High-throughput single-cell TCR - pMHC dissociation rate measurements performed by an autonomous microfluidic cellular processing unit

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Abstract

We developed an integrated microfluidic cellular processing unit (mCPU) capable of autonomously isolating single cells, perform, measure, and on-the-fly analyze cell-surface dissociation rates, followed by recovery of selected cells. We performed proof-of-concept, high-throughput single-cell experiments characterizing pMHC-TCR interactions on live CD8 T cells. The mCPU platform analyzed TCR-pMHC dissociation rates with a throughput of 50 cells per hour and hundreds of cells per run, and we demonstrate that cells can be selected, enriched, and easily recovered from the device.

Microfluidics, or "lab-on-a-chip" technology has the potential to autonomously perform complex laboratory methods. In mammalian cell applications, microfluidic devices automated tasks such as long-term cell culture [1], transfection [2], cell-secretion analysis [3], chemical cytometry [4, 5], cell sorting [6], and many others. In most if not all applications to date, microfluidic devices are used to scale-down and to automate experimental processes, but data processing and analysis are generally performed off-line after experiments have been completed.

Here we describe a fully-autonomous microfluidic cellular processing unit (mCPU) that performs single-cell surface marker dissociation measurements, and runs all necessary fluid handling, microscope, imaging, image processing, and data analysis steps on-the-fly, creating a closed-loop system (Fig. 1a and Supplementary Fig. S1). Machine vision and image analysis is used to feedback and control device operation allowing the device to perform complex fluidic processing steps autonomously. We applied this platform to a proof-of-concept, high-throughput measurement determining the dissociation rates between peptide - major histocompatibility complexes (pMHC) and T cell receptors (TCR) on hundreds of cells, and demonstrate that the platform can automatically select cells based on the measured dissociation rate, and recover these cells from the device for downstream applications.

We applied our mCPU concept to single-cell immunology [7, 8]. Contemporary single-cell techniques like flow cytometry acquire single time point measurements. Although useful for a large number of applications, single-time point measurements are not able to provide kinetic information, which is critical in a number of systems including T cell [9], B cell [10], and synthetically engineered surface receptor [11] interactions. We demonstrate that the mCPU can measure the dissociation rate of the interaction between TCRs on CD8+ T cells and pMHCs. This affinity has been shown to be an important parameter and to correlate with T cell function. Current state-of-the-art methods to study single-cell surface receptor dissociation rate measurements are based on cumbersome, non-automated, and low-throughput approaches [9, 12]. There is therefore a need for novel technology to study biological mechanisms that require precise, single-cell control in time and space.

The mCPU design consists of two fluidic layers, 106 micromechanical valves, and 8 cell isolation chambers in which cells are isolated and analyzed individually (Fig. 1b and Supplementary Fig. S2). Buffer conditions can be changed in each chamber by fast flow exchange without loss of cells by incorporating small sieves and fluidic bypasses in the chamber design (Fig. 1c). Complete automation of the experimental process was achieved via a MATLAB program that controls

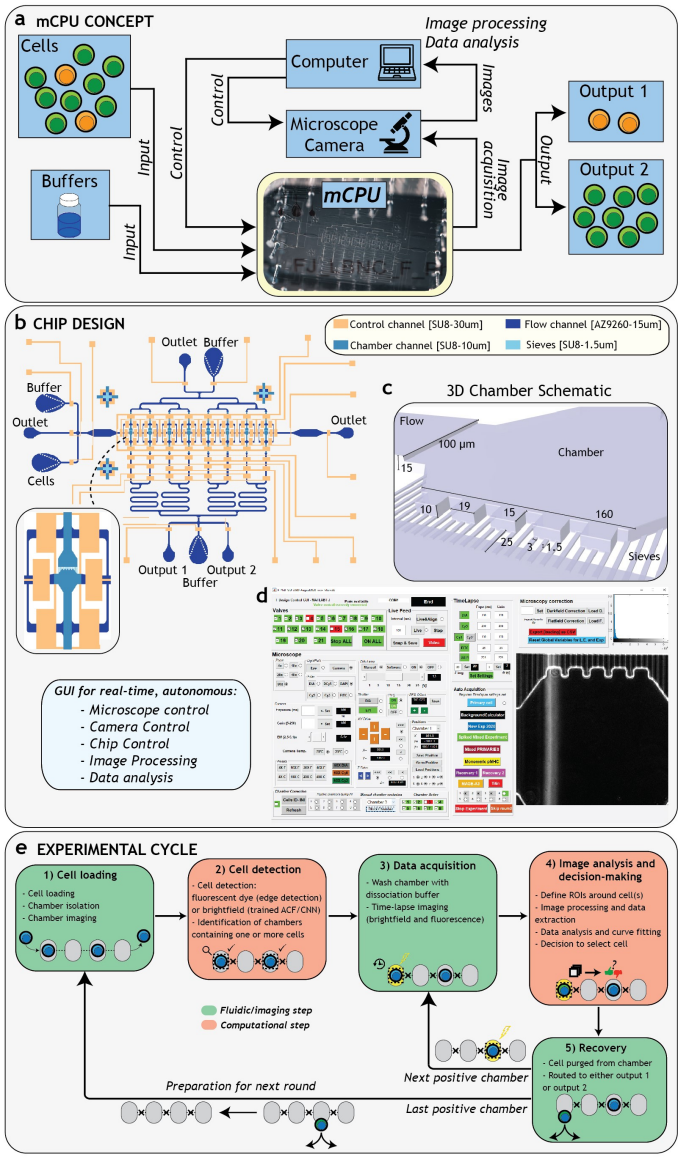

Figure 1: **mCPU concept & design**. **a** A control chart describing the mCPU platform. A microfluidic device forms the core of the mCPU. **b** The microfluidic device design showing the two-layer channel network consisting of a control (orange) and flow (blue) layer. **c** 3D chamber schematic showing chamber design details. **d** Automation is achieved through a custom MATLAB GUI controlling the chip, camera, and microscope, and performs all image processing and data analysis steps in real-time. **e** The mCPU platform performs sequential experimental rounds that are divided into five discrete steps.

the microfluidic device, camera, and microscope (Fig. 1d). To achieve autonomous single-cell experiments, the mCPU platform performs sequential experimental rounds consisting of cell loading and isolation, followed by automatic cell detection in the chambers (Fig. 1e). Buffer exchange is triggered in chambers identified to contain a cell, followed by data acquisition through time-lapse imaging. An elaborate analytical pipeline performs image processing, data extraction, and curve-fitting for real-time extraction of single-cell surface marker dissociation rates (Supplementary Figures S3 and S4). Cells above or below a particular dissociation rate threshold can be selected for recovery. The system then automatically triggers the analysis process in the next cell-containing chamber, or, if no more chambers contain a cell, triggers a new round of cell loading starting the next experimental cycle (Fig. 1e).

Here we used NTAmers which are pMHC multimers that can be rapidly dissociated by imidazole (Fig. 2a) [13]. To perform dissociation rate measurements of T-cell receptor - pMHC interactions, TCR-transduced CD8+ SUP-T1 cells were pre-stained with NTAmer-pMHC multimers followed by on-chip analysis. Cell containing chambers were identified using either brightfield images with trained convolutional neural network (CNN) and aggregate channel features (ACF) object detectors or via fluorescence using live-cell stains (Fig. 2b,c and Supplementary Fig. S5). In 6 experimental runs the mCPU achieved an average throughput of $\sim$50 cells per hour and ran stably and continuously for at least 6 hours (Fig. 2d). Acquisition of dissociation kinetics was performed by rapid ($\leq$5 seconds) buffer exchange introducing an imidazole containing buffer in the chamber and acquired images over a period of 60 to 90 seconds with a 3 second interval (Fig. 2e). Image analysis allowed for automatic determination of the switch from multimeric to monomeric forms by detecting the sudden decrease in PE signal (Fig. 2f). Each event was filtered based on multiple parameters described in the methods section (Supplementary Figures S6 and S7). Monomeric pMHC-TCR dissociation (Alexa647) was then fitted to a one-phase exponential decay model (Supplementary Fig. S8). Finally, the cell (or cells) in the chamber was directed to one of two chip outputs depending on whether it met a minimal dissociation rate threshold (Fig. 2i).

We focused on characterizing a wild-type (WT), low-affinity patient-derived TCR specific for the NY-ESO-1 tumor antigen and a higher affinity variant (DMβ) that was previously generated [14]. We bench-marked our system by measuring both the WT and DMβ variants, as they cover the physiologically relevant dissociation rates ranging from high for WT (short half-lives) to low for DMβ (long half-lives).

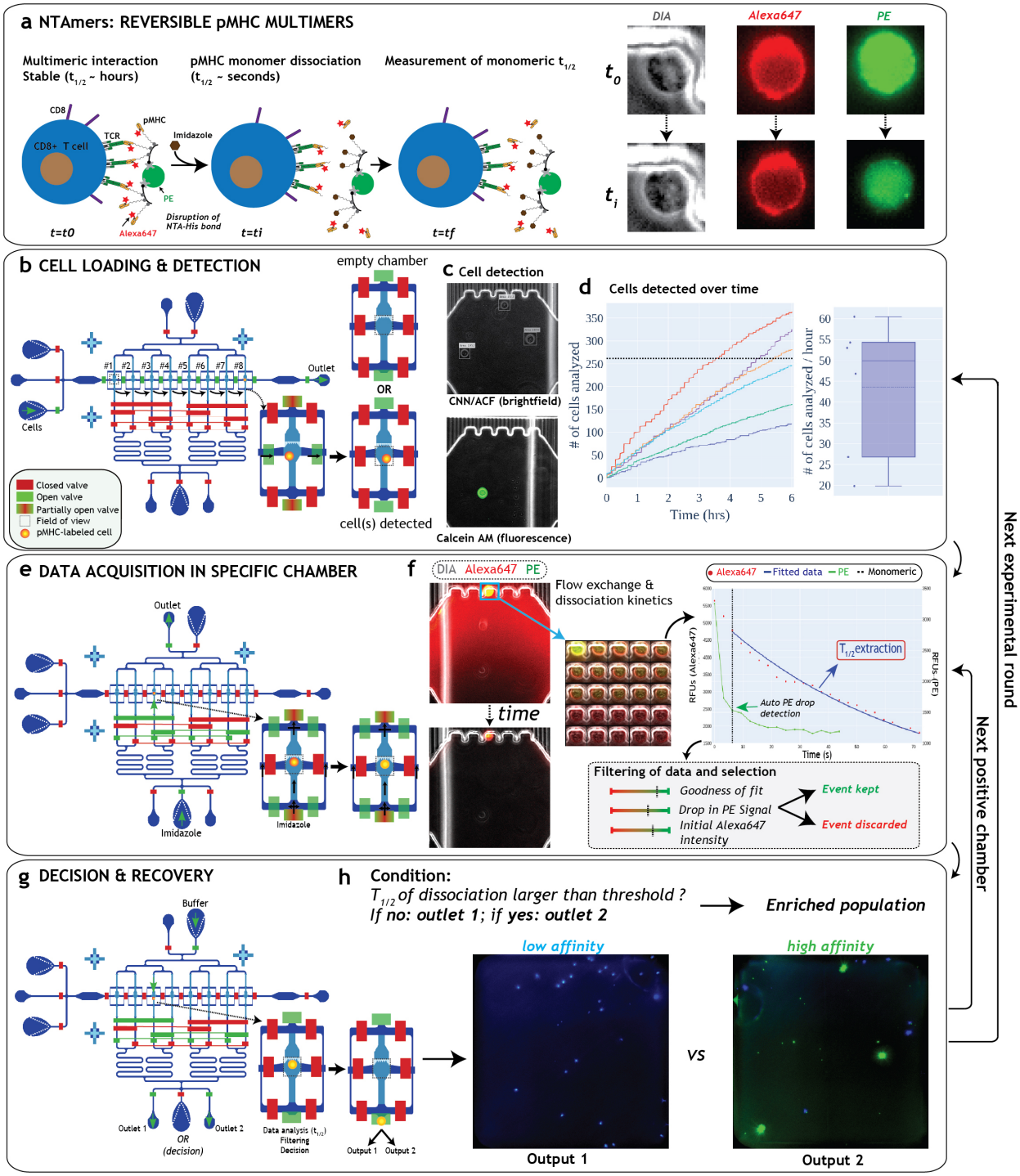

Figure 2: Caption on next page

Figure 2: **mCPU single-cell analysis**. **a** We used pMHC NTAmers [13] for dissociation measurements of cell-surface TCR-pMHC interactions. **b-i** The mCPU performs sequential microfluidic processing steps: cell-loading and detection, buffer exchange and single-cell analysis, and cell selection and recovery **b** Cells are loaded and the eight chambers are isolated and imaged to identify chambers containing one or more cells. **c** Cell detection is performed either in brightfield or fluorescence. **d** The cumulative number of cells recorded and analyzed in all chambers per round of cell loading, showing 6 separate experiments. The average analysis rate was ∼50 cells per hour. **e** Chambers containing one or more cells are then imaged via fluorescent time-lapse imaging and washed with imidazole buffer leading to NTAmer dissocation. **f** Time-lapse acquisition is performed on two fluorescent channels: PE (NTAmer dissociation), and Alexa647 (pMHC - TCR dissociation). Image processing, data analysis, and curve fitting is performed to extract TCR-pMHC dissociation half-lives. **g-h** Filtered data are then compared with user-defined thresholds for cell selection and routing to a specific outlet. The process is then repeated in the next cell containing chamber or a new round of cell-loading is triggered.

In six mCPU runs a total of 908 cells were analyzed, 447 WT and 461 DMβ expressing cells. We obtained average half-lives of 29.1s and 80.5s for WT and DMβ, respectively (Fig. 3a; Supplementary Figures S9 & S10). These half-lives are similar to those obtained by a clonal population-level FACS measurement requiring at least 200'000 cells (27.1s and 75.8s for WT and DMβ respectively, Supplementary Fig. S11) and a previous FACS analysis (17s and 89.7s for WT and DMβ, respectively [15]). The results were repeatable between experiments based on the observed half-life averages (Fig. 3b). In this series of experiments 82% of chambers contained a single cell, 14% contained 2 cells, and 4% contained 3 or more cells identified pre-filtering (Fig. 3c). These experiments demonstrate that the mCPU is capable of autonomously performing large-scale single-cell surface marker dissociation measurements, which should find uses in characterizing T cell, tumor-infiltrating lymphocyte (TIL), or B cell samples, and synthetically generated surface receptor libraries.

The mCPU integrates cell selection and recovery capabilities to extract cells of interest from the mCPU for downstream processing. To test the mCPU's capability of identifying and enriching rare cells from a mixed population we generated a mixture of WT and DMβ expressing cells. The

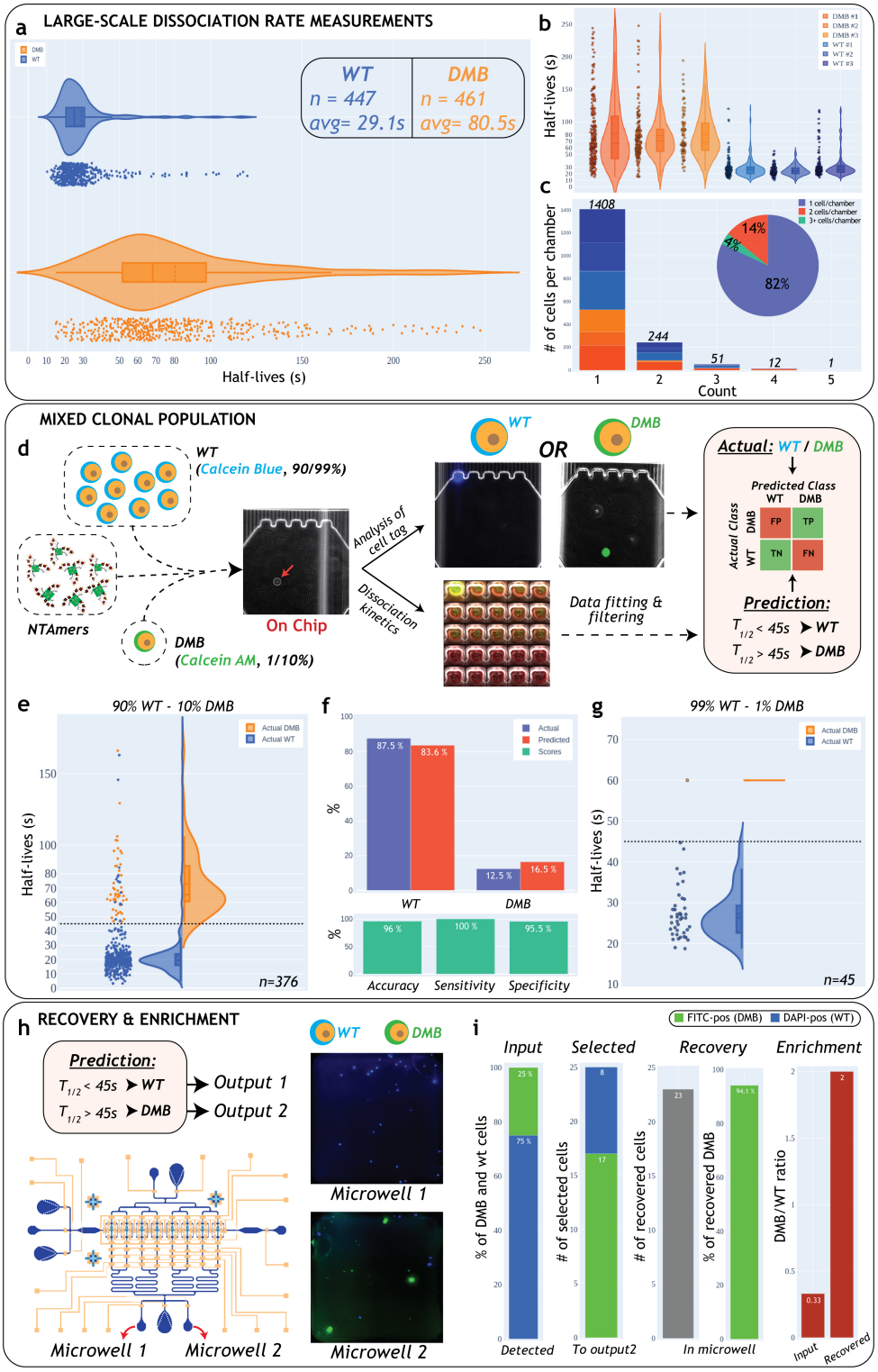

Figure 3: Caption on next page

Figure 3: **Single-cell TCR-pMHC dissociation measurements**. **a** Large-scale single-cell dissociation rate measurements for WT and DMβ expressing cells (aggregate of 3 independent experiments for each clone). **b** Half-live distributions for each experiment. **c** Distribution of cells detected per chamber. **d** Mixed population experiments. The actual WT to DMβ ratio was determined on-chip via Calcein Blue for WT and Calcein AM for DMβ, followed by dissociation rate measurements. **e** 90% WT to 10% DMβ experiment. Measured half-lives for each cell. Data point color indicates cell-type as determined by calcein staining. A half-life threshold of 45s was used to differentiate between high and low affinity cells. **f** WT to DMβ ratio as determined by calcein (actual) and as determined by using half-life measurements with a 45s half-life threshold (predicted). Accuracy, sensitivity and specificity were determined based on these ratios. **g** Experiment with higher dilution of high-affinity cells (99%WT and 1%DMβ) performed to identify a single-high affinity clone. **h** A recovery and enrichment experiment was performed by routing selected cells to a specific chip outlet. Cells were then transferred into microwells for counting. **i** Recovery and enrichment ratios were determined by comparing on-chip input with counted cells in microwells

cells were pre-stained with Calcein Blue and Calcein AM to establish the actual cell identity (Fig. 3d). The mixed population was then analyzed on the mCPU measuring the pMHC dissociation rate as well as identifying the cell-type using Calcein staining. The mCPU analyzed a total of 376 single-cells in three mCPU runs (Fig. 3e). An initial 90% WT to 10% DMβ target ratio resulted in an average observed ratio of 88% WT to 12% DMβ expressing cells as determined by Calcein dye labelling (Fig. 3f). We then sought to identify cells with apparent long half-life pMHC-TCR interactions by setting a half-life threshold of 45s near the lowest observed DMβ half-life, resulting in a sensitivity of 100%. This threshold level in turn yielded a specificity and accuracy of 96%. The mCPU correctly identified all long half-life DMβ clones, as well as a small number of WT clones that also exhibited long half-lives (Fig. 3a). With these encouraging results we further increased the input ratio between WT and DMβ clones by an order of magnitude to 99% to 1%, respectively (Fig. 3g & Supplementary Fig. S12). We let the mCPU run until it detected the first high-affinity cell based on a half-life measurements and were able to identify a DMβ clone after 45 single-cell measurements.

Enabling downstream processing of selected cells requires identification and recovery of selected

cells from the device. We performed an experiment on a mixture of WT and DMβ TCR expressing cells at a ratio of 77% to 23%, respectively (Fig. 3h). We measured half-lives and specifically routed cells with half-lives above a 45s threshold to a recovery outlet. In the outlet we observed a ratio of 33% WT to 67% DMβ expressing cells based on Calcein staining, which represents close to an order of magnitude enrichment, based on the input ratio of DMβ to WT expressing cells of .3 and a post-selection ratio of 2. Cells could be aspirated from the recovery outlet with a pipette and transferred to a multiwell plate and 92% of cells could be successfully transferred with this simple approach (25 cells selected for recovery and 23 cells successfully transferred). We recovered 94% of the selected DMβ cells. Single-cell recovery and transfer to a multiwell plate was also achieved for the single cell identified and selected in the 99% WT and 1%DMβ ratio experiment described above (Supplementary Fig. S12).

By enabling complex, time-dependent experiments the mCPU platform fully automates single-cell analysis workflows, and is expected to find applications in areas where precise temporal and spatial control over single-cells is required. For example, promising application areas are basic immunology such as characterizing T and B cells in response to natural infection and applied immunology including cancer immunotherapy where screening large-numbers of T cells and selection of high-affinity candidates is critical for therapeutic applications. In biotechnology related areas, the mCPU is capable of characterizing native as well as synthetic receptor libraries, which is useful for T cell, B cell, and de novo engineered cell receptor library characterization and screening. In addition to quantifying cell-surface receptor interactions, the mCPU should also be able to measure fast intracellular signaling events, such as calcium dependent signaling. More broadly, we demonstrate the evolution of microfluidic technology towards a truly autonomous "lab-on-a-chip" platform that performs complex fluid handling operations, data acquisition, data analysis, and decision making in a closed-loop system. Such systems could in the near future perform complex and completely unsupervised experiments.

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Competing interests

The authors declare no competing interests.

Author contributions

J.S, G.C and S.M conceived the idea to perform single-cell pMHC-TCR dissociation rate measurements. J.S provided the NTAmers, cell clones, performed FACS analysis, and contributed to the methods section. S.J.M. and F.J. invented, designed, and optimized the microfluidic device and methods. F.J. implemented the microfluidic design, wrote software, and performed experiments. F.J. and S.J.M analyzed data and wrote the manuscript.

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