Accurate 3D cell-based assay - targeted on *ex vivo* -

■ Background/Objective

3D cell-based assays using spheroids and *ex vivo* tumor fragments have recently been paid much attention in preclinical drug discovery and drug efficacy evaluation. However, size uniformity as well as cell viability need to be significantly improved for obtaining accurate and reproducible data.
On-chip™ Sort (On-chip Biotechnologies Co., Ltd., Tokyo, Japan) can select intended samples from a population based on scattered light and fluorescence. YAMAHA CELL HANDLER™ (YCH) can select and transfer targeted samples from one plate to another. In this report, we describe a method for preparation of precise 3D cell-based assay by combining the two instruments.

■ Results

Microscopic validation of tumor cell fragments

Figure 1 shows the state of tumor fragments following the sample preparation described in Materials and Methods. After 3 days culture, the tumor fragments obtained were morphologically spherical but not uniform in size.

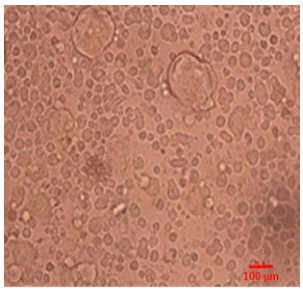

Figure 1. Microscopic image of 3D HT-29 fragments. See Materials and Methods for details.

Sorting and evaluation of 3D tumor cell population

Figure 2 shows dot plots for cell sorting by On-chip™ Sort. Using the disposable microfluidic chip with 350 µm-wide microchannel, the cell sorter was capable of sorting cells and cell clusters up to approximately 300 µm in diameter, which is not attainable with a conventional Jet-in-air type cell sorter. Size distribution profiles were analyzed using YCH before and after sorting (Table 1). The number of small cell fragments (≤ 75 µm) were significantly decreased from about 29,038 to 2,493, while cell clusters of the target size range (≥ 100 µm) were relatively retained (1,868 to 448).

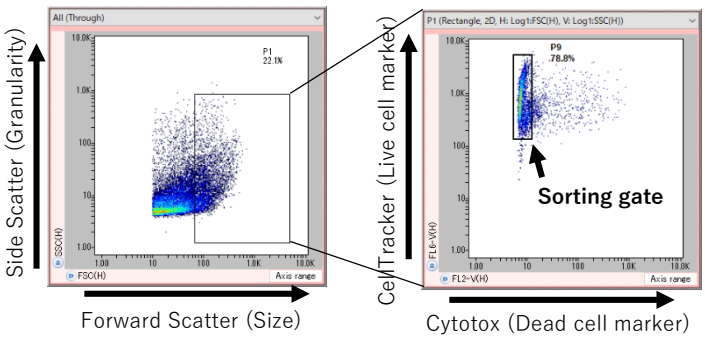

Figure 2. Dot plots for cell cluster sorting.

Table 1. Size distribution of tumor fragments before and after sorting.

Size range	Number of fragments	
	Before	After
≤ 75 mm	29,038	2,493
≥ 100 mm	1,868	448

Assessment of assay plate

Figure 3 shows images of the assay plate wells after plating single 3D tumor fragments via YCH (A) and manually (B). Each 3D tumor fragment in a well is indicated by green line. Quantitative and qualitative efficiencies of YCH in such tumor fragment plating were evaluated in comparison to those of manual plating (Table 2).

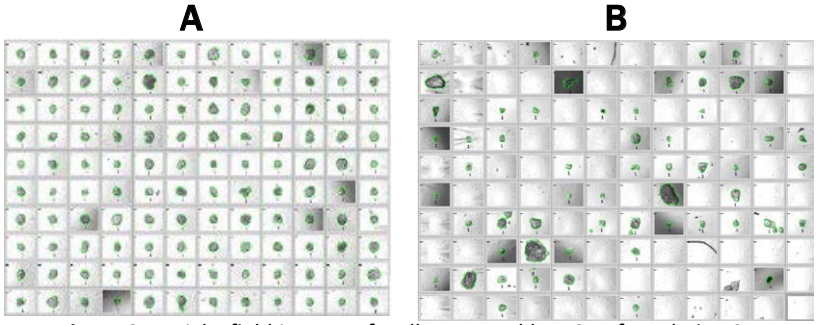

Figure 3. Bright-field images of wells captured by YCH after plating 3D tumor fragments via YCH (A) and manually (B).

Almost all the wells were filled with single and uniform 3D cell fragments using YCH, while manual dispensing resulted in only one-thirds of the wells filled but with poor uniformity. It was demonstrated that a plate for precious 3D cell-based assay can be successfully prepared using YCH.

Table 2. Comparison of sample plates prepared manually and by YCH.

	YCH	Manual
Wells with single tumor fragment	118/120 (98%)	42/120 (35%)
Empty wells	0/120 (0%)	62/120 (52%)
Average area of fragment (μm^2)	8,938±2,733	6,699±8,056

Evaluation of size and viability of 3D tumor fragments

The wells with single tumor fragment were divided into two groups and further incubated for 3 days with or without 1 μM of Staurosporine. In case of the assay plate prepared by YCH (Fig. 3A), both viability and diameter decreased in the drug treatment group with statistical significance of $p < 0.05$ compared to non-treat group (Fig 4A). On the other hand, no significant difference in the diameter was apparent between non-treat and drug treatment groups, when manually prepared assay plate (Fig. 3B) was used for this drug treatment (Fig 4B). These results indicate that the samples prepared using YCH were uniform and alive, and hence YCH enables accurate assessment of 3D cell-based assays.

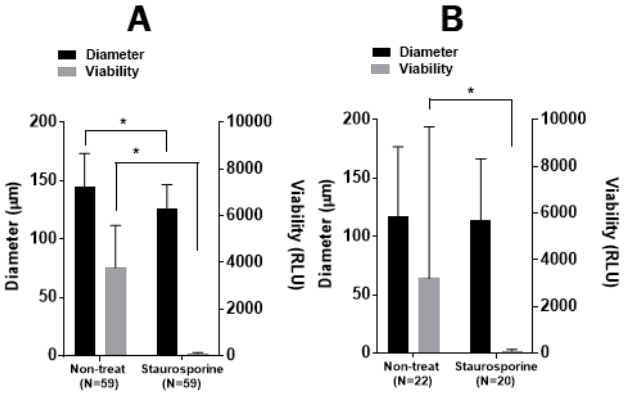

Figure 4. Effect of Staurosporine on the diameter and viability of 3D cell fragments prepared using YCH (A) and manual method (B). Bars represent mean + SD of those fragments with or without Staurosporine (1 μM) (*$p < 0.05$).

■ Conclusion

On-chip™ Sort allows for the enrichment of desired samples even from *ex vivo* tumor fragments. YCH efficiently and accurately isolates target single fragments into individual wells in an automated fashion, and hence secure statistically significant sample size with improved tumor size uniformity, while saving time and reagents. The results demonstrated the combined use of those instruments increases accuracy and quality of 3D cell-based assay, and prove YCH plays a key role in such assays.

■ Material & Methods

Tumor sample preparation: HT-29 xenografts which had been grown subcutaneously in nude mice for 3 weeks were used for the following preparation. The xenograft tissues were enzymatically treated with 0.26 U/mL of Liberase™ (Roche Diagnostics) and 10 mg/mL of DNase I at 37°C for 30 minutes after mincing with a scalpel. The resultant tumor cell clumps were passed through filters with pore size of 300 μm and 200 μm. The resultant tumor fragments obtained were resuspended with culture medium (a mixture of equal parts of DMEM with 10% fetal bovine serum and StemPro® hESC SFM (Thermo Fisher Scientific)), incubated at 37°C under 5% CO_2 for 3 days.

Tumor fragment separation: Tumor cells and fragments harvested were first stained with live/dead fluorescent dyes (CellTracker™ Red CMTPX Dye (Thermo Fisher Scientific) for live cells, and IncuCyte® Cytotox Green (Sartorius) for dead cells) and suspended with an equal volume of On-chip™ Sample buffer 2X (On-chip Biotechnologies). Live cell clusters with a particular size range (100 μm ± 25 μm) were sorted with On-chip™ Sort using 2D Chip Z1000-w350 (On-chip Biotechnologies). Sort gate setting was: forward scatter (FSC) >70 (corresponding to the cell size approximately >100 μm in diameter), FL2 intensity (for Cytotox Green) <10, and FL6 intensity (for CellTracker Red) >100. Tumor size population before and after the sort was evaluated using YCH imaging analysis.

Assay plate preparation: Sorted fragments were collected to a Precision Chamber™ (Yamaha Motor) and then applied to YCH for further identification, picking and plating of tumor fragments of interest to a microtiter plate (384-well Black/Clear Round Bottom Ultra-Low Attachment Spheroid Microplate (Corning)). At the same time, for comparison purpose, the sorted fragments were plated with limiting dilution method (Manual method).

Viability assay: Viability assay (CellTiter-Glo® 3D Cell Viability Assay (Promega)) was performed on the prepared test plates to determine the cell viability and drug efficacy.

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