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A New Automatic Cell Isolation System for

Flow Cytometry: Cell Isolation Unit and Staining Reagent Kit

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***Abstract*—Flow cytometry is well-known cell analysis method and useful to gain quantitative information from cells in blood, however, it is not widely used for solid tissues in clinical settings. This is partly because it takes a long time to prepare samples and the operation can be complicated. To resolve these problems, we developed a new automatic cell isolation system which consists of cell isolation unit and staining reagent kit specialized for flow cytometry. With this new system, cell isolation can be done more rapidly and easily.**

By using this method, we could determine optimum condition to disintegrate porcine colon tissue and stain cells stably in 6 minutes. This result indicates that our method can provide analysis data within 10 minutes. We also evaluated our method in colorectal cancer patients, and the result was promising. All the data suggests that this method can support and facilitate rapid diagnosis.

I. Introduction

Intraoperative histopathological investigation plays an important role, however, it can be difficult to perform for frozen section diagnosis. Also, pathological diagnosis could be challenging because it usually uses only a part of specimen for diagnosis. Moreover, due to the shortage of pathologists, on site intraoperative rapid diagnosis is available only in limited hospitals and workload of pathologists increases. Therefore, simplified method that can provide quantitative information of cells from whole specimen is desired to increase the accuracy of the diagnosis. DNA ploidy analysis by using flow cytometry is suitable for this purpose [1]-[5], however, it may not be most suitable for rapid analysis considering its reproducibility, preparation time, required cell isolation technique, and complicated operation of flow cytometer.

We developed an automatic cell isolation system with cell isolation unit and staining reagent kit specialized for flow cytometry. In this study, porcine colon tissue was used to evaluate the performance of this system. This system can determine optimum condition to obtain a sufficient number of cells and stain cells stably by controlling condition of pipetting, including aspiration and discharge speed, stop time between aspiration and discharge, and the number of pipetting.

In addition, we analyzed fresh tissue samples of colorectal cancer patients by using the proposed system with the optimized condition to evaluate the efficacy of the system in clinical settings. We observed a notable difference between DNA ploidy pattern of cancer and normal tissues. The evaluation result indicates that our proposed system can be used as a rapid DNA ploidy analysis intraoperatively.

II. Materials and Method

A. Cell isolation unit

Fig.1 shows a cell isolation unit we have developed. Its dimensions are W 125 × H 257 × D 250 mm, with a weight of 4.3 kg. It consists of 3 user interface parts; display, setup key, and sample stage. Table 1 shows parameters and setting range which are user-configurable.

Pipetting was performed using modified pipette shown in Fig.2. The pipette tips have two small holes placed diagonally at 4 mm distance from its bottom. These holes work as flow paths of liquid and pressure when tissue gets stuck in the tip and blocks a main path.

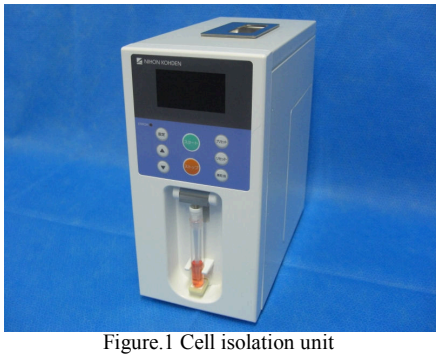

Figure.1 Cell isolation unit

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TABLE.1 CONFIGURABLE PARAMETERS OF PIPETTING

Parameters	Unit	Setting range
Pipetting flow	µL	100 - 1000
Aspiration speed	µL/sec	100 – 1000
Aspiration interval	sec	0 – 10
Discharge speed	µL/sec	100 – 10000
Discharge interval	sec	0 – 10
Number of pipetting	times	10 - 500

Figure.2 Pipetting tip

B. *Cell Staining Reagent Kit*

Fig.3 shows a cell staining reagent kit that we have developed. Ingredients of the cell staining reagent are 0.1mg/mL propidium iodide (Sigma-Aldrich Japan, Tokyo, Japan), 0.05mg/mL RNaseA (Wako pure chemical industries, ltd., Osaka, Japan), and 0.2% tritonX-100 (Kishida Chemical Co., ltd., Osaka, Japan). First, these ingredients are mixed together in water solution, then dispensed into test-tubes, and lyophilized in a vacuum freeze dryer (Kyowa Vacuum Engineering. Co., Ltd., Tokyo, Japan) to turn it into pellet. Our preliminary experiments showed that cells can be stained in 6 minutes in dark at room temperature by using this reagent kit.

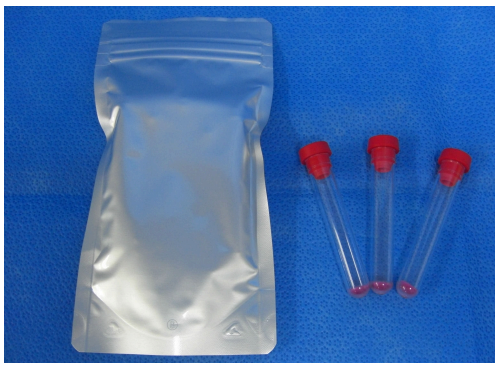

Figure.3 Cell staining reagent kit

C. *Specimen and protocol for DNA ploidy analysis*

Fig.4 schematically depicts the procedure for this method using our new system and reagent. In this study, porcine colon tissue was used as a specimen. First, for preparation of the specimen, porcine colon tissue was cut by φ4mm disposable biopsy punch (BF-40F, Kai Industries, Co., ltd., Gifu, Japan). And second, diluents (ISOTNAC, Nihon Kohden Corp., Tokyo, Japan) and specimen were put into the staining reagent. Then the prepared specimen was placed on the sample stage with a pipette tip attached to it.

We used two parameters, discharge speed and the number of pipetting, to evaluate the efficacy of this method. First, discharge speed was controlled within the range of 2000 to 6000 µL/sec to assess if this method has sufficient mechanical power and pressure to disintegrate tissues. The number of pipetting was fixed at 170 times to complete the processing for the rapid DNA ploidy analysis in 6 minutes. And second, the number of pipetting was controlled within the range of 50 to 400 times with discharge speed fixed at 4000 µL/sec to determine an optimum condition. Other parameters were constant values in all experiments. In addition to this experiment, evaluation of manual pipetting was also performed as reference.

After pipetting, the specimen was filtered by a mesh with a diameter of 40 µm, and then measurement was performed by using flow cytometer (EPICS-XL, Beckman Coulter, Inc., Fullerton, CA, USA).

1. Reagent dissolution
   - 2.5mL of buffer solution is added to the test-tube containing freeze-dried reagent. The reagent have to be stirred and dissolved well.
2. Tissue injection
   - A piece of tissue with about $2mm^3$ is put into a test-tube.
3. Cell Isolation & staining
   - A test tube is placed on sample stage of the pipetting device.
   - Cell isolation and staining are carried out for 6 minutes in dark.
4. Filtration
   - Cell suspension is filtered by a ϕ 40um nylon mesh.
5. Measurement & analysis

Figure.4 Process flowchart of the method by using our new device and reagent

D. *Method for analysis of DNA histograms*

Fig.5 shows the correlation between the number of cells and fluorescent intensity. Fluorescent intensity is considered to be equivalent to DNA content. Gating was performed using scattergram to eliminate doublet factor for this analysis before counting the number of cells. According to the guideline [6],

the intensity of the fluorescent peak of the stained normal human peripheral blood mononuclear cells should be set at the value of 200. Cancer or other unusual cells with different DNA content were observed at each corresponding position.

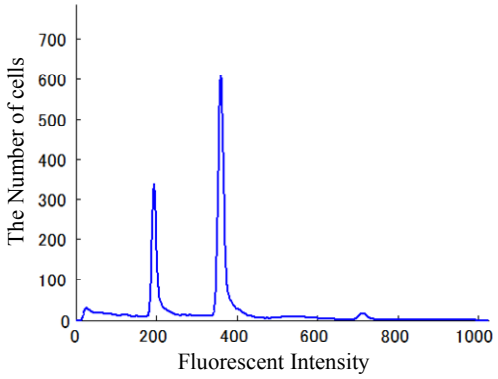

Figure.5 Example of DNA histogram: Measurement was conducted with EPICS-XL (Beckman Coulter). After excitation with 488 nm laser, the fluorescence of 610 ± 10 nm was selectively detected and measured at low sample flow rate (15 µL/min).

E. Clinical study

Clinical study was conducted at the Cancer Institute Hospital. Specimens were taken from cancer and normal sites of colorectal cancer patients. Each specimen was divided into two pieces, one for the DNA ploidy analysis by flow cytometer and the other for hematoxylin and eosin (HE)-stained sample. Evaluations of our new method were performed by comparing histogram data with pathological diagnosis from HE-stained samples. Pathological diagnosis was made by an experienced pathologist.

III. RESULTS

Stability of the staining reagent was assessed. Table.2 shows the stability of this reagent when stained normal human peripheral blood mononuclear cells was used as reference material according to the guideline [6]. Compared to the method recommended by the guideline, our method shows equivalent standard deviation (SD), and no significant difference was observed with F-test value of 0.66 (> 0.05).

TABLE.2 COMPARATIVE CHART OF GUIDELINE AND OUR METHOD

	Guideline	**Our method**
Measured value of fluorescent intensity (N = 5)	192.9	203.6
	193.5	203.3
	192.4	204.3
	192.8	204.6
	192.6	204.1
Average	192.8	204.0
Standard deviation	0.42	0.53
F-test value	0.66 (> 0.05)	

Fig.6 shows the correlation between the number of obtained cells and discharge speed. This graph indicates that discharge speed has little impact on cell isolation procedure, although the number of obtained cells mildly changed.

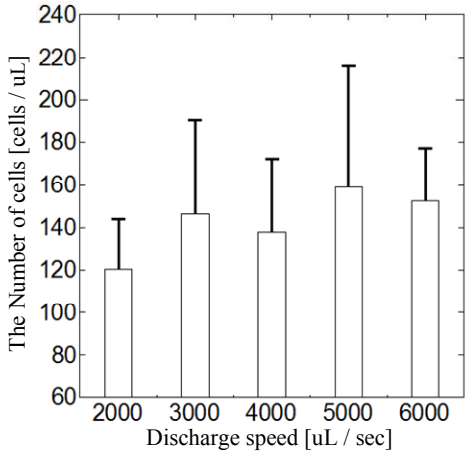

Figure.6 Correlation between discharge speed and number of cells: Other parameters were as follows.
Pipetting flow: 800uL, aspiration speed: 2000 µL/sec, aspiration interval: 0.5 sec, discharge interval: 1.0 sec, pipetting number: 170 times.

Fig.7 shows the correlation between the number of obtained cells and the number of pipetting. Positive correlation between the number of obtained cells and the number of pipetting was observed (R^2=0.995). This result suggests that the same amount of cells can be obtained by 100 times of pipetting as obtained manually.

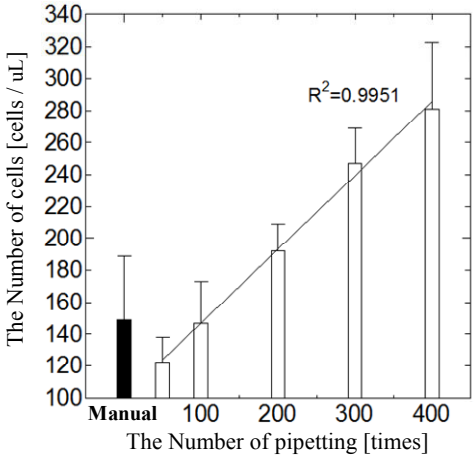

Figure.7 correlation between the number of cells and the number of pipetting: Other parameters were as follows.
Pipetting flow: 800 µL, aspiration interval: 0.5 sec,
Discharge speed: 4000 µL/sec, discharge interval: 1.0 sec

Fig.8 shows typical histogram of normal and cancer tissues, and their corresponding histopathological features. For DNA analysis, fresh tissue samples were treated by using the automatic cell isolation system with the same condition as the aforementioned evaluation. A peak with high-DNA content was observed at the histogram of cancer tissue.

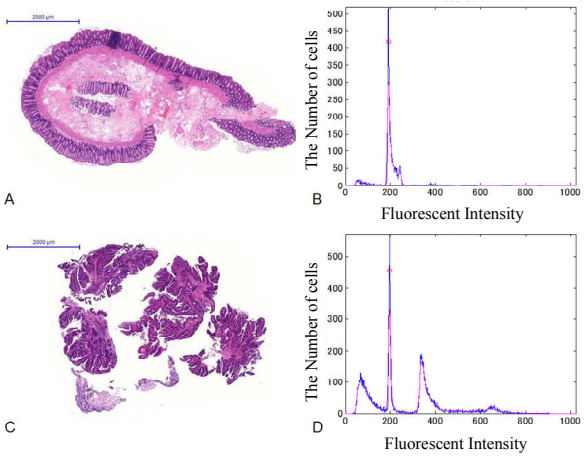

Figure.8 Histopathological features and DNA histograms of tissues from a colorectal cancer patient.
A: Histopathological feature of a normal tissue sample as a reference of fig.8B, B: DNA histogram of a normal tissue sample, C: Histopathological feature of a cancer tissue sample as a reference of fig.8D, D: DNA histogram of a cancer tissue sample

IV. DISCUSSION

The proposed method has been shown to be useful as a quick analysis method as it takes only 6 minutes to obtain adequate performance, while conventional method takes about 30 minutes only to prepare samples.

As shown in Figure 2, fluorescent intensity value is higher in our method in comparison with in the guideline, because propidium iodide content of our reagent is higher than that of the guideline. Despite such difference between the reagent of guideline and our method, the stability of the proposed staining reagent kit was also confirmed to be sufficient with the small SD value. In addition, the new reagent requires only 1 step for the preparation. Therefore, this method is less likely to be affected by human error, indicating that the reproducibility can be improved by using this reagent kit.

Fig.6 shows that discharge speed has little impact on the efficiency of cell isolation. Although there was some dispersion in the number of cells, equivalent results were obtained at discharged speed above 2000 µL/sec. In terms of the performance stability of pipetting and reproducibility of the data, we used 4000 µL/sec as a fixed value.

Fig.7 shows that the performance of cell isolation largely depends on the number of pipetting, and there was a positive correlation between the number of pipetting and the number of cells. On the other hand, processing time also increased as the number of pipetting increased (e.g. 7 min at 200 times and 14 min at 400 times of pipetting). A sufficient number of cells were obtained by pipetting of over 100 times. Based on the dispersion rate and processing time shown by this study, we have determined that the optimum number of pipetting would be 170 times. With this condition, cell isolation from φ4 mm of porcine colon tissue can be done within 6 minutes.

For the clinical evaluation data (fig.8), typical histograms of normal and cancer tissue were obtained. A peak with high-DNA content was detected in the histogram of cancer tissue. Thus existence of cancer cells could be identified from the DNA ploidy analysis, and these results were also in good agreement with the pathological evaluation.

We have demonstrated that a sufficient number of cells for DNA ploidy analysis can be obtained by using our method; however, further work is required to define a clear relationship between isolated cells and cells in tissues.

V. CONCLUSION

In this study, we evaluated our developed automatic cell isolation system consists of cell isolation unit and staining reagent kit specialized for flow cytometry. For the reagent, stability in staining cells and usability were confirmed. By using the automatic cell isolation unit, the optimum condition for cell isolation from fresh tissue was determined by using porcine colon tissue. The number of pipetting was considered to have a large impact on the performance of cell isolation, and we have concluded that 170 times of pipetting would be optimum. With this proposed method, tissue disintegration and cell isolation takes only 6 minutes, thus DNA ploidy analysis can be done in 10 minutes. Therefore, this method can be useful as a rapid method for intraoperative histopathological investigation.

Also, the clinical study result was well correlated with pathological diagnosis. All the results show that our proposed method has a potential application of enhancing intraoperative histopathological investigation. .

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