells spiked in mouse blood (final cell concentration: 2 x10^4/mL) were analyzed on the particle size analyzer by different combinations of size and shape parameters to standardize suitable parameters for the detection of SP2/O cells in the presence of blood cells. The data are presented as scatterplots with variable parameters by volume. The scatterplot of SP2/O cells spiked in mouse blood, circularity (%) versus inner diameter (μm),
confirms that SP2/O cells are larger (inner diameter >10 μm). In Figure 2h and 2i, scatterplots of blood cells and SP2/O cells (2x10^3 cells/mL of PBS) are presented (plotted by volume), respectively, confirming that the inner diameter of most of the blood cells are less than 10 μm, while that of cancer (SP2/O) cells is 11.6 μm (average).
Figure 3. Scatterplots of cancer and normal cells with respect to inner diameter (µm) and circularity (%) by volume. Scatterplot of (a) LLC, (b) B16, (c) K562, (d) HeLa, (e) A549, (f) Colon-26, (g) Jurkat, (h) Molt-4, (i) MDA-MB-468, (j) MDA-MB-157, (k) MC38, (l) red blood cells, (m) platelets, (n) white blood cells, (o) lymph node cells, and (p) splenocytes (cell aggregates are shown in the inset).
Figure 4. Size and shape parameters of normal and cancer cells analyzed by the particle size analyzer. Analysis of (a) size parameters of normal and cancer cells such as inner diameter, area diameter, mean diameter, width, length and max distance and b) shape parameters such as solidity, convexity, luminance, luminance RSD, circularity, elongation and roundness, respectively in arbitrary units (a.u.). The data are presented as the mean ± S.E.M with “*” p<0.05 considered as significant and “NS” as non-significant.
**Figure 5.** Software (“CCF ver. 1.0”) designed to discriminate cancer cells and WBCs based on brightness diagram of cell’s interior. Particle size analyzer images of WBC (a), lymph node cell (b), SP2/O cancer cell (c) and LLC cell (d), were sectioned horizontally and vertically. The brightness functional curves of sections, which pass through the center of gravity of each cell (bold plain and bold dashed lines) analyzed by counting inflection point method is shown for WBC (e and i), lymph node cell (f and j), SP2/O cancer cell (g and k) and LLC cell (h and l) (1 pixel corresponds to 0.185 μm). Arrows indicate inflection points in the graphs. Counts (inflection points) are presented for WBCs (m), lymph node cells (n),
SP2/O cancer cells (o) and LLC cells (p) (dashed lines indicates the appropriate threshold (11 IFP) between cancer cells and normal cells).
Figure 6. Analysis of single cells (LLC) and clusters (LLC and B16 cells) spiked in saline and mouse blood on particle size analyzer and “CCF ver. 1.0”. Particle size analyzer images of (a) LLC cells spiked in 0.9% saline and (b) a mixture of LLC cells and mouse
blood (LLC cells encircled). (c) Scatterplot of LLC cells spiked in mouse blood (LLC cells are shown in the inset). (d) Top 10 single cells (inner diameter) observed in Figure (c). (e) Confirmation of cells (Figure 6d) as cancer or normal cells via software analysis (cancer cell detection threshold >10, dashed line), “**” indicates cancer cell. Scatterplots of LLC (f) and B16 (g) clusters (insets depicting largest clusters), respectively. (h) and (g) LLC and B16 clusters spiked in mouse blood (insets depicting largest clusters), respectively.
Figure 7. Cancer cell detection in *in vivo* B16 tumor xenograft model. Scatterplot of splenocytes of (a) normal mouse, and (b) B16 tumor bearing mouse. Top single cells observed in scatterplot of (c) normal mouse (No. 1-8), and (d) B16 tumor bearing mouse (No. 9-20) and *in vitro* cultured B16 cancer cell lines (No. 21C-24C). The results of “CCF ver. 1.0” analysis for verification of cells presented in (c) and (d) as normal/cancer cells are summarized in (e) normal mouse (No. 1-8), and (f) B16 tumor bearing mouse (No. 9-20) and...
*in vitro* cultured B16 cancer cell lines (No. 21C-24C) (cancer cell detection threshold >10 is indicated with a dashed line), “*” indicates cancer cell.
Figure 8. Detection of CTC cluster-like particles in the blood of cat and dog diagnosed with cancer. Scatterplots of (a) normal dog (TU-0001-D), (b) dog suffering from cancer (TU-0002-D) Images of the clusters detected by the particle size analyzer are shown as insets in the scatterplots. (c) Top single cells/particles observed in scatterplot of cancer bearing dog (b) and “CCF ver. 1.0” analysis of cells/ cells/particles are shown in (d). Scatterplots of (e) normal cat (TU-0003-C), and (f) cat suffering from cancer (TU-0004-C). Images of the clusters detected by the particle size analyzer are shown as insets in the scatterplots. (g) Top single cells/particles observed in scatterplot of cancer bearing cat (f) and “CCF ver. 1.0” analysis of cells/particles are shown in (h) (cancer cell detection threshold >10, dashed line), “*” indicates cancer cell/cluster.
Figure 9. Detection of human lung adenocarcinoma A549 cells spiked in human blood by the particle size analyzer and “CCF ver. 1.0”. Analysis of human whole blood (a) typical scatterplot of human whole blood, (b) top single cells observed in the scatterplot (1-7), and (c) “CCF ver. 1.0” analysis of cells. Analysis of human white blood cells, (d) typical scatterplot of human white blood cells, (e) single cells observed in the scatterplot (1-8), and (f) “CCF ver. 1.0” analysis of cells. Analysis of A549 cells (g) typical scatterplot of A549 cells, (h) top single cells observed in the scatterplot (1-8), and (i) “CCF ver. 1.0” analysis of cells. Analysis of A549 cells and human white blood cells (j) typical scatterplot of a spiked sample, (k) single cells observed in the scatterplot (1-10), and (l) “CCF ver. 1.0” analysis of cells. Analysis of A549 cells and human whole blood (m) typical scatterplot of spiked sample, (n) single cells observed in the scatterplot (1-8), and (o) “CCF ver. 1.0” analysis of cells. Cancer cell detection threshold is >10, indicated as dashed line, “*” indicates cancer cells.
Table 1. Inflection points observed for cancer and non-cancer cells as analyzed by the CLE value point method of “CCF ver. 1.0” (See Figure 5).

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