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RESEARCH LETTER

Large Particle Fluorescence-Activated Cell Sorting Enables High-Quality Single-Cell RNA Sequencing and Functional Analysis of Adult Cardiomyocytes

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The advent of single-cell RNA sequencing (scRNA-seq) has offered great opportunities for studying cardiac pathology at single-cell resolution. To date, however, scRNA-seq of adult cardiomyocytes remains limited owing to technical difficulties in single cardiomyocyte isolation. Adult cardiomyocytes are large (≈125×25 µm), rod-shaped cells whose structure precludes isolation by fluorescence-activated cell sorting (FACS) or commercial single-cell microfluidic platforms.[1] Here, we report on successful isolation of viable single cardiomyocyte through use of large particle FACS (LP-FACS), designed for the isolation of large objects, including worm/fly embryos and cell clusters. Our study demonstrates that LP-FACS enables rapid, high-throughput isolation of cardiomyocytes.

To generate viable cardiomyocytes, we performed Langendorff perfusion of cannulated 12-week-old mouse heart with a standard protocol. We first attempted to isolate cardiomyocytes with conventional FACS, using a commercial cell sorter with a 130 µm microfluidic channel. Live and dead cardiomyocytes were identified by DAPI staining. Despite selecting DAPI-negative cardiomyocytes, the vast majority of isolated cells were dead or had distorted structures Figure [A], suggesting that the small flow size (relative to cardiomyocyte size) leads to terminal damage of live cardiomyocytes.

We next attempted to isolate cardiomyocytes through LP-FACS, using a channel size of 500 µm (COPAS-FP-500, Union Biometrica). Notably, live, rod-shaped myocytes could be easily separated from other cells with the time-of-flight (measuring axial length) and optical extinction (measuring optical density) parameters Figure [B]. When sorted, this population was enriched with healthy-appearing myocytes with intact sarcomeres Figure [B′]. Unlike conventional FACS, where only ≈5% of DAPI-negative cells were rod-shaped, ≈85% of the LP-FACS-isolated cardiomyocytes were rod-shaped Figure [C]. Area measurement demonstrates that LP-FACS isolates cardiomyocytes across the spectrum of normal size ranges (bioRxiv: https://doi.org/10.1101/654954). Additionally, LP-FACS could separate cardiomyocyte populations based on endogenous fluorescence (bioRxiv: https://doi.org/10.1101/654954). These support the methodological superiority of LP-FACS for cardiomyocyte sorting.

To test whether isolated cardiomyocytes can be used for transcriptomic assays, we isolated RNA from bulk-sorted cardiomyocytes (500–1500 cells) and assessed RNA quality using the Advanced Analytical Fragment Analyzer system (bioRxiv: https://doi.org/10.1101/654954). Samples presented with RNA quality number of 10.0 indicating maximal RNA quality.

We subsequently performed a proof-of-concept scRNA-seq experiment with LP-FACS-isolated adult cardiomyocytes. The resulting cells selectively expressed

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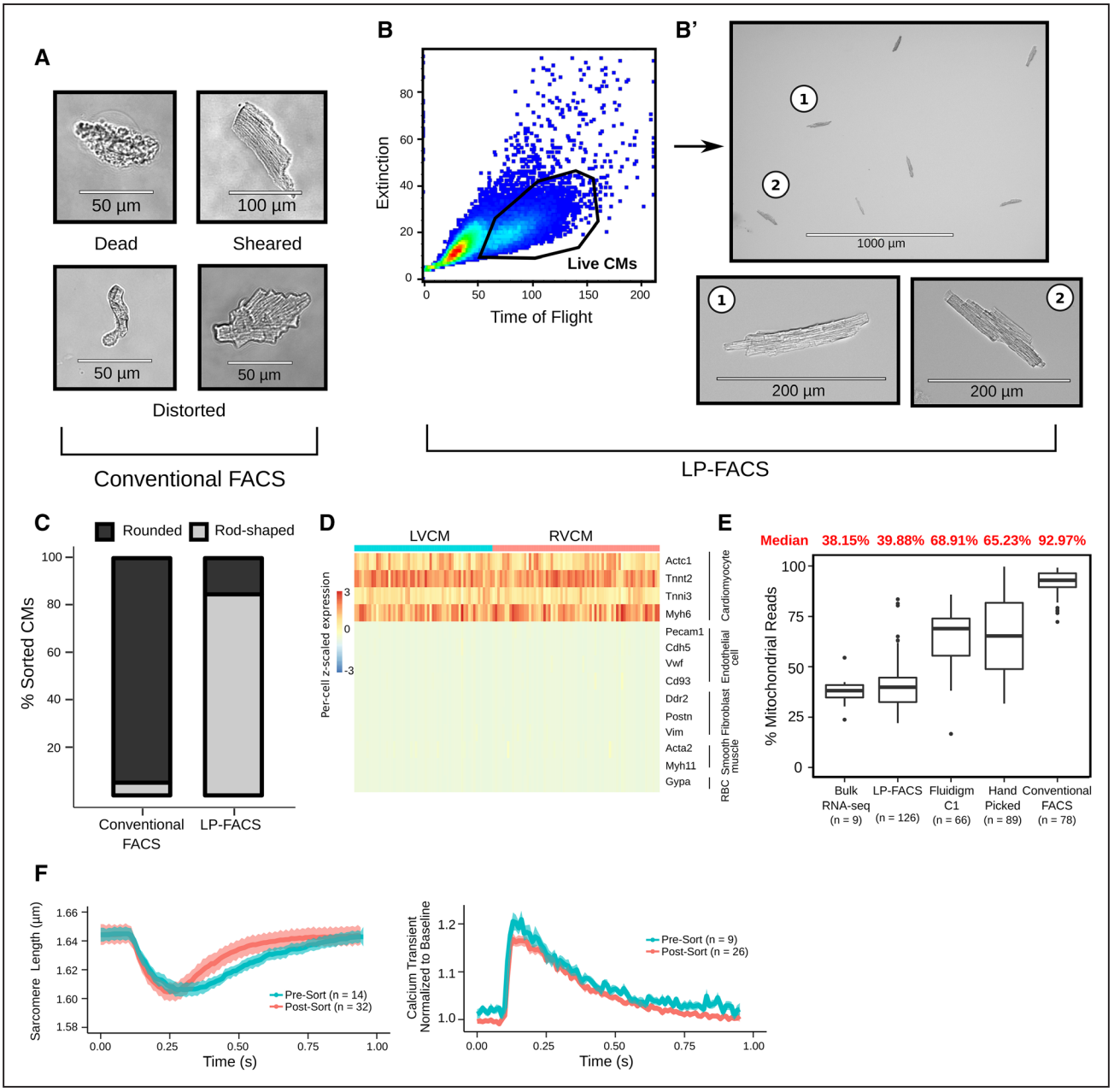

Figure. Morphological, transcriptomic, and functional analysis of single cardiomyocytes isolated by LP-FACS. **A**, Cardiomyocyte (CM) images obtained from conventional FACS, representing the vast majority of sorted cells. **B**, CM isolation through large particle FACS (LP-FACS). **C**, Percent rod-shaped CMs from conventional FACS vs LP-FACS. CMs of 200–400 were analyzed per sorting approach. **D**, Heatmap of genes representing cardiac cell types. **E**, Comparison of percentage of mitochondrial reads resulting from methods indicated. **F**, Sarcomere shortening (**left**) and calcium transient (**right**) traces for pre-and post-sorted CMs. Dark lines/shaded areas represent mean/SE, respectively. LVCM indicates left ventricular CM; and RVCM, right ventricular CM.

cardiomyocyte markers Figure [D]. We assessed the quality of our sequenced cardiomyocyte libraries by analyzing the percentage of reads originating from mitochondrial transcripts. In a damaged cell, cytoplasmic RNA leaks out of the cell, while RNAs in the mitochondria are retained. Thus, a high percentage of reads mapped to mitochondrial genes indicates likely cell damage. In LP-FACS-isolated cardiomyocytes, mitochondrial transcripts accounted for ≈40% of reads, consistent with bulk studies[2] Figure [E]. However, mitochondrial transcripts from previous single-cell studies[3–5] showed abnormally high percentages of sequenced reads. These high percentages indicate likely cellular damage/rupture, supporting the use of LP-FACS to generate improved quality cardiomyocyte scRNA-seq libraries.

Furthermore, we detected ≈3700 genes at a subsampled read depth of 200 000 reads, comparable to or superior than other cardiomyocyte scRNA-seq data sets (bioRxiv: https://doi.org/10.1101/654954). We successfully recovered rod-shaped cardiomyocytes fixed using

80% ethanol through LP-FACS and performed scRNA-seq (bioRxiv: https://doi.org/10.1101/654954). This can be useful in experiments where immediate sorting is infeasible.

We next investigated the functional properties of LP-FACS-isolated cardiomyocytes using Ionoptix (1 Hz/20 V/15ms). Post-sort cardiomyocytes maintained sarcomeric shortening and calcium transients comparable to presorted cardiomyocytes Figure [F]. In particular, several important parameters, including fractional shortening, change in calcium transient, and calcium transient decay τ among others, were equivalent pre- and post-sort (bioRxiv: https://doi.org/10.1101/654954). These support maintenance of contractile function in LP-FACS-isolated cardiomyocytes. While differences in cardiomyocyte relaxation kinetics were detected, we believe that minor technical differences contributed to this, and further optimization of sorting conditions, such as applying strict selection criteria of presorted cardiomyocytes, will eliminate the functional discrepancies.

Together, our data demonstrate the methodological superiority of LP-FACS for isolating single adult cardiomyocytes, compared with existing methods. To our knowledge, LP-FACS is the only approach that enables both generation of high-quality scRNA-seq libraries and allows for isolation of cells for functional analysis. It is important to note that successful use of LP-FACS requires a protocol for dissociating hearts into a single-cell suspension. Future improvements in dissociation methods, particularly for human tissues, will facilitate improved recovery of single cardiomyocytes via LP-FACS. We envision this technology will enable researchers to integrate transcriptomic and functional data for a range of cardiac disease models at the single-cell level.

ARTICLE INFORMATION

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Disclosures

All animal studies were performed in accordance with institutional guidelines/regulations of the ACUC at Johns Hopkins University. Supplementary data are at https://doi.org/10.1101/654954 and GEO (GSE133640). The other authors report no conflicts.

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