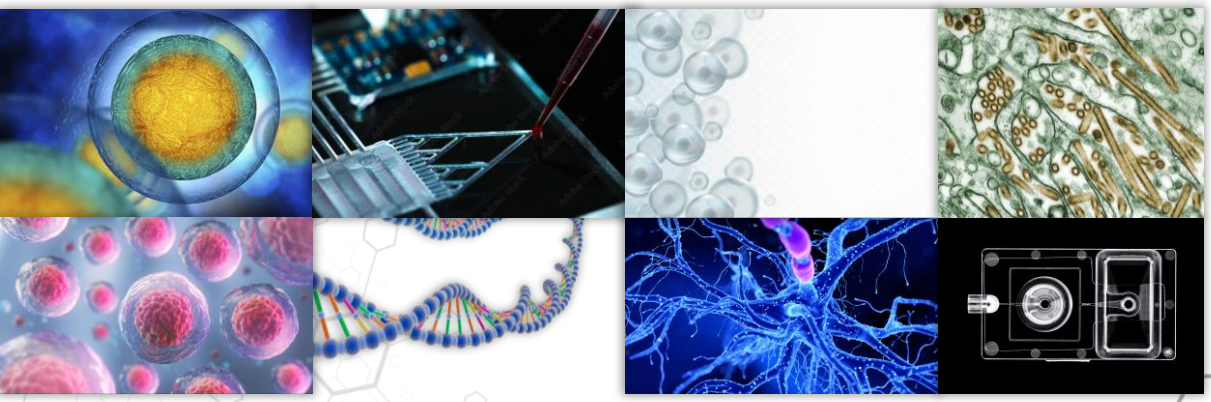
A Cell Sorter for Reducing Sorter Induced Cellular Stress (SICS)

On-chip Sort

~ Innovative technology for sorting high-quality cellular samples ~



On-chip Biotechnologies Co., Ltd.

Introduction

A commonly used technique in the biomedical field is flow cytometry-based cell sorting. This allows cells of interest to be isolated in a heterogenous sample. Cells can be easily stained and passed through a cell sorter to be analysed or sorted, which is useful if the necessity is only sorting. In many cases where cells are used for further downstream analyses, researchers have noticed changes in their cells post-sorting (i.e., morphological changes, delayed cell growth, decrease in cell viability and alterations in gene expression). Therefore, we hypothesized that the post-sorting cell changes are a result of “sorter-induced cellular stress” (SICS; Lopez, P., *CYTO2018*).

SICS arose from the cell sorting technique used in most cell sorting systems known as Jet-in-air method. With this method, the use of high pressure for manipulation of sample, the large pressure differentials inside and outside the nozzle, the use of electric charges to deflect droplets into collection chamber, and high-speed collisions during collection, can all lead to SICS. Therefore, as a prevention for post-sorting cell changes with conventional cell sorter, we have developed the world’s first microfluidic chip-based cell sorter, On-chip Sort. On-chip Sort employs a unique cell sorting mechanism to eliminate all the damaging steps involved in cell sorting on FACS to significantly reduce SICS.

What is On-chip Sort?

On-chip Sort uses a small disposable microfluidic chip (Fig. 1) (with either 80 µm or 150 µm flow channel width) as the core of its technology. Sample and sheath fluid are loaded into the sample (yellow area in Fig. 2) and sheath reservoirs (blue area in Fig. 2) on the chip, respectively. Unlike conventional cell sorters, which a specific sheath fluid has to be used, On-chip Sort allows the use of any sheath fluid appropriate to your cells (e.g., culture medium, fresh water, sea water, oil).

Cell sorting on On-chip Sort works on the basis of the ‘Flow shift’ mechanism. When a detected target cell reaches close to the sorting area, a short liquid pulse is generated by pressurization of air to deflect the target cell (red dot in Fig. 3) into the collection reservoir (white left arrow in Fig. 3; and red area in Fig. 2). Non-target cells (white dots in Fig. 3) flow in a continuous flow to the waste (green area in Fig. 2). This sorting method differs to that of other cell sorters where it does not involve the use of high pressure, nozzle, electric charges or high-speed collision. The contamination between samples is prevented by the use of disposable chips. Contamination of the sorter itself is also avoided as all the liquids (sample, sheath and waste) remain inside the chip during analysis and sorting.

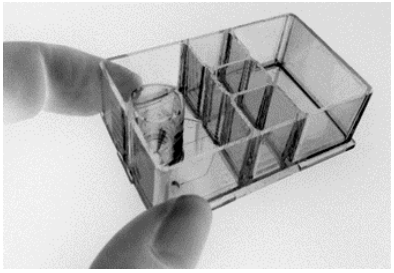


Fig. 1 Our small and disposable microfluidic chip for analysis and sorting.

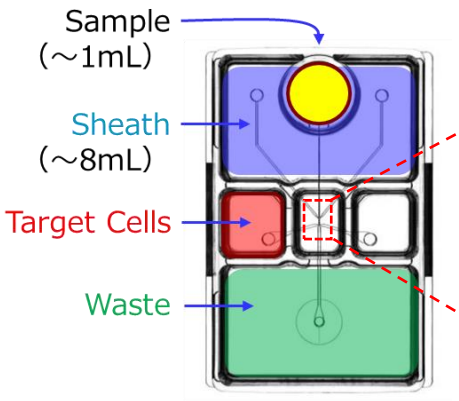


Fig. 2 Reservoirs where initial sample, sheath fluid, waste liquid and collected sample are on the chip.

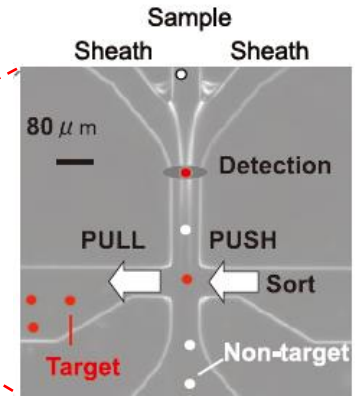


Fig. 3 Enlarged image of sorting area of the chip and the Flow shift sorting mechanism.

Damage-free cell sorting

In order to compare the effects of sorting on relatively robust HeLa cells between a conventional cell sorter (Jet-in-air method) and On-chip Sort (Flow shift method), 100 HeLa cells were sorted on both sorters. After sorting, collected cells were cultured in wells of a 96-well plate and the cell numbers were counted daily. Cells sorted by On-chip Sort began to grow two days after sorting, and cell number doubled on day 3 (Fig. 4). On the other hand, the cells sorted by conventional sorter only started growing three days after sorting, with a slower growth rate as compared to those sorted by On-chip Sort (Fig. 4).

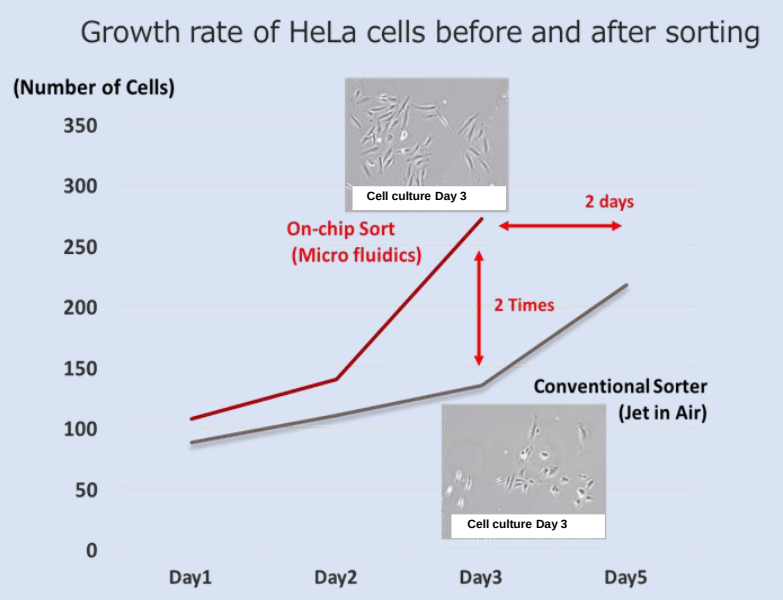


Fig. 4 Growth curves of HeLa cells after sorting. The images were taken on day 3.

In addition to HeLa cells, the morphology of eosinophils that are highly susceptible to physical damage, were also compared between a conventional sorter and On-chip Sort after sorting. Fig. 5 shows the morphologies of eosinophils from peripheral blood stained with HE and fixed by CytoSpin after sorting by On-chip Sort (left) and a conventional sorter (right). Post-sorting morphological differences were clearly observed. Eosinophils sorted by On-chip Sort were intact, with cellular structures maintained. However, those sorted by conventional sorter were ruptured. It is speculated that sorting by conventional sorter has induced damages to the cell membranes.

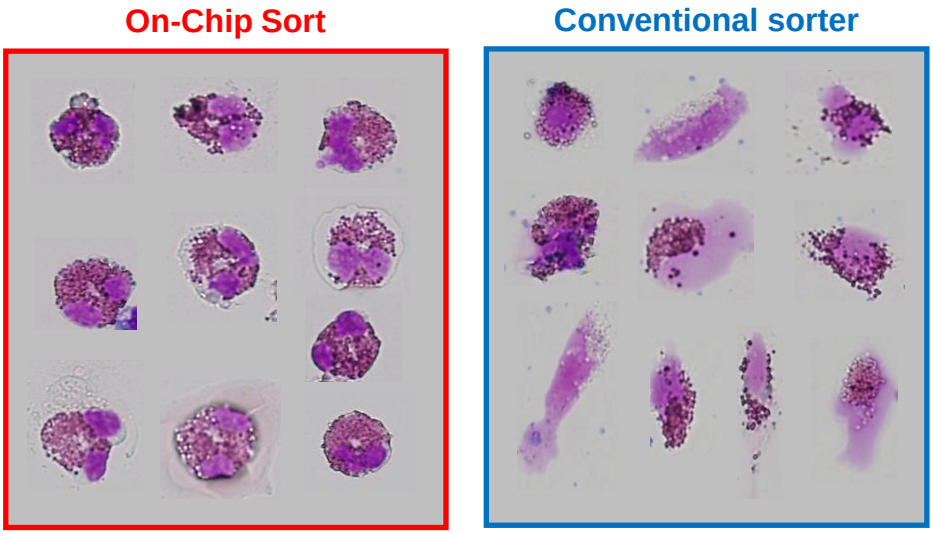


Fig. 5 Morphology of peripheral blood eosinophils stained with HE and fixed by CytoSpin after sorting by On-chip Sort (left) and a conventional sorter (right).

Damage-free sorting of sperm

Sperm cells are known to be extremely sensitive to SICS, and a large number of sperm that are sorted using a conventional sorter are not suitable for use in *in vitro* fertilization (IVF). The effect of sperm sorting on On-chip Sort was evaluated using parameters such as motility, fertility and developmental ability. The overall procedure of the experiments is presented on Fig. 6. Oocytes and sperm were retrieved from C57BL/6L mice and sperm were pre-incubated in BSA-free Toyoda Yokoyama Hosi medium. Pre-incubated sperm was sorted using On-chip Sort with calcium-enhanced human tubal fluid (mHTF) as sheath fluid. IVF was performed on a dish with the collected sperm and retrieved oocytes in mHTF. In order to determine the developmental ability of the cultured two-cell embryos produced by IVF using collected sperm, embryo were transferred to Jcl:ICR mice.

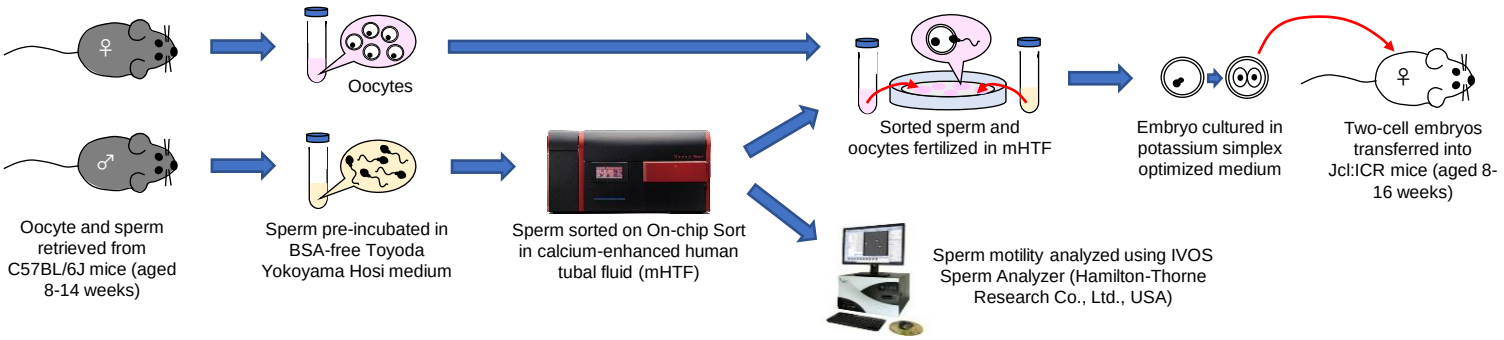


Fig. 6 Overall experimental procedure.

Sperm sorting on On-chip Sort was performed based on signals from forward scattered light (FSC) and side scattered light (SSC). The sorted sperm had lower motility than unsorted sperm (Fig. 7A), but the fertilization rate showed no significant difference between unsorted and sorted sperm (Fig. 7B). The sorted sperm were fertilized with oocytes, and the fertilized eggs developed normally up to the blastocyst stage in *in vitro* culture, which was comparable to those that were unsorted (Fig. 7C). Normal pups were obtained after embryo transfer, and birth rate was similar for both that used unsorted and sorted sperm (Fig. 7D). On-chip Sort has shown the mouse sperm isolation capability while maintaining normal fertilization and developmental ability.

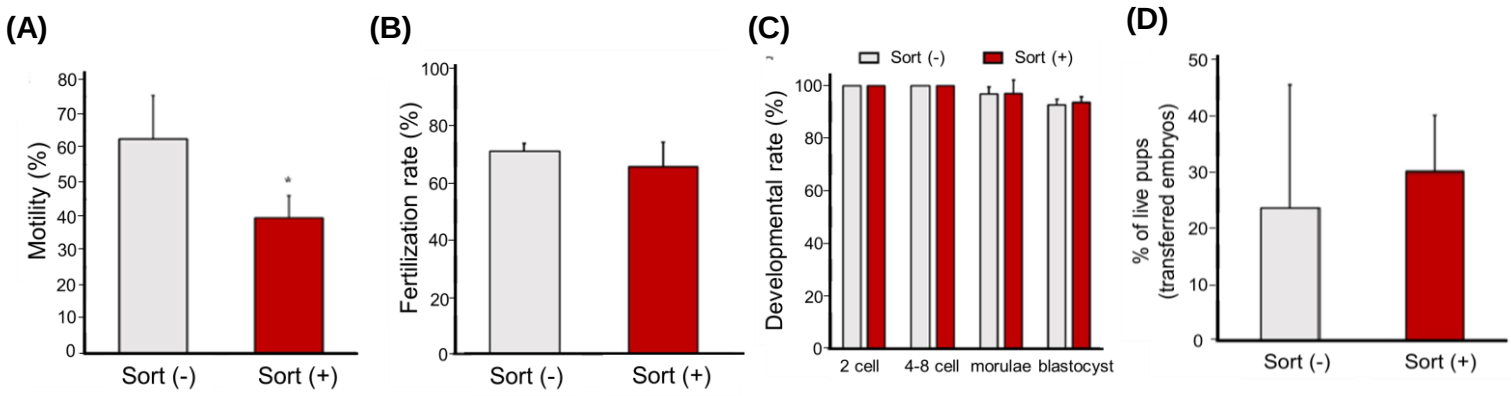


Fig. 7. (A) Motility of sperm with and without sorting. (B) Fertilization rate of sperm with and without sorting. (C) Developmental rate of sperm with and without sorting at several developmental stages. (D) Birth rate of live pup derived from unsorted and sorted sperm.

Sorting of spheroids

Three-dimensional (3D) cell cultures are progressively used for research in regenerative medicine, tissue engineering, drug discovery, toxicity testing, drug discovery and cancer research. Three-dimensional cell cultures are preferred over two-dimensional cell cultures or single cells, because they can imitate *in vivo* architecture of natural organs and tissues. Cultured spheroids can be heterogenous in size so it is necessary to isolate them into a uniformly sized population for downstream applications. When sorting spheroids on conventional sorters, spheroids can be damaged and deformed due to high shear stress and high-speed collision, and in many cases, spheroids do not even pass through the nozzle. On the other hand, On-chip Sort is capable of performing damage-free sorting of spheroids up to 140 µm in diameter. Fig. 8 shows sorting of 100 µm spheroids from a heterogeneous population of spheroids using On-chip Sort. Smaller spheroids (45 µm) were also sorted using On-chip Sort (Fig. 9) and cultured over several days. The cultured spheroids tripled in size on Day 10 (Fig. 9). These results suggest that On-chip Sort is not only capable of sorting spheroids, but also capable of keeping spheroids viable after sorting.

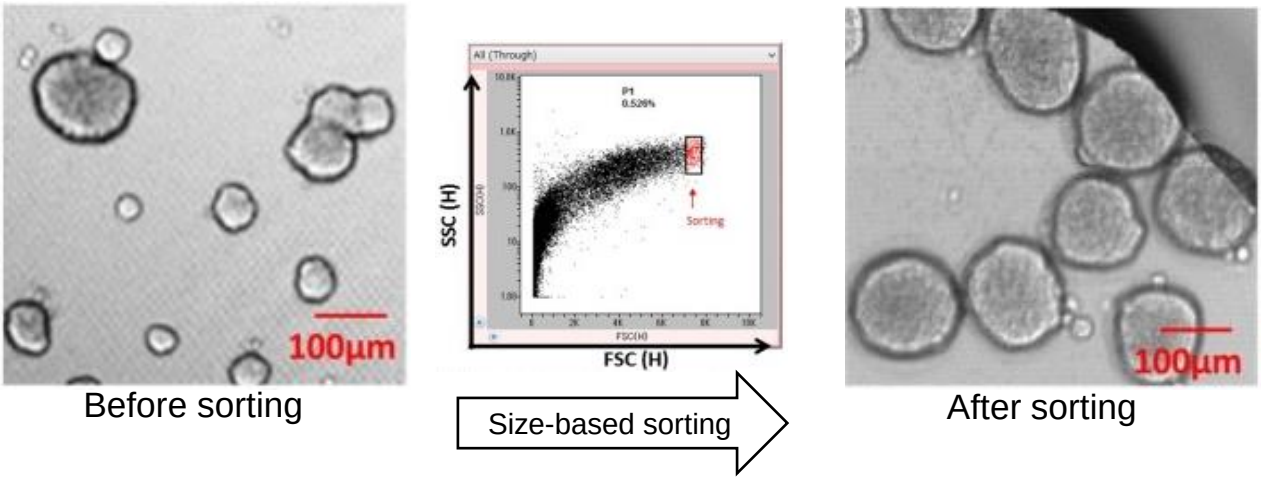


Fig. 8 Sorting of 100 µm spheroids using On-chip Sort.

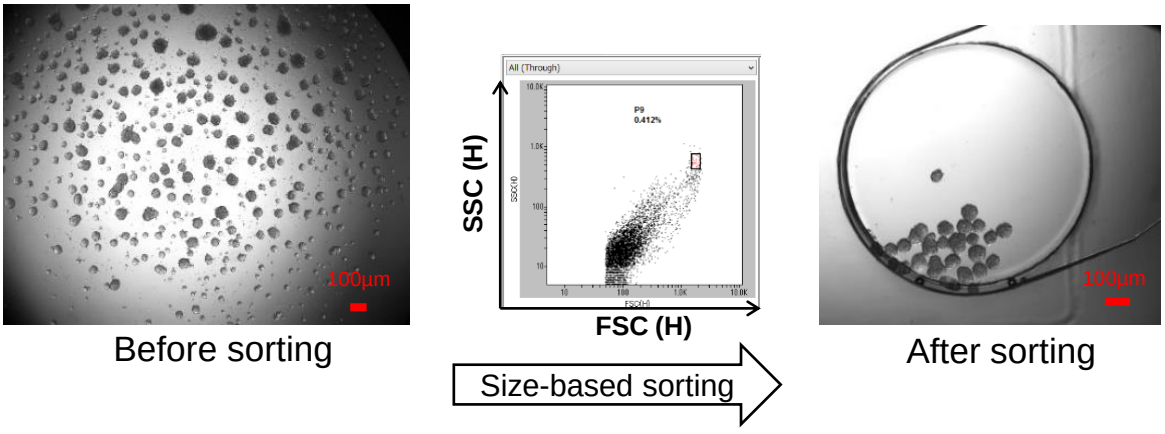


Fig. 9 Sorting of 45 µm spheroids using On-Chip Sort.

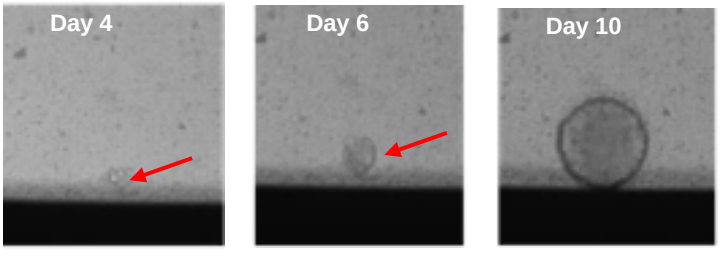


Fig. 10 Growth of 45 µm sorted spheroid from Fig. 8 over time. Red arrows indicate cell cluster.

Effect of cell sorting on gene expression

Many researchers sort cells into sub populations before analyzing the gene expression. In order to examine the effect of cell sorting on the difference in gene expression patterns between On-chip Sort and a conventional sorter, 50,000 cells of Caco-2 and HeLa cells were sorted on both sorters. The change in gene expression level for each cell type was determined by microarray analysis using unsorted cells as control. Fig. 11A and B show that both cell types sorted by On-chip Sort had less alteration in gene expression than those sorted by the conventional sorter. Furthermore, the number of genes with no change in gene expression level was 3-5 folds higher after sorting by On-Chip Sort (Fig. 11C) than by the conventional cell sorter.

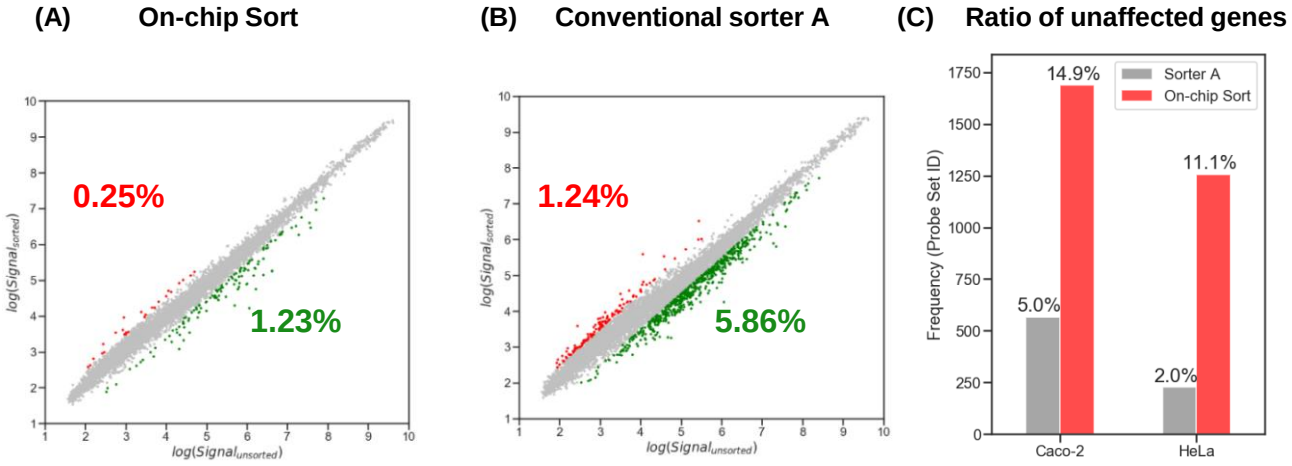


Fig. 11 Comparison of large changes in gene expression levels between sorted and unsorted HeLa cells (control) on (A) On-chip Sort and (B) conventional sorter A. In both (A) and (B), red dots represent up-regulated genes and green dots represent down-regulated genes. The numbers in percentages indicate percentages of all genes up-regulated (red) and down-regulated (green). (C) A histogram of unaffected gene expressions for Caco-2 and HeLa cell after sorting by On-chip Sort and conventional sorter A. The numbers in percentage indicate the fraction of unaffected genes over total genes for all properties (apoptosis, cell adhesion, cell cycle, etc.) combined. The ratio of expression change is defined as the signal intensity of sorted cells/signal intensity of unsorted cells.

Fig. 12 shows the gene expression patterns of different processes. The processes displayed, namely proliferation, cell cycle, transcription factor, apoptosis, reactive oxygen and oxidative stress, were the ones that showed clear differences after sorting by On-chip Sort and conventional sorter. These differences in gene expression patterns between On-chip Sort and conventional sorter (Fig. 12) supported the difference in growth rates between the two sorters shown in Fig. 4. Note that precautious measure must be taken when carrying out downstream analysis of sorted cells as transcription factors are likely to be affected. Although the gene expression analysis was done within 30 to 60 min after sorting for both types of cells, the result may differ depending on the post-sorting processes. Overall, differences in gene expression after sorting between On-chip Sort and conventional sorter can be observed.

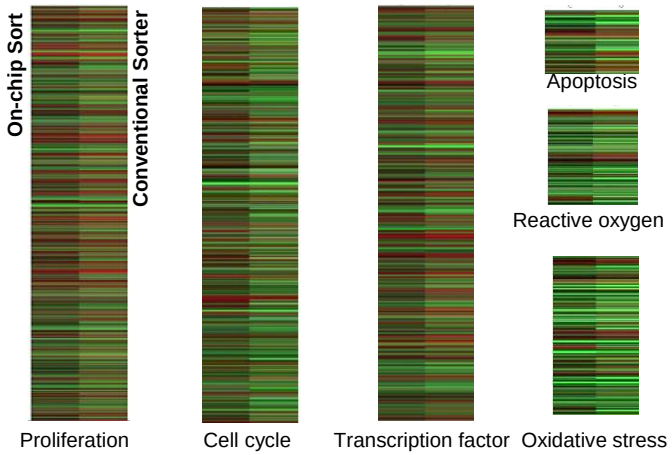


Fig. 12 Gene expression patterns of different processes. In each process, left and right columns represent data of On-chip Sort and conventional sorter, respectively. Black color indicates no change in gene expression, red color indicates up-regulation, and green down-regulation.

Microarray analyses data obtained with Dr. Yasuda and Dr. Hagiwara from Cell Innovator Co., Ltd., and Dr Tashiro from Kyushu University.

single-cell RNA sequencing for neurons through gentle cell sorting

Gentle isolation of a target cell population using fluorescence activated cell sorting is an important step for very sensitive downstream applications such as gene expression analysis. The damage induced to cells during cell sorting on electrostatic droplet-based cell sorters can often cause gene expression changes, cell damage and cell death, which can cause gene expression artefacts and extraneous RNA contaminations, and hence false gene expression profiling results. To overcome this problem, our microfluidic chip cell sorter, On-chip Sort, is used for damage-free sorting of fragile and sensitive cell types for effective downstream single-cell genomic analysis. In this study, mouse dorsal raphe (DR) *Pet1* neurons were purified by On-chip Sort and subsequently analyzed by droplet-based single-cell RNA sequencing (scRNA-seq) to characterize their molecular diversity in a high throughput and high resolution manner. The results of this study can contribute to the understanding of the DR functional organization, which can bring more insight into field of neuroscience, and potentially to the development of targeted therapeutics.

Fig. 13 shows the experimental workflow. Brains of mice aged 6 to 10 weeks from two genotypes were dissected (Fig. 13A) and DR cells were dissociated (Fig. 13B). DR-*Pet1* cells marked by EGFP expression were sorted by On-chip Sort (Fig. 13B). Sorted neurons were run through the 10X Genomics Chromium Single Cell 3' v3 protocol, then by Illumina NextSeq 500 sequencing (Fig. 13C). Single-cell RNA-seq data analysis was performed with R Package Seurat. After stringent data filtering and analysis, 2,350 single cell libraries were further analyzed (Fig. 13C).

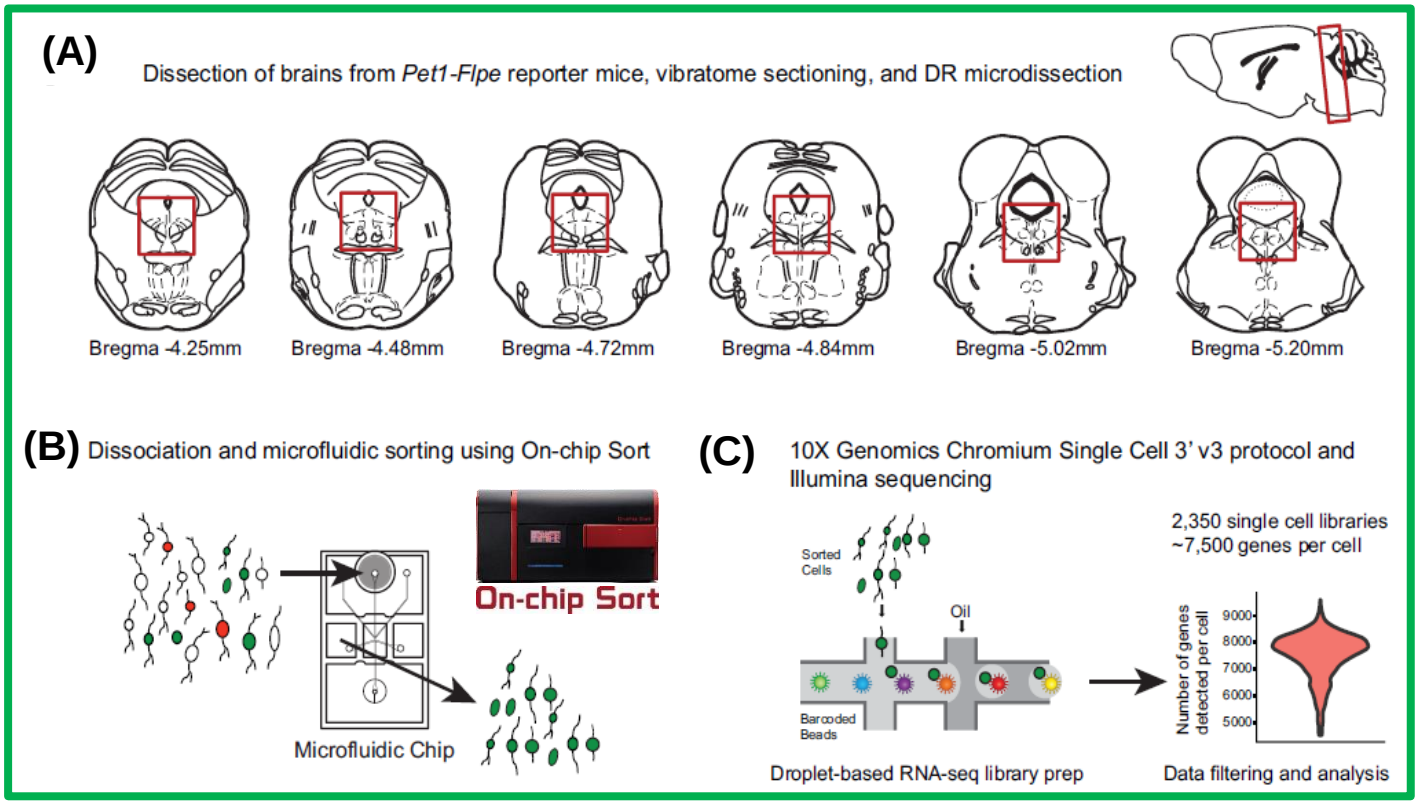


Fig. 13 Schematic diagram of the experimental workflow: (A) dissection of mouse brains, (B) dissociation of DR cells and fluorescence-based sorting using On-chip Sort, and (C) library preparation using 10X genomics, scRNA-seq using Illumina sequencing and data analysis using R package Seurat.

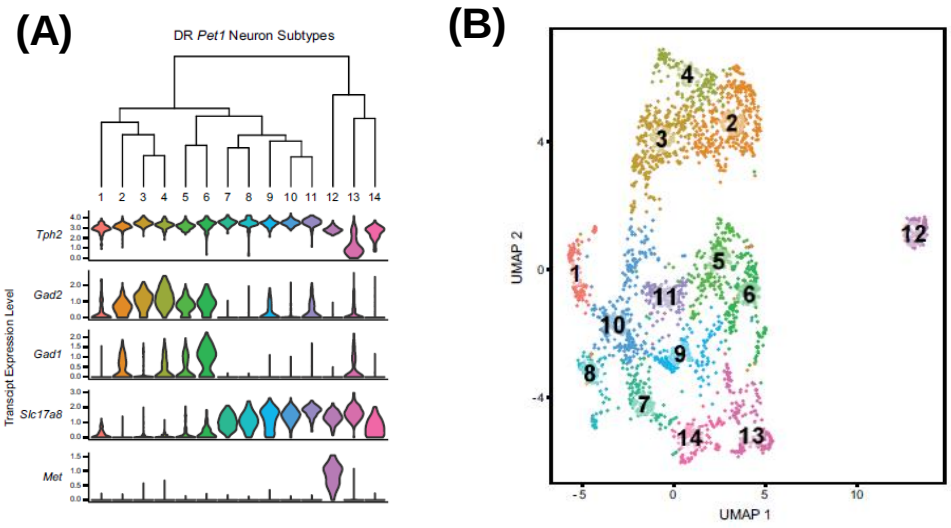


Fig. 14. Characterization of molecular diversity of single DR neurons by clustering analyses. (A) Hierarchical clustering of DR-*Pet1* neuron subtypes (clusters) identified by Louvain clustering, violin plot depicting the log-normalized expression of a common set of genes. (B) UMAP visualization of single-neuron transcriptome community/similarity structure. Each colour represents a discrete cluster, with the same clustering parameters used in (A).

Many of the DR neurons sorted by On-chip Sort were observed to be viable and had intact processes emanating from their somas, indicating that On-chip Sort was capable of gentle sorting. In order to characterize the molecular diversity of DR-*Pet1* neurons, genes with significantly variable transcript expression across single neurons were identified and based on these gene expression differences, 14 distinct molecularly-defined subtypes of *Pet1* neurons were classified in mouse DR using clustering analyses shown in Fig. 14A-B. Overall, gentle sorting of DR neurons using On-chip Sort is an important preliminary step upstream of scRNA-seq to ensure the delivery of improved single cell transcriptomic results.

Summary

Due to the wide selection of sheath fluids for sorting and the 'Flow shift' sorting mechanism, On-chip Sort has shown significant reduction in post-sorting changes to cells as a result of SICS as compared to those of conventional sorters. On-chip Sort demonstrated to have better cell viability, less physical damage and less changes in gene expression than conventional sorters after sorting. It also has the capability to sort spheroids and perform multi-step sorting for further purification of cellular samples.

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