

Supplemental Experimental Methods

Fabrication and design of elastomeric arrays of microwells

Silicon masters for 50 μm wells were produced by photolithography (Stanford Microfluidics Foundry, Palo Alto, CA). Each chip fits on a standard glass slide (75 x 25 mm^2 , Corning, Lowell, MA) and has an array of 72 x 24 blocks of wells. Each block of wells contains a 7 x 7 grid of 50 x 50 x 50 μm^3 microwells. For every four blocks, a channel was included to facilitate liquid removal by aspiration from the device. Approximately 5 mL of polydimethylsiloxane (PDMS; Sylgard 184 Silicone Elastomer Kit, Dow Corning, Midland, MI) was injected into a mold so that the final device has a thickness of 1 mm and is attached to a standard glass slide. PDMS was mixed at a mass ratio of 10:1 elastomer base to curing agent, deaerated for 20 min under vacuum, and cured for 2 hr at 80 °C.

Cell culture

Epstein-Barr virus transformed human hybridoma 4D20 was a generous gift from James Crowe (Vanderbilt University). Cells were cultured in RPMI 1640 (Mediatech, Manassas, VA) supplemented with 20% fetal bovine serum (FBS; PAA Laboratories, New Bedford, MA), 2 mM L-glutamine (Mediatech), and 1x Penicillin-Streptomycin (Mediatech). The cell lines were maintained in 25 mm^2 canted-neck flasks (BD Falcon, BD Biosciences, San Diego, CA) in 5% CO_2 at 37 °C and were split every three days to 2.5×10^5 cells/mL.

Cell preparation and loading

Cells were split the day before their use in experiments. For cell labeling, the cells were first washed once with phosphate buffered saline (PBS; Mediatech), then resuspended in 1 mL PBS with 1 μL of the labeling dye (CellTracker Violet BMQC, Life Technologies, Carlsbad, CA) according to the manufacturer's recommended concentration. Labeling was carried out at 37 °C for 30 min. Cells with high-viability were isolated with Ficoll-Paque Plus (GE Healthcare Biosciences, Pittsburgh, PA) and then treated with 30 $\mu\text{g}/\text{mL}$ bovine pancreatic RNase A (Sigma-Aldrich, St. Louis, MO) at 37 °C for 30 min. The cells were then washed three times with 10% FBS in RPMI and once with PBS before they were resuspended in 5 mL PBS. After these steps, more than 98% of the cells remained viable as determined by the cellular exclusion of trypan blue (Life Technologies). Each array of microwells was cleaned by a 30 s plasma treatment (Plasma Cleaner PDC-32G, Harrick Plasma, Ithaca, NY) and blocked in 0.5% BSA in PBS for 30 min at room temperature before loaded with cells by gravity such that about 50% of the wells had cells in them.

RT-PCR amplification from single cells

Primers and dual-labeled TaqMan probes for housekeeping genes were designed using the online software RealTimeDesign (Biosearch Technologies, Novato, CA). The primer and probe sequences (Biosearch Technologies) were as follows, from 5' to 3':

TCCAGCGTACTCCAAAGATTCAG (B2M forward),
GAAACCCAGACACATAGCAATTCAG (B2M reverse),
FAM-CTCACGTCATCCAGCAGAGAATGGA-BHQ1 (B2M probe),
TTGCCCTCAACGACCACTTTG (GAPDH forward),
GAGGTCCACCACCCTGTT (GAPDH reverse),
FAM-TCCTGGTATGACAACGAATTTGGCTACA-BHQ1 (GAPDH probe),
GATGCAGAAGGAGATCACTGC (ACTB forward),
GCCGATCCACACGGAGTA (ACTB reverse),
FAM-CAAGATCATTGCTCCTCCTGAGCGC-BHQ1 (ACTB probe),
GGTCCTGTGCTGGTGAAC (4D20 Heavy Chain forward),
GCTCACACCCATTCTATCATTG (4D20 Heavy Chain reverse),
Quasar670-CACAGAGACCTCACGGTGACCT-BHQ2 (4D20 Heavy Chain probe).

The reaction mix used the qScript One-Step Fast qRT-PCR kit with ROX (Quanta Biosciences, Gaithersburg, MD). It contained 1x One-Step Fast Master Mix with ROX, 1 μM of each primer, 200 nM of probe, 1x qScript One-Step Fast RT, 80 U of SUPERase-In RNase Inhibitor (Life Technologies), and 0.05% NP40 (Sigma) in a total volume of 40 μL per array. The reaction mix was applied to the microwells and spread using a pipet tip before the device was sealed onto another glass slide. Excess reaction mixture was removed along the sides and the entire device was placed on an Eppendorf Mastercycler Gradient (Eppendorf, Hamburg, Germany) with a glass slide adaptor (*in situ* Adapter, Eppendorf). Mineral oil (Sigma) was added to improve the heat conductivity between the adaptor and the device. The thermocycling profile was 40 min at 50 °C, 2 min at 95 °C, 12 cycles of 40 s at 95 °C and 1 min at 65 °C, and 38 cycles of 40 s at 95 °C and 1

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min at 60 °C, with the lid maintained at 50 °C. It was common to observe dried wells around the perimeter of the array.

Microscopy

After the thermocycle, the array of microwells was imaged on an automated epifluorescent microscope (Observer.Z1; Carl Zeiss GmbH, Jena, Germany) at 10x magnification (Objective EC “Plan-Neofluar” 10x/0.3, Carl Zeiss GmbH). A broad spectrum light source was produced by a xenon lamp in a Lambda DG-4 (Sutter Instrument, Novato, CA) and passed through a “Pinkel” quad-band filter set (Semrock, Rochester, NY) for specific excitation bandwidths. Images were captured using an EM-CCD digital camera (C9100-13, Hamamatsu Photonics, Hamamatsu, Japan). The entire system was controlled using the software AxioVision version 4.7 (Carl Zeiss GmbH).

Data analysis

Images generated by automated microscopy were analyzed using custom software. The location, the number of cells, and the fluorescence intensity of each channel were tabulated in a text file. These information were filtered and plotted using MATLAB (MathWorks, Natick, MA). The data were filtered to remove wells with more than four cells (inaccurate measures of the well intensity). Wells with large variation in the reference channel (greater than two standard deviations from the mean reference signal) were also removed to eliminate wells with no liquid and wells with a high degree of covariance in fluorescence. For each block of wells, the mean gene-specific fluorescence intensity of empty wells (I_{empty}) was calculated and used to determine the relative fluorescence of every well ($I_{\text{well}}/I_{\text{empty}}$). A histogram was plotted to bin the relative fluorescence intensities. The histogram peak for $I_{\text{well}}/I_{\text{empty}}$ of empty wells was fit to a Gaussian curve to compute estimated values for the mean and standard deviation of negative reactions. The threshold value on the relative fluorescence for positive reactions was set to be three standard deviations above the mean. From this value (e.g., $I_{\text{well}}/I_{\text{empty}} = 1.4$), the sensitivity, specificity, and positive predictive value were determined for each gene.

Combination of microengraving and RT-PCR

Detailed procedures for microengraving can be found in Ogunniyi *et al.* Nature Protocols (2009) vol. 4 (5) pp. 767-82. Briefly, cells were labeled with CellTracker Violet, loaded into the microwells, and imaged. The microwells were then sealed with a glass slide that was functionalized with anti-IgG1 antibodies at 37 °C. After 2 hr, the glass slide was separated from the microwells and captured IgG1 was detected following the application of a secondary antibody (goat-anti-human-IgG1) conjugated with Alexa Fluor 647 (Life Technologies). RT-PCR was then performed on the cells in the microwells. Data from the microscopy, microengraving, and RT-PCR were collected and filtered. Only wells that contained a single live cell initially, and had a single cell after RT-PCR (detected by non-specific staining with the reference dye, ROX) were tabulated. Spots on the microarray generated by microengraving that had a signal-to-noise ratio greater than 2 for more than 55% of its pixels and a coefficient of variation less than 80 were considered positive for IgG1 secretion.