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High throughput single cell gene expression analysis using SH800Z cell sorter

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Background

Historically, flow cytometry of multivariate populations has involved analysis of cellular phenotype at the population level rather than at the single cell level. Therefore, genetic analysis of populations sorted by flow cytometry used mean values from a heterogeneous population. But understanding of heterogeneity of cells and searching of molecular makers are difficult by the traditional flow cytometry population level analysis. The recent emphasis on developing methods for studying cells at a single cell level underlines the importance of single cell analysis. And high throughput methods that offer the ability to mine data on a larger scale are especially important for single cell analysis.
There are several way to handling single cells, manual picking, robotic picking, capturing in micro fluidics, droplet sorting and all that. Sorting of flow cytometry is one of best way, because a large amount of single cells which are selected by using specific biological markers can be sorted in high speed sorting. Only the problem was that sorting of flow cytometry was not easy for the users, adjusting the optical condition, detecting proper sorting conditions and targeting well plate precisely. But SH800Z cell sorter resolved these difficulties and offers the ability of high throughput well sorting function. In this study we demonstrate the utility of sorting and single cell gene expression analysis of single cells in a 384 well plate format using SH800Z cell sorter. In the single cell analysis method, to avoid loss during RNA purification sample volume should be reduced to the minimum. As a result accuracy of sorting position is important for achieving accurate single cell gene expression analysis downstream of cell sorting.

Material and Methods

SH800Z plate sorting technology:

Angled Sorting Plate
To maximize the possibility to sort the sample into the lysis buffer SH800 PCR plate holder is angled at 4 degrees(Fig1-(B)). In case lysis buffer volume is 0.4µl the margin to get the sample into tube is 0.054[mm] without angled holder, the margin to get the sample into tube is 0.738[mm] with angled holder.

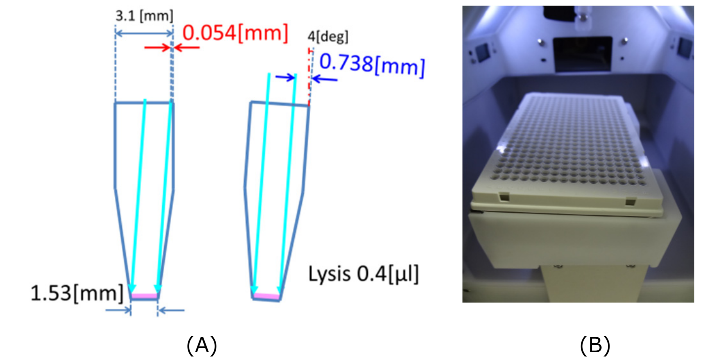

Figure 1: Angled holder benefit
(A) Comparison between vertical standing PCR plate cone and angled PCR plate cone. The margin of sort stream to sort the lysis in the cone, in case of vertical standing margin is 0.054[mm] very close to the edge of cone, and in case of angled all stream can hit into the lysis with 0.738[mm] margin. (B) picture of angled holder for PCR plate.

High accuracy position adjustment function
SH800Z performs automatic setup function to adjust the plate position. But in case of very strict experiment setting, for example single cell analysis of 384 well plates with very small lysis volume, expected accuracy will be over the auto setup position accuracy. Then SH800 software provides adjustment function to user. User can adjust plate position with 0.1 [mm] increments in between using 4 corner wells of the plate.

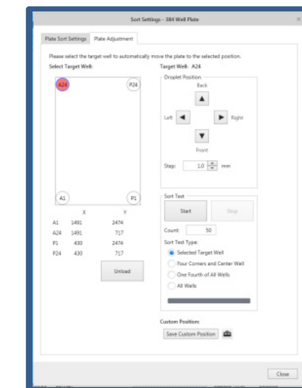

Figure 2: SH800 software plate adjustment user interface
SH800 software provides 4 corner position adjustment method for ease of use. Test sort function, only droplet will be sorted, and adjustment user interface are included.

High precision and high speed movement
The time required to sort into 384 wells is not dictated by sorting speed but by speed of plate stage movement and communication with the analysis software. SH800Z realizes high speed plate sorting with intelligent control software and high speed stage.

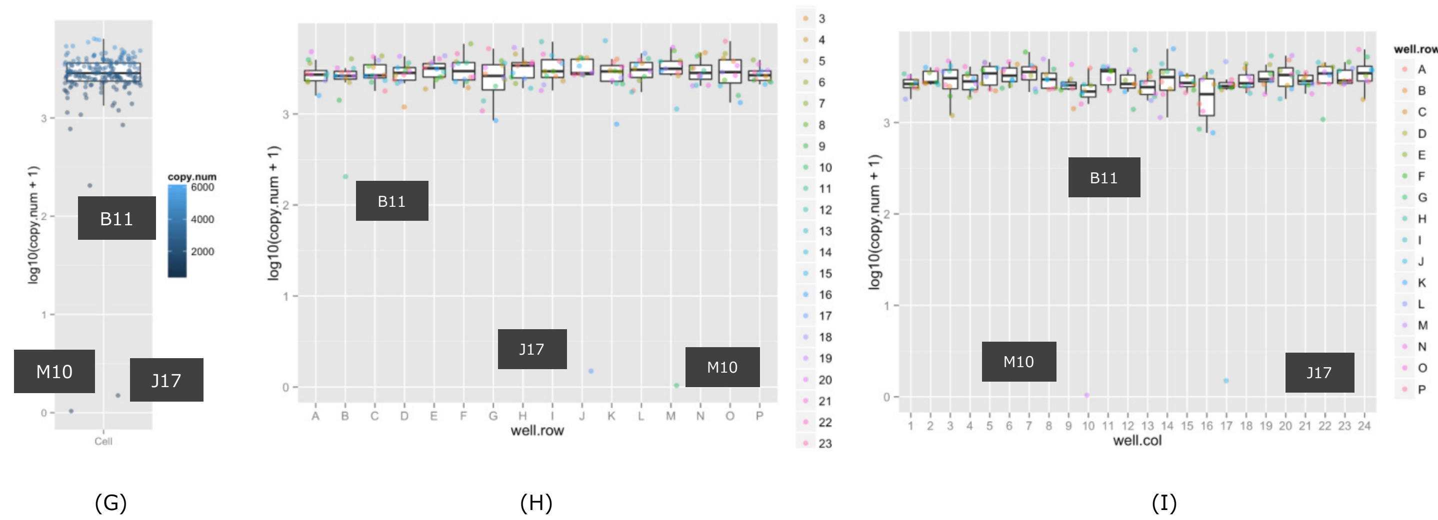

Figure 4: sample layout and qPCR results
(A)Sample layout in 384 wells, sorted single cells in 192 wells, sorted sheath drops in 96 wells, negative control(NTC) in 32 wells and standard controls in 16 wells, (B) Amplification curve of single cell wells, (C) Amplification curves of sheath wells, (D) Amplification curves of negative control wells, (E) Amplification curves of standard sample wells, standard samples were , 4 of 1copy sample, 2 of 5 copies sample, 2 of 25 copies sample, 2 of 125 copies sample, 2 of 625 copies sample and 2 of 3125 copies sample, (F) Standard curves from standard controls, (G) Calculated copy number of single cells, expression level of single cell = absolute count of qPCR X 2 (ssDNA) X 6 (dilution ratio), (H) row wise calculated copy number of single cell sample, (I) column wise calculated copy number of single cell samples.

Discussion

In this study we showed combination of SH800Z 384 well plate sorting function and amplification-free single-cell qPCR method provided high throughput single cell gene expression analysis method. Even for the small volume lysis (0.4µl) SH800Z could sort samples precisely and amplification-free single-cell qPCR method measured the absolute copy number stably. Ease-of-use of SH800Z allows wide range of users to do high throughput single cell analysis researches. This will bring them opportunity to discover new. As the next step by using combination of SH800Z 384 well plate sorting function and single cell sequencing method will provide high throughput single cell sequencing results.

Reference

1. Sasagawa et al.: Quartz-Seq: a highly reproducible and sensitive single-cell RNA sequencing method, reveals non-genetic gene-expression heterogeneity. Genome Biology 2013 14:R31.

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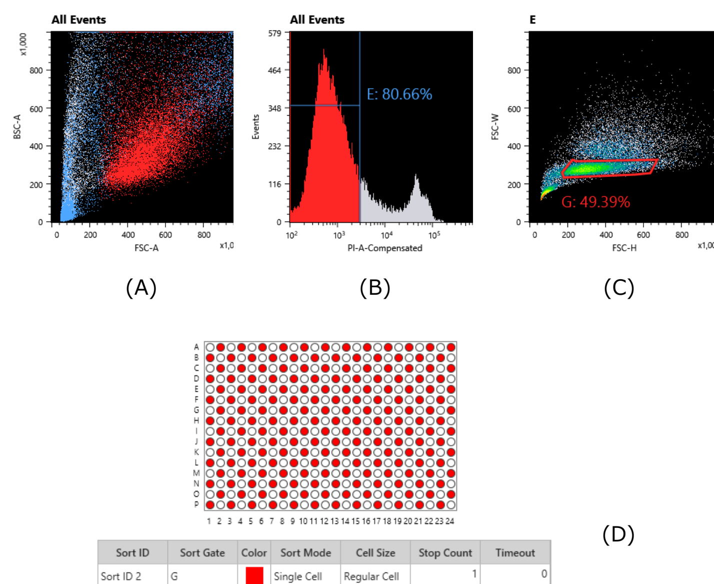
Cell staining and sorting strategy:

Cell staining

Mouse embryonic stem (ES) cells were suspended in PBS and these cells were stained with PI (1μg/ml) to differentiate between live and dead cells.

Sorting strategy

FSC was used as a trigger (5% threshold) and live cells (PI-A histogram), and singlet live cells were identified (FSC-H vs. FSC-W). The target population was identified by gate G as singlet gate. Single cells from target population were sorted into 0.4μl of RNA lysis buffer of 384 wells. To evaluate the sorting accuracy and in this study each single cell was sorted into the staggered wells (total 192wells).



Sort ID	Sort Gate	Color	Sort Mode	Cell Size	Stop Count	Timeout
Sort ID 2	G		Single Cell	Regular Cell	1	0

Figure 3: Sorting strategy

(A) FSC-A vs. BSC-A of all events, (B) PE-A histogram, gate E was gating live cells, PI negative. (C) gate G was doublet discriminator using FSC-H vs. FSC-W. (D) cells are sorted into alternative wells of 384 well plate using single cell mode. Sorting gate was gate G.

Amplification-free single-cell qPCR:

We used modified method of amplification-free single-cell qPCR which was described in previous study*1. Single cells were individually collected into 0.4 μl of lysis buffer (0.5% NP40 and 1 U/μl RNasin (Promega) plus RNase inhibitor) in 384-well PCR plate by using SH800Z. After sorting, the 384-well PCR plate was centrifuged at 2000 X g and 4 ℃ for 2 minutes, and mixed at 2,000 rpm for 15 seconds. Subsequently, 2.6 μl of RT buffer (0.6 μl of 5× VILO Reaction Buffer (contains random primers) and 0.3 μl of 10× SuperScript Enzyme Mix; both Invitrogen) was added to each well. These plates were incubated as follows: 25 ℃ for 10 minutes, 42 ℃ for 60 minutes, and 85 ℃ for 10 minutes. Then 12 μl of nuclease free water was added and mixed well. To a 384-well qPCR plate, we added 3.5 μl of the qPCR solution (1.4× QuantiTect SYBR Green PCR Master Mix (Qiagen), 5 pmol forward primer, and 5 pmol reverse primer) and 1.5 μl of 1/6 diluted cDNA from single-cell were added.

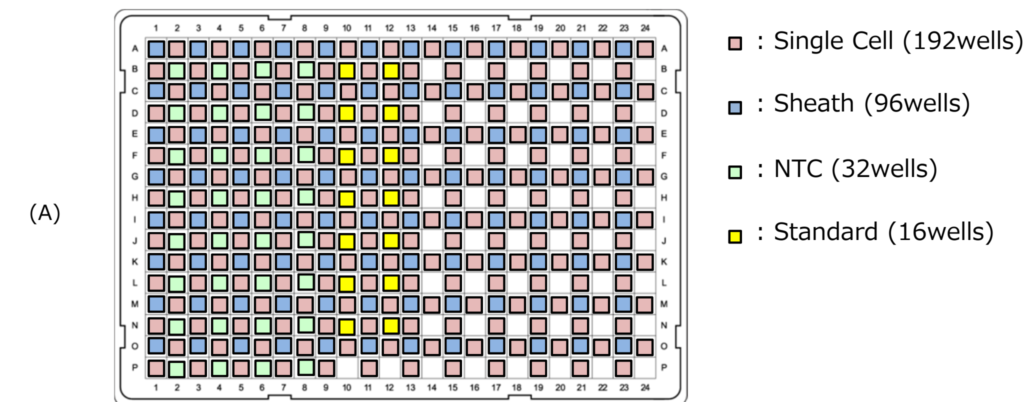
The qPCR plate was incubated at 95 ℃ for 15 minutes. Subsequently, qPCR was performed for 45 cycles, which consisted of 95 ℃ for 15 seconds, and 60 ℃ for 1 minute. The data were collected at 60 ℃. The standard curve for absolute values was constructed using a 5-fold dilution series of mouse genomic DNA as standard (3125, 625, 125, 25, 5, 1 copies) .

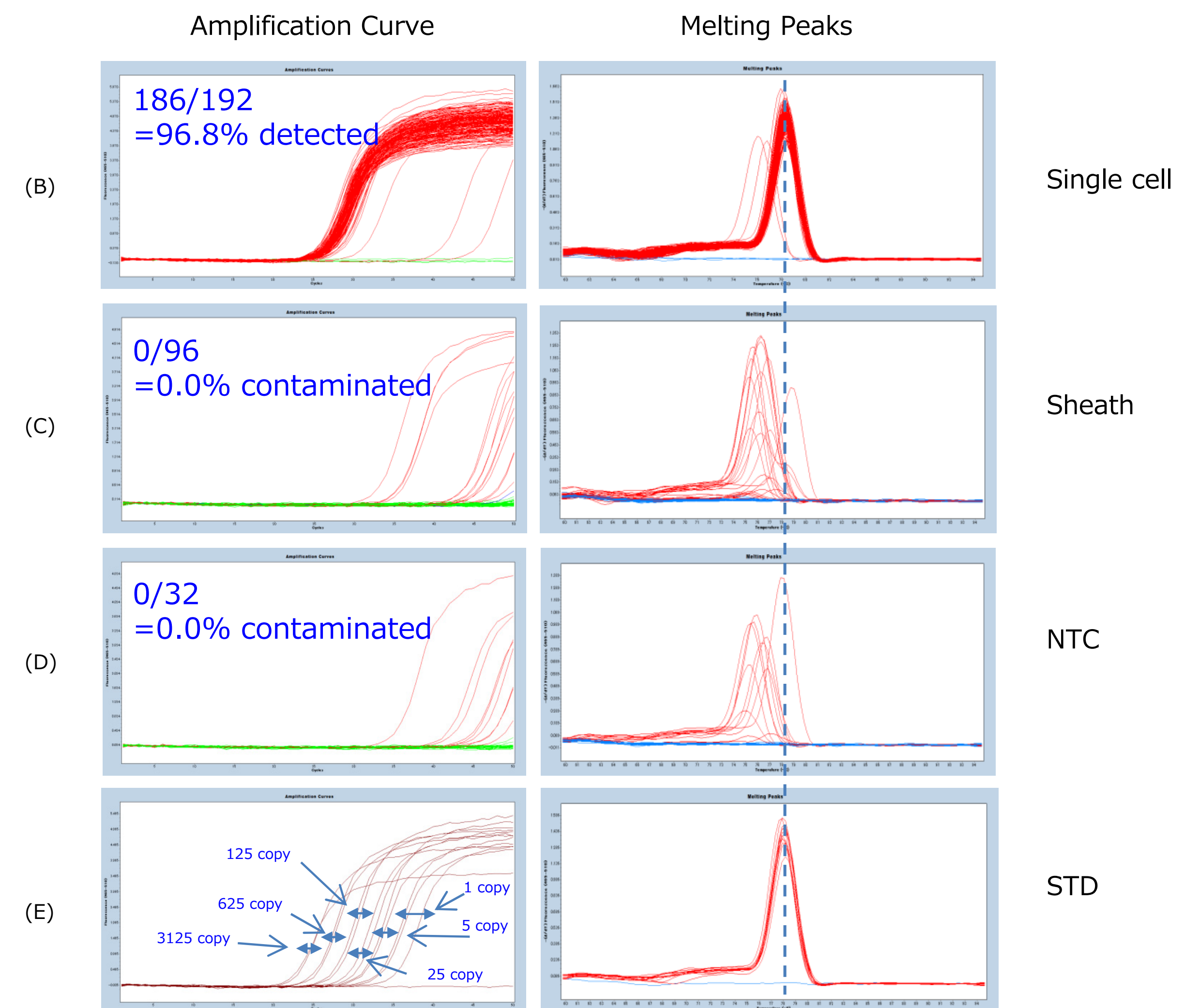
Results

Evaluation of 384 well single cell sorting performance:

To evaluate the performance of single cell sorting into 384 well plate by using SH800Z, we performed amplification –free single-cell qPCR analysis detecting *Gnb2|1* (Accession No. NM_008143), a housekeeping gene of mouse ES cells. To evaluate success rate of single cell analysis single cells were sorted into 192 wells. To evaluate contamination rate by sorting error empty sheath droplets was targeted in the empty wells interspersed between the sorted wells. The sheath drops were set by sort test function of SH800 software function. Furthermore to evaluate absolute quantitative analysis performance of amplification-free single–cell qPCR analysis 32 of non-sorted wells (only lysis buffer) were prepared for non template control (NTC) of RT-qPCR. Single copy control sample were sorted into 4 wells (B10, B12, D10, D12), 5 copies control sample were sorted into 2 wells (F10, F12), 25 copies control sample were sorted into 2 wells (H10, H12), 125 copies control sample were sorted into 2 wells (J10, J12), 625 copies control sample were sorted into wells(L10, L12) and 3,150 copies control sample were sorted into 2 wells(N10, N12) into 384 well plate.The results of amplification-free single-cell qPCR analysis demonstrated. That success rate of sorted cell was 96.8% (186/192). Error wells were M2, M10, O12, B11, J17 and H11. The amplification curves are shown (fig4-(B)). Moreover contamination rate from sorting samples was 0.0%, no gene expression was detected in sheath and NTC wells (fig4-(C), fig4-(D)). Since the sheath wells and NTC wells were located between the single cell wells, any inaccuracy in sorting position would be detectable. Such cross contamination was not detected. Absolute quantitative analysis of single cell gene expression showed good results, R^2=0.9785 efficiency=1.831 (ideally 2.0). Absolute quantification using the standard curve method indicated that the expression level of *Gnb2l1* was about 3000 copies per cell (mean = 3051.35, S.D = 995.50, CV = 0.33)(fig4-(G)). These results showed amplification–free single-cell qPCR method achieved analysis of absolute quantitative *Gnb2|1* gene expression clearly. Moreover both row-wise and column-wise analysis demonstrated that this analysis method is effective (fig4-(H) and fig4-(I)).

(A)





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