Dear Editor,

Cell-based high-throughput screening (HTS) is an important strategy for discovering a new medicine. Assays suitable for HTS should be sensitive, robust, and economical. However, the readout of conventional HTS assays is restricted to gross phenotypes, including bulk economical. However, the readout of conventional HTS assays should be sensitive, robust, and cost-effective. For HTS, single-cell RNA sequencing (scRNA-seq) has been combined with several cell-labeling strategies, including cellular hashing (e.g., sci-Plex) and CRISPR/Cas9 (e.g., Perturb-Seq). In addition, in-cell reverse transcription (RT) reactions can label cells using barcoded primers and significantly increase the throughput of scRNA-seq. Our previous works of mouse cell atlas and human cell landscape showed that Microwell-seq 1.0 is a sensitive, robust, and cost-effective scRNA-seq technology with advantages of low batch effects and high cell-type compatibility. Combining in-cell RT and Microwell-seq 1.0, we established Microwell-seq 2.0 for cost-effective and high-throughput HTS with single-cell transcriptional profiling (Fig. 1a; Supplementary Fig. S1).

We carried out a series of optimizations to considerably improve the sensitivity of Microwell-seq 2.0. We established a TaqMan qPCR-based optimization system to speed up the process and dramatically reduce the cost (Supplementary Fig. S2). The CT value was used for preliminary evaluation of different reaction conditions, and next-generation sequencing (NGS) was used for verification. In the workflow of Microwell-seq 2.0, cells were fixed and barcoded (round 1) in RT reactions using well-specific RT primers, corresponding to the given perturbations. We tested two RT temperatures (42 and 55 °C), of which 42 °C-RT had a higher cell recovery rate (Supplementary Fig. S3a, b). In 55 °C-RT, the cells were sticky and hard to collect and load. For in-cell RT, reverse transcriptase needs to be resistant to inhibitors that may carry over from fixation and complex intracellular environments. Maxima RTase showed the highest sensitivity, consistent with previous work (Supplementary Fig. S3c). One hour of incubation at 42 °C was necessary for the RT reaction. Additional 42 °C incubation and plate-rotation did not significantly increase the RT efficiency (Supplementary Fig. S3d, e). Furthermore, we found that the best sensitivity was obtained using 25 T poly-T primers (with and without -VN) (Supplementary Figs. S3f, g, S4c, d). We found that 1 M betaine did not improve the RT efficiency (Supplementary Fig. S3h). We also replaced KCl in Maxima RT buffer with NaCl, which improved RT sensitivity as previously reported (Supplementary Fig. S3i). After RT pre-indexing, all cells were pooled and loaded into the agarose plates. In Microwell-seq 1.0, an agarose plate with 105 microwells was used, which can trap only 10,000 individual cells per experiment. To load multiple cells, we increased the aperture and depth of the microwells (Supplementary Figs. S1e, S3j). Moreover, we adopted a honeycomb-like arrangement to reduce the space gap so that each plate can accommodate more microwells. An agarose plate of Microwell-seq 2.0 with 70,000 wells can contain up to 700,000 individual cells, which can meet the demand of high-throughput screening. The optimization of the
microwell plate also improved the adaptability for various cell types with different sizes. After cell loading with centrifugation, most of the wells were filled, and multiple cells occupied the same well (Supplementary Fig. S1f). Then, barcoded magnetic beads were loaded and trapped into most of the wells. After cell and bead loading, lysis...
buffer was used for cell lysis and hybridization. Formamide-based lysis buffer (2.0 lysis buffer) was more suitable for hybridization of DNA–DNA than 1.0 lysis buffer (Fig. 1b, c; Supplementary Figs. S3k, S4a, b). Hybridization with 50% formamide and 5× SSC improved the sensitivity. Neither T4 buffer nor PEG resulted in better hybridization (Supplementary Fig. S3l, m). Barcoded oligonucleotides on the beads captured and labeled cDNA (round 2) by ligation. Then, we tested three ligation systems: Ampligase, T4 ligase, and E. coli ligase (Supplementary Fig. S3n). We chose T4 Ligase, which can ligate hybridization substrates with 1–2 nt gaps. After ligation, it was necessary to digest the bead oligonucleotides that did not capture cDNA (Supplementary Figs. S3o, S4e, f). To add the PCR handle for cDNA amplification, we performed second-strand synthesis10 (Supplementary Fig. S3p). To prevent multiple displacement amplification (MDA), excess dN-TSO primer was removed before polymerization of Klenow Exo- (Supplementary Figs. S3q, S4g, h). After second-strand synthesis, barcoded cDNA was enriched by PCR and fragmented by customized Tn5 transposase with two identical insertion markers (Supplementary Fig. S5 and Table i). By harnessing the power of Microwell-seq 2.0, we analyzed massively multiplexed chemical perturbation of human embryonic stem cells (hESCs) at single-cell resolution. We selected 16 small molecules widely used to target the key pathways in stem cell biology (Supplementary Table S3). We exposed H9 cells (hESCs) to each of 48 combinations for 48 h in duplicate (Supplementary Table S4). Cells from each well were fixed separately and subjected to in-cell RT for cell labeling followed by single-cell transcriptional profiling using Microwell-seq 2.0. After sequencing and filtering, we obtained 108,782 single cells (mean UMI 536, mean gene 454, mean read 1169). We used uniform manifold approximation and projection (UMAP) to visualize these data and defined five clusters with specific markers (Supplementary Fig. S5 and Table S5). Small-molecule combinations were specifically distributed in five clusters (Supplementary Fig. S6). Next, we used partition-based graph abstraction (PAGA) to show cell transitions in chemical perturbation (Fig. 1f–h; Supplementary Figs. S7, S8). Both Repsox and SB435142 are ALK inhibitors (Repsox: ALK5, ALK4, ALK7; SB435142: ALK5, TGFβR1). Microwell-seq 2.0 sensitively identified their different perturbation effects (Fig. 1h). PD173074, PD0325901, CHIR-99021, and Retinoic acid (RA) j A gene expression heatmap shows top differentially expressed genes for small-molecule combinations in i Yellow corresponds to high-expression levels; purple and black correspond to low-expression levels.
with the expression of VIM (a general marker of mesenchymal fate), FST (a marker of myogenic differentiation), FGFBP3, and CCND1 (canonical Wnt/β-catenin transcriptional target) (Fig. 1j). With the perturbation of expression of retinoic acid, cluster W switched to cluster R with the expression of SKAP2 (retinoic acid-induced protein 70) and PRTG (a marker of neuroectodermal development). Some small molecules (such as CHIR-99021) alone can significantly affect gene expression. However, some small molecules (such as retinoic acid) need to be combined with others to produce obvious perturbations. Multiplexed Microwell-seq 2.0 enables a detailed molecular dissection of chemical perturbations during hESC differentiation with complex small-molecule combinations.

The pre-index strategy with Microwell-seq is not limited to scRNA-seq. We show that the same method can be used to enhance the throughput of single-cell ATAC-seq for HTS. Here, we also established Microwell-2.0-ATAC-seq (Supplementary Fig. S9) with a potential for multimodal HTS.

In summary, these results illustrated the high sensitivity and robustness of Microwell-seq 2.0 in cell-based screening. Our method may pave the way for a more cost-effective multi-dimensional and high-throughput drug screening assay.

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Data availability
All raw and processed datasets are available from the NCBI GEO database (GSE175413).

Conflict of interest
G.G. has submitted a patent application related to the Microwell-seq 2.0 method reported in this paper. The other authors declare no competing financial interests.

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G.G. conceived the project. H.C. and Y.L. performed Microwell-seq experiments. H.C. and X.F. performed the cell culture and chemical perturbation. L.Y. performed qPCR. X.H., Z.S., L.M. and J.L. performed scRNA-seq data analysis. G.Z. performed Microwell-2.0-ATAC-seq experiments. Y.F. and Q.G. performed ATAC data analysis. All authors analyzed the data and contributed to manuscript preparation. H.C. and G.G. wrote the manuscript; all authors contributed to the revision.

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