Cellular Barcodes for Efficiently Profiling Single-Cell Secretory Responses by Microengraving

Supporting Information

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This document contains the following supplementary figures and information:

Supplementary Methods

Fabrication of arrays of nanowells

Arrays of nanowells comprising 50 μm cubic wells (84,672 wells/array) were prepared on 75 × 25 mm^2 glass slides (Corning, Lowell, MA) following previously reported protocols[1] with minor adaptations. To fabricate the arrays, the silicone elastomer poly(dimethylsiloxane) (PDMS) (Sylgard 184 Silicone Elastomer Kit; Dow Corning, Midland, MI) was mixed at a 10:1 ratio of base:catalyst, degassed under a vacuum at room temperature for 1 h, and then injected into a mold containing a microfabricated silicon master. The PDMS was cured at 80°C for 4 h and subsequently released from the mold to produce a glass slide-backed array of nanowells.

Shortly before use, the arrays of nanowells were treated with oxygen plasma (Plasma Cleaner PDC-001; Harrick Plasma, Ithaca, NY) for 2 min to sterilize the array and render the PDMS hydrophilic. Following plasma treatment, the arrays were stored in phosphate-buffered saline (PBS) and then washed and blocked with serum-containing media prior to depositing cells onto the array.

T helper (Th) cell biasing

Naïve $CD4^+$ T cells were isolated from fresh PBMCs by negative selection (EasySep Human Naïve $CD4^+$ T Cell Enrichment Kit; STEMCELL Technologies). Purity was routinely >95%. Cells were plated at 50,000 cells per well in 96-well U-bottom plates and activated with anti-CD3/28 Dynabeads (Life Technologies, Carlsbad, CA). Th0, Th1, and Th2 cultures were maintained in Xvivo20 (Lonza, Walkersville, MD) and activated with a 2:1 bead:cell ratio. Biasing conditions for each subset were as follows: Th0- unsupplemented; Th1- 20 ng/mL IL-2 (Peprotech, Rock Hill, NJ), 10 ng/mL IL-12 (R&D Systems, Minneapolis, MN), and 10 μg/mL anti-IL-4 (BD Biosciences, Franklin Lakes, NJ); Th2- 20 ng/mL IL-2 (Peprotech), 20 ng/mL IL-4 (R&D Systems), 10 μg/mL anti-IFN-γ (BD Biosciences), and 10 μg/mL anti-IL-12 (BD Biosciences). On day 3, cells were counted, split to a concentration of 5×10^6 cells/mL, and re-fed with fresh media. On day 5, the beads were magnetically removed, and the cells were re-fed and split. Cells were left resting for 2 days before being restimulated for another 5 days as described above. After resting for another 2 days, cells were used for functional assays.

Detection of secreted proteins by microengraving

Microengraving was performed using previously reported protocols[1,2] with minor adaptations. Poly(L-lysine)-coated glass microscope slides were coated with a capture antibody against human IgG (ZyMax; Invitrogen) and a set of 3 of the following capture antibodies, as detailed in the text: anti-IFN-γ (Mabtech, Mariemont, OH), anti-MIP-1β (R&D Systems), anti-IL-2 (R&D Systems), anti-IL-6 (BD Biosciences), anti-TNF-α (BioLegend, San Diego, CA), or anti-IL-4 (BioLegend). The capture antibodies were diluted in borate buffer (pH 9) to a concentration of 10 μg/mL for each antibody immediately prior to being applied to the glass slide.[3] Coating was performed at room temperature for 1 h or at 4°C overnight. Slides were then blocked in 1.5% bovine serum albumin (BSA; EMD Chemicals, Gibbstown, NJ) / PBS-TWEEN20 (.05%; Sigma-Aldrich) (PBST) or in non-fat milk (3% w/v in PBST) for 30 min, washed once in PBS, dipped in water, and spun or blotted to remove excess fluid.

Immediately prior to microengraving, the cell-loaded arrays of nanowells were rinsed with FBS-free media with 0.01% human serum (containing IgG) to provide a positive background

signal in every well. This uniform background signal facilitated the registration of the array during image analysis of the captured protein microarrays. Capture antibody-coated glass slides were placed face-down on top of the cell-loaded arrays, and compression was applied using a microarray hybridization chamber (Agilent, Santa Clara, CA). The clamped arrays were returned to the incubator for 1 h to allow the capture of secreted proteins onto the antibody-coated glass slide. The resulting protein microarrays of secreted products were then separated from the PDMS array, washed in PBS, blocked with 1.5% BSA-PBST or 3% milk, and hybridized (45 min, room temperature) with detection antibodies against the analytes of interest. Solutions of detection antibodies were prepared at 1 μg/mL for each antibody in 0.1% BSA-PBST. The following detection antibodies were used: anti-hIgG-AlexaFluor700, anti-IFNγ-AlexaFluor555, biotinylated anti-MIP-1β (in combination with streptavidin-AlexaFluor647 (1 μg/mL; Invitrogen) applied during an additional 30 min hybridization step), anti-IL-2-AlexaFluor594, anti-IL-6-AlexaFluor555, anti-TNF-α-AlexaFluor488, and anti-IL-4-AlexaFluor647 (all from the same manufacturers as the paired capture antibodies listed above).

The resulting microarrays of secreted proteins were imaged with 5-μm resolution using a commercial microarray scanner (GenePix 4200AL; Molecular Devices, Sunnyvale, CA). The microarrays were analyzed using commercial image processing software (GenePix Pro 6, Molecular Devices). The median fluorescence intensity (MFI) in each channel was calculated for each spot on the array to determine the relative intensity of secretion from the cells in the corresponding nanowell. Data were filtered to exclude spots with saturated pixels or high coefficients of variation (>100). Spots with a high signal-to-noise ratio (>1), low relative local background, and MFI > [MFI of local background spots + 2 standard deviations] were marked as positive spots. Background correction was performed on a per-block (7 × 7 block of nanowells) basis using a custom-written script in MATLAB (R2010b; MathWorks, Natick, MA).

Calculation of the classification accuracy of cellular barcoding

Groups of barcoded cells were loaded into separate wells of a 96-well flat-bottom plate; each well contained a collection of cells with a single, known barcode. Calcein violet (2 μM) was then added to identify viable cells. The cells in each well were imaged, and the intensities of each viable cell's barcoding dyes were determined. Images were acquired within 30 min of when the images were acquired in the microengraving assays measuring secretion. The data from all cells were then pooled to produce histograms of the distribution of fluorescence intensities for each barcoding dye. Barcode classifications for each cell were assigned based on the intensity thresholds determined from these histograms.

The classification accuracy was calculated as the percentage of cells that received a given barcode (i.e., total number of viable cells in a given well) that were correctly classified as having the given barcode. For example, consider a case in which barcode **1** was applied to a group of cells, which were then loaded into a single well of a 96-well plate. If 100 total cells were analyzed from this well, and if the imaging and analysis procedure classified 98 of these cells as being labeled with barcode **1**, then a classification accuracy of 98% would be assigned to barcode **1**.

The analysis described above was performed by imaging cells with uniformly applied barcodes in separate wells of a 96-well plate. In actual experiments, however, cells with different barcodes are mixed and imaged in nanowells. In these mixed settings, it is possible that two adjacent cells with different barcodes could be misclassified as one double-positive cell. Therefore, to quantify the accuracy of classifying double-positive cells in typical nanowell experiments, we manually

reviewed the images of randomly selected putative double-positive cells and recorded how frequently their classification as a double-positive cell was incorrect.

References

(1) Ogunniyi, A. O.; Story, C. M.; Papa, E.; Guillen, E.; Love, J. C. *Nat Protoc* **2009**, *4*, 767-782.

(2) Han, Q.; Bradshaw, E. M.; Nilsson, B.; Hafler, D. A.; Love, J. C. *Lab Chip* **2010**, *10*, 1391-1400.

(3) Ronan, J. L.; Story, C. M.; Papa, E.; Love, J. C. *J Immunol Methods* **2009**, *340*, 164-169.

Supplementary Figures

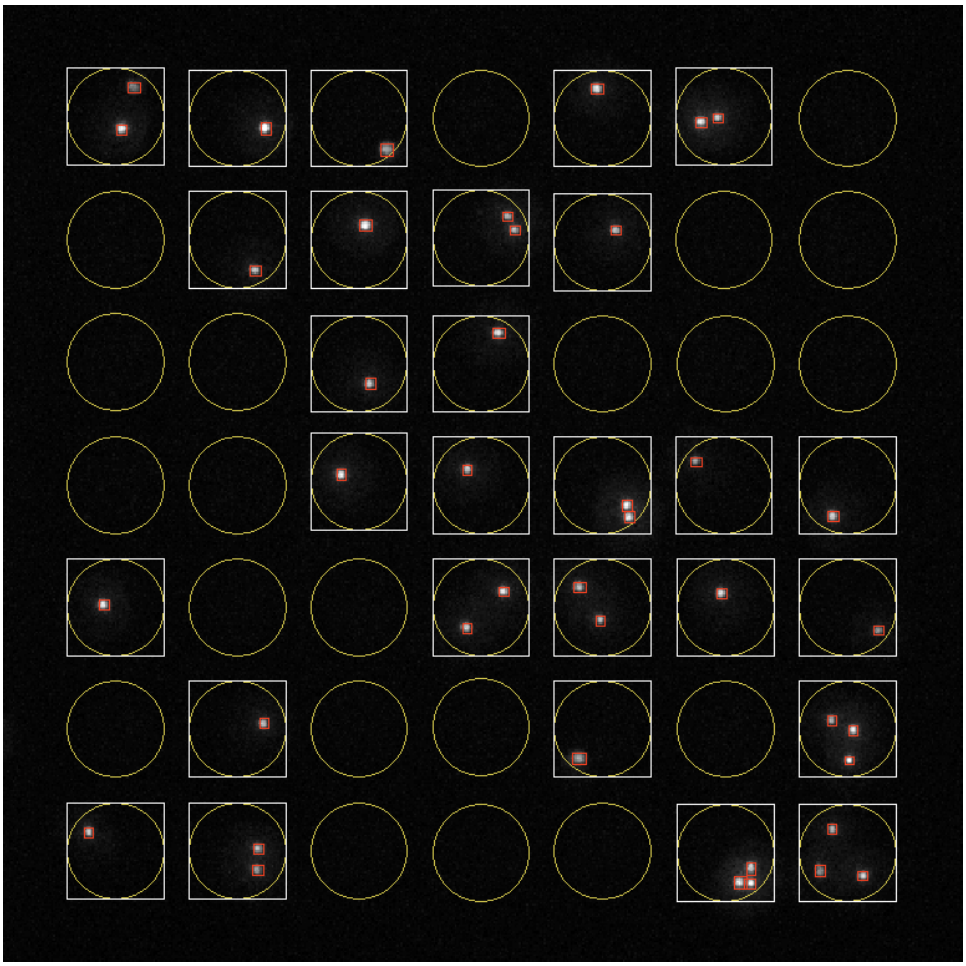

Figure S-1. Representative image of automated segmentation and counting of cells using Enumerator (written in MATLAB). The positions of the wells are determined from the transmitted light image (not shown), and the segmentation of the cells within the wells is determined from the fluorescence signal of the viability dye (calcein violet; shown in white). Red boxes mark cells that were identified by the segmentation algorithm. The barcode of each identified cell is determined from the intensities of the fluorescent cellular barcoding dyes associated with the cell (not shown).

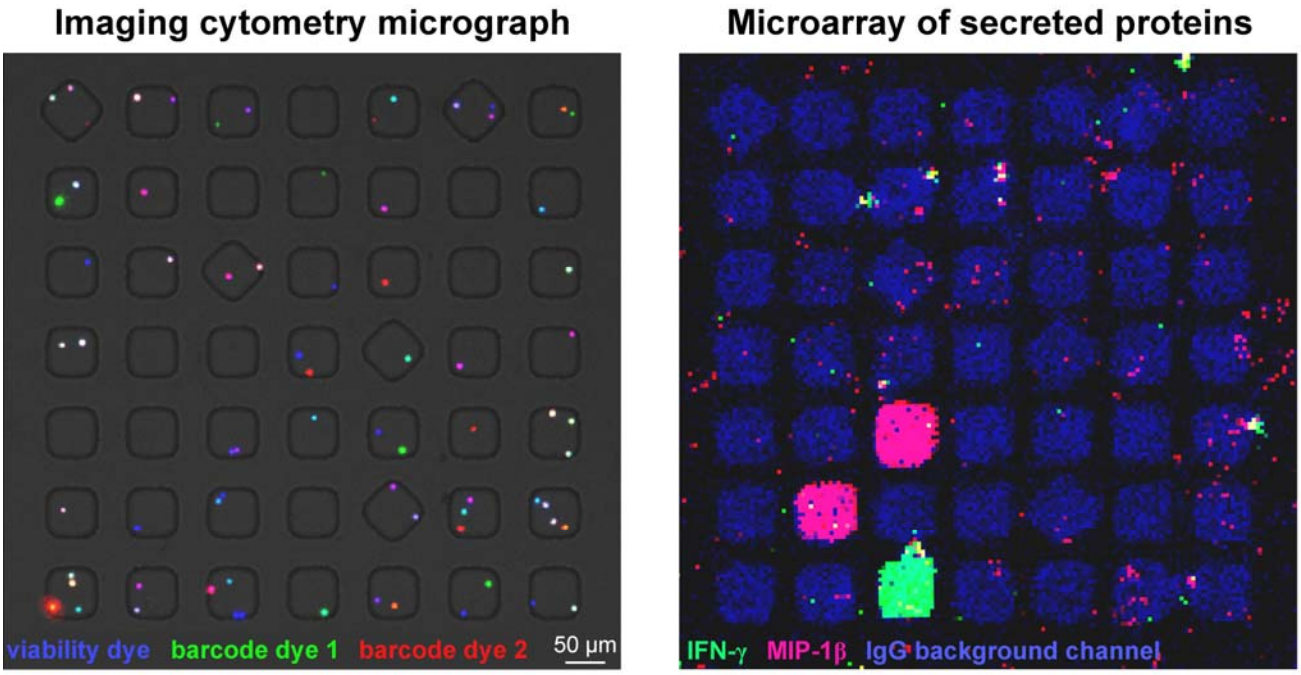

Figure S-2. Representative composite micrographs of imaging cytometry (left) and corresponding microarray of secreted proteins (right) from a 7 × 7 block of nanowells containing barcoded cells. In this example, calcein violet was used as the viability dye, carboxyfluorescein diacetate succinimidyl ester (CFSE) was used as barcode dye 1, and CellTracker Red (CTR) was used as barcode dye 2.

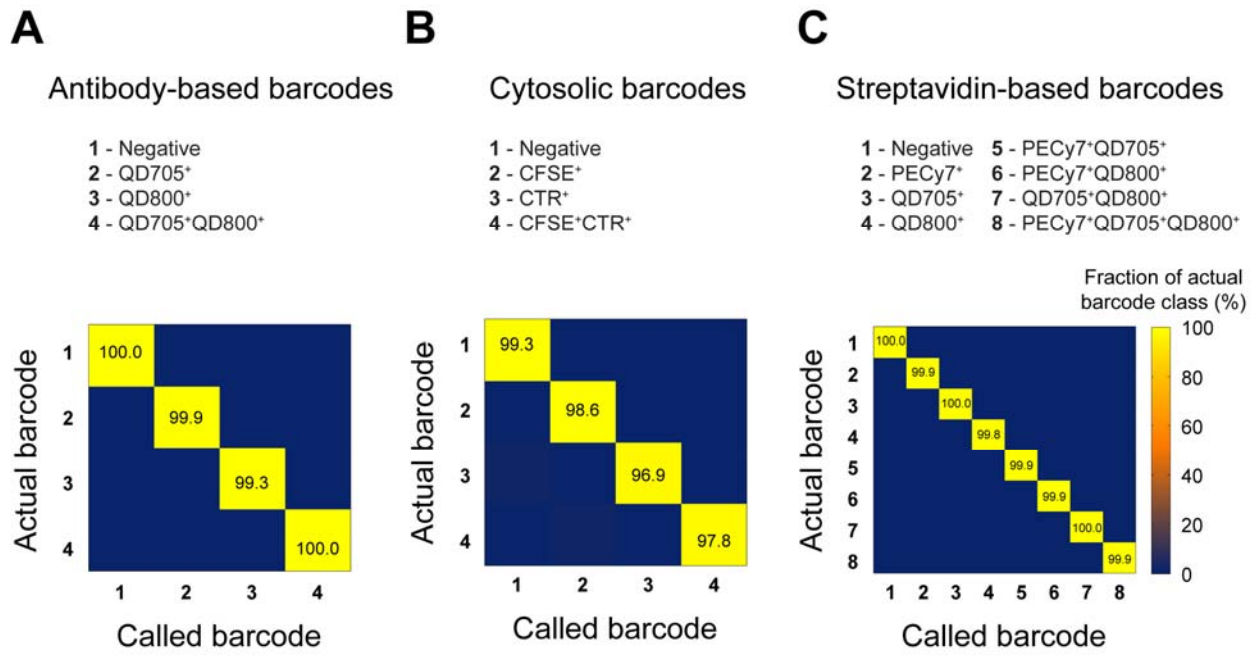

Figure S-3. Classification accuracy of cells labeled with (A) antibody-based barcodes, (B) cytosolic barcodes, or (C) streptavidin-based barcodes.

Table S-1. Accuracy of classifying double-positive cells.

Barcode set	Double-positive cells manually reviewed (#)	Correctly classified double-positive events (#)	Accuracy of classifying double-positive cells (%)
Antibody	50	49	98
Cytosolic	50	50	100
Streptavidin	50	48	96

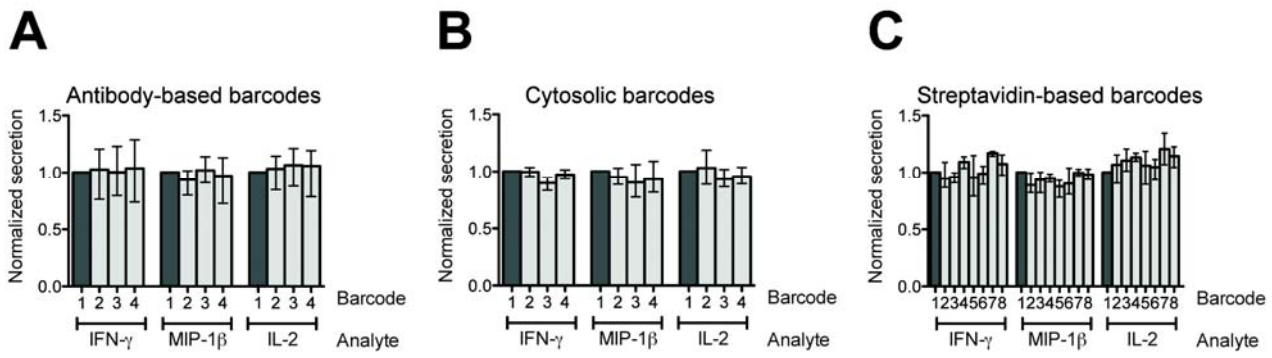

Figure S-4. Dye rotation experiments to validate that the application of barcoding dyes does not affect the short-term secretory profiles of cells. Secretory responses were compared among uniformly stimulated T cells that received different (A) antibody-based barcodes, (B) cytosolic barcodes, or (C) streptavidin-based barcodes. For each analyte, the frequency of secretion observed from cells with different barcodes was normalized to the unlabeled group of cells (Barcode 1; dark grey). The mean and range of three replicates are shown. For each analyte and barcode set, there was no significant difference ($P > 0.05$) in normalized secretion among the different barcodes (one-way analysis of variance (ANOVA)).

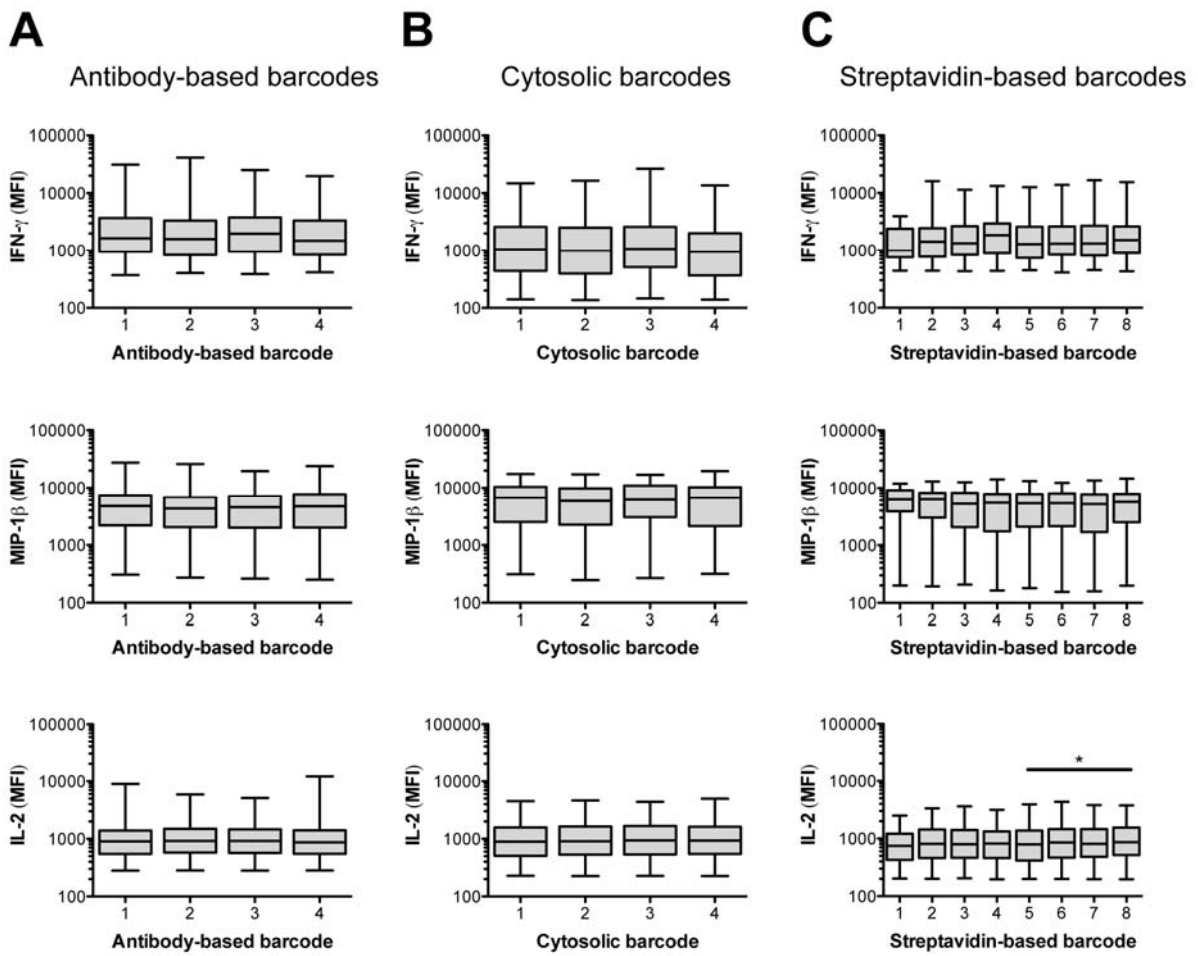

Figure S-5. Intensities of secretion from secretion-positive single cells that were exposed to a uniform stimulation (PMA/ionomycin) and labeled with (A) antibody-based barcodes, (B) cytosolic barcodes, or (C) streptavidin-based barcodes. Boxes indicate the median and the 25th and 75th percentiles, and whiskers indicate the min and max. MFI, median fluorescence intensity. *$P < 0.05$, Kruskal-Wallis test followed by Dunn's post-test. Note: The borderline-significant ($P = 0.039$) difference in the intensity of secreted IL-2 from cells labeled with the streptavidin-based barcodes was only observed in one of nine replicates of the microengraving process; in all other replicates, there was no significant difference ($P > 0.05$) among the streptavidin-based barcodes.

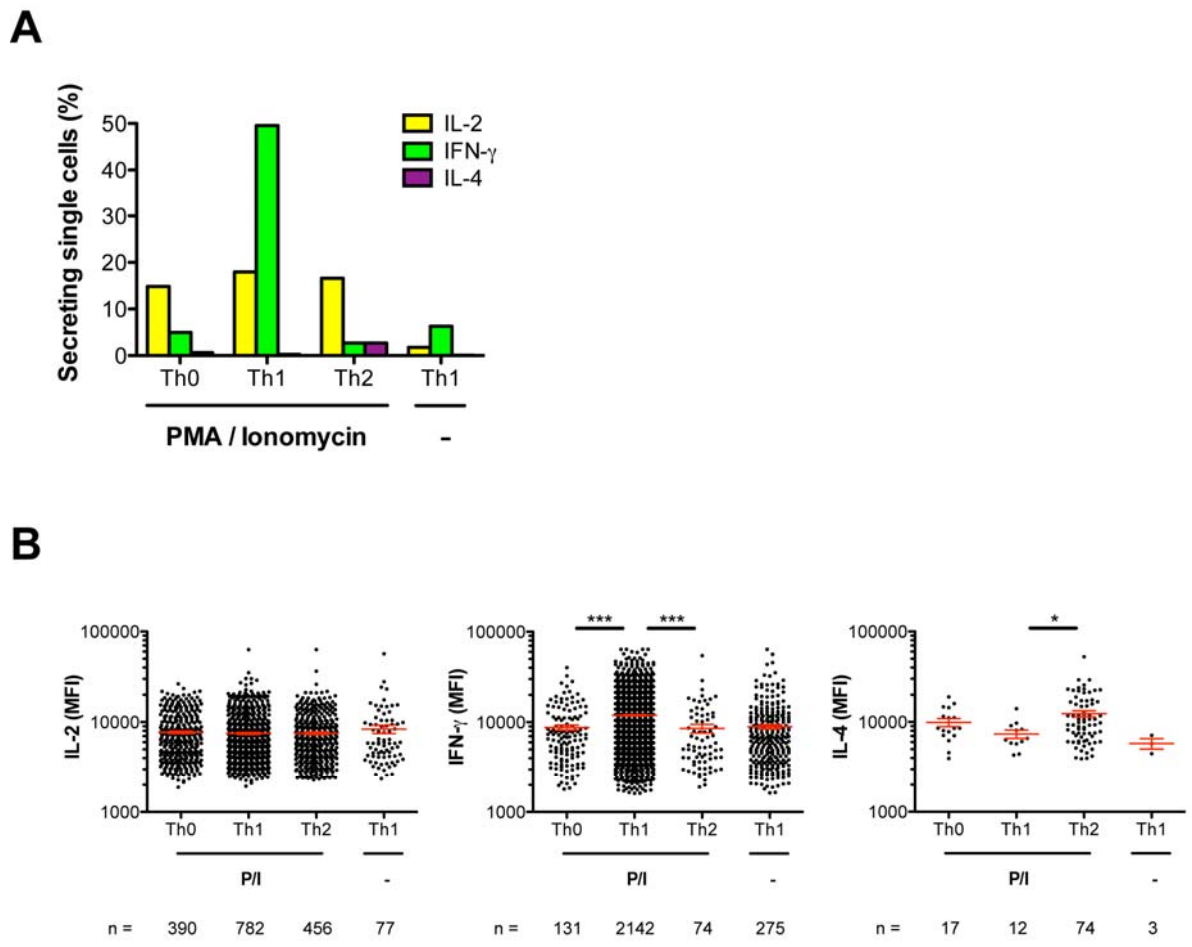

Figure S-6. Single-cell secretory responses from barcoded $CD4^+$ T helper (Th) cells biased to Th0, Th1, or Th2 and then stimulated with PMA/ionomycin (P/I) or left unstimulated (-). (A) Percentage of secreting single cells from each population of Th cells. (B) Intensities of secretion from single Th cells. Only positive secretion events are shown. Red lines indicate the mean and the standard error of the mean. $*P < 0.05$, $***P < 0.0001$, Kruskal-Wallis test followed by Dunn's post-test comparing the three groups of Th cells that were stimulated with PMA/ionomycin. The cells were labeled with cytosolic barcodes in this experiment.