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Supporting Information for

Janus Nanoparticles for T Cell Activation: Clustering Ligands to Enhance Stimulation

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EXPERIMENTAL SECTION

1. Materials and cells

Silica particles (0.5 μm in diameter, 5% w/v) were purchased from Cospheric (Santa Barbara, CA). Bovine serum albumin (BSA), L-ascorbate, 1, 4-dioxane were purchased from Sigma-Aldrich (St. Louis, MO). Biotinylated bovine serum albumin (BSA-biotin), N-hydroxysuccinimide ester alkyne (NHS-alkyne), and sodium azide (laboratory grade) were obtained from Thermo Fisher Scientific (Waltham, MA). Biotinylated anti-human CD3 (aCD3) OKT antibody and biotinylated anti-human CD28 (aCD28) antibody were purchased from eBioscience (San Diego, CA). Streptavidin (SA) Alexa 568 conjugate was from Invitrogen (Grand Island, NY). Sylgard 184 silicone elastomer kit was obtained from Dow Corning (Midland, MI). (3-Bromopropyl) trimethoxysilane was from Gelest Inc. (Morrisville, PA). N, N- Dimethylformamide (DMF) and copper(II) sulfate pentahydrate were obtained from Macron Fine Chemicals (Center Valley, PA) Phospholipids 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (DOPC) and 1,2-dipalmitoyl-*sn*-glycero-3-phosphoethanolamine-N-lissamine rhodamine B sulfonyl (RhB-PE) were purchased from Avanti Polar Lipid (Alabaster, AL). Tris (3-hydroxypropyltriazolylmethyl) amine (THTPA) was bought from Click chemistry Tools (Scottsdale, AZ). Potassium phosphate monobasic (KH_2PO_4) and dimethyl sulfoxide (DMSO) were from J.T. Baker (Center Valley, PA). Fluo-4 AM for calcium imaging was purchased from Life Technologies (Grand Island, NY). Jurkat T cells (clone E6-1) were a gift from Prof. Jay T. Groves (University of California, Berkeley) and originally purchased from ATCC (Manassas, VA). Jurkat T cells were cultured in RPMI 1640 complete growth media supplemented with 10 % fetal bovine serum (FBS), 1 mM sodium pyruvate, 100 units/mL penicillin, and 100 μg/mL streptomycin. Calcium-containing imaging buffer (121 mM NaCl, 6 mM $NaHCO_3$, 5.4mM KCl, 5.5 mM D-glucose, 0.8 mM $MgCl_2$, 25 mM HEPES, 1.8 mM $CaCl_2$, pH 7.35) was used for all live-cell imaging experiments. Ultrapure water (resistivity of 18.2 MΩ·cm) was used to make buffers.

2. Azide-functionalization of silica particles

Silica particles (500 nm in diameter) were pretreated with piranha solution (H_2SO_4: 30% H_2O_2 v:v = 3:1), rinsed with deionized water for several times, and then functionalized with azide following the protocol from a previous study[1] with minor modification. Briefly, 64 mg of piranha-etched silica particles were dispersed in 10 mL 1, 4-dioxane in three-neck round-bottom flask. 98.6 μL (d=1.293 g/mL) of 3-bromosilane was added to the particle solution and kept to react at 75 °C overnight under continuous stirring and the protection of Ar flux. After the reaction completed and cooled down to room temperature, bromo-functionalized silica particles were spun down, washed with ethanol three times, and redispersed in 7 mL of N, N- dimethylformamide (DMF). 0.052 g (0.8 mmol) of sodium azide dissolved in 0.5 mL of deionized water was added to the particle to react at 80 °C overnight under continuous stirring. After the reaction completed and cooled down to room temperature, particles were washed with DMF, ethanol, and then DMF, twice for each solvent. Particles were finally dispersed in DMF for storage and further usage.

3. Fabrication of bifunctional aCD3/aCD28 Janus particles

The bifunctional aCD3/aCD28 Janus particles were fabricated using a microcontact printing (μCP) method followed by click chemistry conjugation reaction. Prior to the functionalization, monolayers of azide functionalized particles were made on pre-cleaned glass microscope slides with the solvent evaporation method as described elsewhere.[2] The microcontact printing (μCP) procedure has been described in our previous study.[3] To prepare the polydimethylsiloxane (PDMS) stamps, monomers and curing agent from the Sylgard 184 silicon elastomer kit were mixed at 10:1 (w/w) ratio in petri dish. The resulting mixture was degassed and cured at 70 °C in a vacuum oven for 12 hours. PDMS stamps were pretreated with piranha solution (H_2SO_4: 30% H_2O_2 v: v = 1:1) for 3 min to increase the surface hydrophilicity. The surface

hydrophilicity is critical for the spreading of protein solutions on the PDMS stamp and the subsequent transfer of the proteins to particles. The etched stamp was rinsed with deionized water and incubated with 30 μg/ml BSA-biotin in 1× phosphate buffered saline (PBS) for 20 min. The stamp was then washed with 1×PBS and deionized water and dried quickly under a stream of filtered air. Immediately after drying, the PDMS stamp "inked" with BSA-biotin was pressed against the particle monolayer at a pressure of 1.5×10^4 Pa for 2 minutes. The stamp was peeled off after the printing. Particles were partially embedded in the stamp. Copper-catalyzed click chemistry was used to conjugate aCD28 antibody to the exposed azide-functionalized surface of particles on the PDMS stamp. 100 μL of click chemistry solution (0.1 M KH_2PO_4: DMSO v: v = 95:5) was added on each PDMS stamp, followed by the addition of 1 μL of alkyne functionalized anti-CD28 antibody (2 μM in PBS), a mixed solution of 1.25 μL of copper sulfate (20 mM in water) and 2.5 μL THTPA (50 mM in water), and 10 μL of L-ascorbate (100 mM in water) sequentially. The mixed solution remained as a large droplet on the PDMS stamp. The solution was gently pipetted up and down three time to make sure all reagents are well mixed. The L-ascorbate solution was prepared fresh for each reaction. The click chemistry reaction proceeded for an hour at room temperature. Particles were harvested by briefly sonicating PDMS stamp in 10 μM BSA in 1× PBS solution and kept in the solution overnight at 4 °C for further passivation. For aCD3 conjugation, particles were rinsed with 1× PBS solution and incubated sequentially with 100 nM streptavidin for 1.5 hours and 10 nM biotinylated anti-CD3 antibody for 1.5 hours.

4. Fabrication of uniformly coated aCD3/aCD28 particles

For amino functionalization, piranha-etched silica particles were incubated in anhydrous ethanol containing 3 wt% (w/v) (3-Aminopropyl) triethoxysilane (APTES) for 30 minutes, washed with ethanol three times, and then annealed in the oven at 120 °C for 2 hours. For biotinylation, amine-functionalized particles were mixed with 2 mM biotin N-hydroxysuccinimide ester (biotin-NHS) in sodium bicarbonate buffer (pH = 8.2) and the reaction was kept at room temperature for 1 hour. To functionalize particles with antibodies, 10 μM BSA was reacted with particles for 1 hour, 100 nM streptavidin Alexa Fluor-568 conjugate for 1.5 hours, sequentially. Finally, mixture of biotinylated anti-CD3 antibody, biotinylated anti-CD28 antibody and biotinylated BSA was added for functionalizing particles for 1.5 hours. The molar ratio of BSA-biotin to antibody-biotin in the mixture was varied to obtain different surface density of aCD3 and aCD28 on the surface of particles. For uniformly coated particles with same number of anti-CD3 (U#), the ratio was anti-CD3: anti-CD28: BSA = 0.0625: 0.4375: 0.5. For uniformly coated particles with matched anti-CD3 density (Ud), the ratio was anti-CD3: anti-CD28: BSA = 0.29: 0.32: 0.39. Total concentration of the mixture was retained same at 4.5 nM. For Uniformly coated particles only with anti-CD3 on the surface, the ratio of antibodies and BSA was following. For uniformly coated particles with same number of anti-CD3 (U#), the ratio was anti-CD3: BSA = 0.15: 0.85. For uniformly coated particles with matched anti-CD3 density (Ud), the ratio was anti-CD3: BSA = 0.5: 0.5.

5. Quantification of ligand patch size on Janus particles

For dual-color imaging of the bifunctional aCD3/aCD28 Janus particles, aCD28 conjugated with Alexa Fluor-568 was used and aCD3 was labeled with secondary antibody conjugated with Alexa Fluor-488. Particles were imaged using an OMX 3D-Structured Illumination Microscopy (3D-SIM) Super-Resolution system (Light Microscope Imaging Center, Indiana University). 1.4 NA Olympus 100 × oil objective was used with immersion oil (refractive index = 1.516). Images were taken along the z-position by 0.125 μm increment and stacks of images were deconvoluted using SoftWoRx (Applied Precision) software. In SIM images, each aCD3 patch appears as an arc. Arc angle of the patch, θ, was estimated using the following equation:

$$\theta = 2arcsin(a/2r) \quad \text{..... (1)}$$

In equation (1), a is the arc chord of each anti-CD3 patch, which was directly measured from SIM images, and r is the particle radius. After obtaining the arc angle θ, surface area of each aCD3 patch was calculated using the following equation:

$$S_{patch} = 2\pi r^2(1 - \cos(\theta/2)) \quad \text{..... (2)}$$

6. Quantification of surface density of antibodies

The surface densities of aCD3 and aCD28 were measured using a quantitative fluorescence imaging method as described in our previous work.[3, 4] The first step was to obtain a calibration curve that relates the fluorescence intensity of each particle to the total number of fluorescent dyes per particle. The calibration sample was lipid bilayer-coated silica particles (500 nm). The advantage of using lipid bilayer is that the surface density of the fluorescent dyes on particles can be precisely controlled by the molar fraction of fluorescent lipids in the lipid mixture. 100 nm Lipid vesicles composed of unlabeled DOPC (1, 2-Dioleoyl-sn-glycero-3-phosphocholine) and Rhodamine B-labeled PE (RhB-PE) were made using a vesicle extrusion method (a schematically illustrated protocol is available on AvantiLipids website). To prepare lipid bilayer coated particles, lipid vesicles in Phosphate buffer (10 mM) were mixed with piranha-etched silica particles for 2 hours at room temperature with brief vortexing once every 15 minutes. Surface density of RhB-PE on particles was calculated by considering the molar fraction of RhB-PE and that each DOPC lipid occupies an average surface area of ≈ 0.70 nm^2/lipid and a vesicle membrane contains two monolayers of lipids.[5] From the calculation, surface density of RhB-PE in the lipid vesicle calibration samples were 25, 40, 100, 200, 300, 600 RhB-PE/μm^2. The molar fraction of RhB-PE was varied in a series of lipid vesicle samples to achieve different surface density: 8.8×10^{-6} (25 RhB-PE/μm^2), 1.4×10^{-5} (40 RhB-PE/μm^2), 3.5×10^{-5} (100 RhB-PE/μm^2), 7.0×10^{-5} (200 RhB-PE/μm^2), 1.1×10^{-4} (300 RhB-PE/μm^2), 2.1×10^{-4} (600 RhB-PE/μm^2). The lipid bilayer-coated particles were imaged using a Nikon A1R-A1 microscope in epi-fluorescence imaging mode (Light Microscope Imaging Center, Indiana University). Imaging acquisition settings were kept the same for all calibration samples to enable comparison of fluorescence intensity between samples. The average fluorescence intensity per pixel was measured in Image J for at least 100 particles for each calibration sample, and plotted as a function of the surface density of RhB-PE. The data were fit with a linear regression in Origin (**Figure S2**).

The second step was to measure fluorescence intensity per pixel of antibody-coated particles. Both Janus particles and uniformly coated particles were functionalized with either aCD3 or aCD28 conjugated to Alexa-568. Particles were imaged with a Nikon A1R-A1 microscope in epi-fluorescence imaging mode (Light Microscope Imaging Center, Indiana University) using the same acquisition setting as calibration particles. Average fluorescence intensity per pixel for each particle sample was measured in the same procedure as described above.

The last step was to obtain a scaling factor to take into account the spectral difference between RhB and Alexa 568.[3] Aqueous solutions of RhB and Alexa Fluor-568 at the same concentration were imaged using fluorescence microscopy under the same acquisition setting. Scaling factor (F) was obtained as the ratio of the fluorescence intensity of the two dyes:

$$F = I_{RhB}/I_{Alexa568} \quad \text{..... (3)}$$

The scaling factor, ratio of fluorescence intensity per pixel, was determined to be 1.007. The dye labeling efficiency of aCD3 was also determined to be 2.2 ($E_{Ab} = 2.2$) using UV-Vis measurements with a NanoDrop Spectrophotometer. By taking into account all parameters and the linear equation from the

calibration curve ($y = 2.42x + 381$), we obtained the surface density of aCD3 or aCD28 using the following equation:

$$Surface\ density\ of\ aCD3\ or\ aCD28 = 2.42 \times (F \times I_{Alexa568})/(S_{patch} \times E_{Ab}) + 381 \quad \ldots (4)$$

In this equation, S_{patch} means surface area of protein patch, $I_{Alexa568}$ is average fluorescence intensity of per pixel, F is the scaling factor, and E_{ab} is the labeling efficiency of antibodies.

7. Characterization of T cell activation

Jurkat T cells were serum starved in serum-free cell media at 37 °C for 2 hours. To load cells with the intracellular calcium indicator Fluo-4 AM, 1 million cells were incubated with 5 μg/mL Fluo-4 in serum-free cell media at 37 °C for 30 minutes, rinsed, and then incubated in serum-containing cell media at 37 °C for an additional 30 minutes to allow complete de-esterification of Fluo-4. The Fluo-4 loaded T cells were re-suspended in imaging buffer and added to an imaging chamber at 37 °C before the addition of Janus particles. Time-lapse dual-color epi-fluorescence images were acquired on a Nikon Eclipse Ti microscope system equipped with an Andor iXon3 EMCCD Camera. Either a Nikon Plan Apo 40×/0.95 N.A objective or a Nikon Plan Apo 100×/1.49 N.A TIRF objective was used for the imaging. Images were acquired with 100-millisecond exposure time, 5-second interval time and a total duration of 4200 seconds. To enable direct comparison between samples, imaging parameters, such as laser intensity and settings of EMCCD, were kept constant for all experiments. Fluorescence intensity of single T cells was analyzed using a custom Matlab script as previously described.[6] Briefly, the MatLab image processing script detects the outline of individual cells and calculates the integrated fluorescence intensity for each cell. Cells not in contact with any particle were not included in the data analysis. Since cells may exhibit different levels of fluorescence intensity due to the heterogeneous amount of Fluo-4 loaded into cell cytosol, fluorescence intensity of single cells was normalized based on the basal intensity to enable direct comparison between cells. Basal fluorescence intensity was determined by the median of fluorescence intensity from first 20 frames of images.

To quantify T cell activation, a few parameters, including threshold of T cell activation, response fraction, and average fluorescence amplitude, were defined by following a previously reported analysis method.[7]

(i) Threshold of activation was first determined to distinguish actual calcium signals from background, because oscillation of calcium concentration in cytoplasm of T cells in the absence of stimulus is not negligible and heterogeneous among different cell lines.[7, 8] We defined probability of detection (PD) to indicate the percentage of activated T cells in the presence of stimuli, and probability of false alarm (PFA) to describe the percentage of cells showing false activation signals in the absence of stimuli. To choose a proper threshold that minimizes the false detection (PFA) while retaining the true activation signal (PD), the Receiver Operating Characteristic (ROC) curve analysis was used. In this ROC analysis, PD and PFA were computed at six different thresholds with 0.5 increment of normalized fluorescence intensity (a.u.) and the threshold that gave the maximum value of $PD \times (1-PFA)$ was chosen. To determine PD, calcium influx of T cells was tracked after being activated by uniformly coated particles with anti-CD3. Calcium influx of T cells without particle-stimulus determined PFA. The threshold was determined to be 1.5 (normalized fluorescence intensity) in our experiments (**Figure S3**).

(ii) Response fraction and average fluorescence amplitude were used to quantify the duration and strength, respectively, of calcium signals. The response fraction for each cell was defined as the fraction of time when the Fluo-4 intensity of a T cell is above the activation threshold. The average fluorescence amplitude was defined as the mean of normalized fluorescence intensity during the entire imaging duration. Both

response fraction and average fluorescence amplitude were analyzed for single cells and results from at least 50 cells for each sample were included in all distribution plots shown in figures. We considered samples with different cell activation efficiency only when both response fraction and average fluorescence amplitude were significant different with $p < 0.05$ (student's t test).

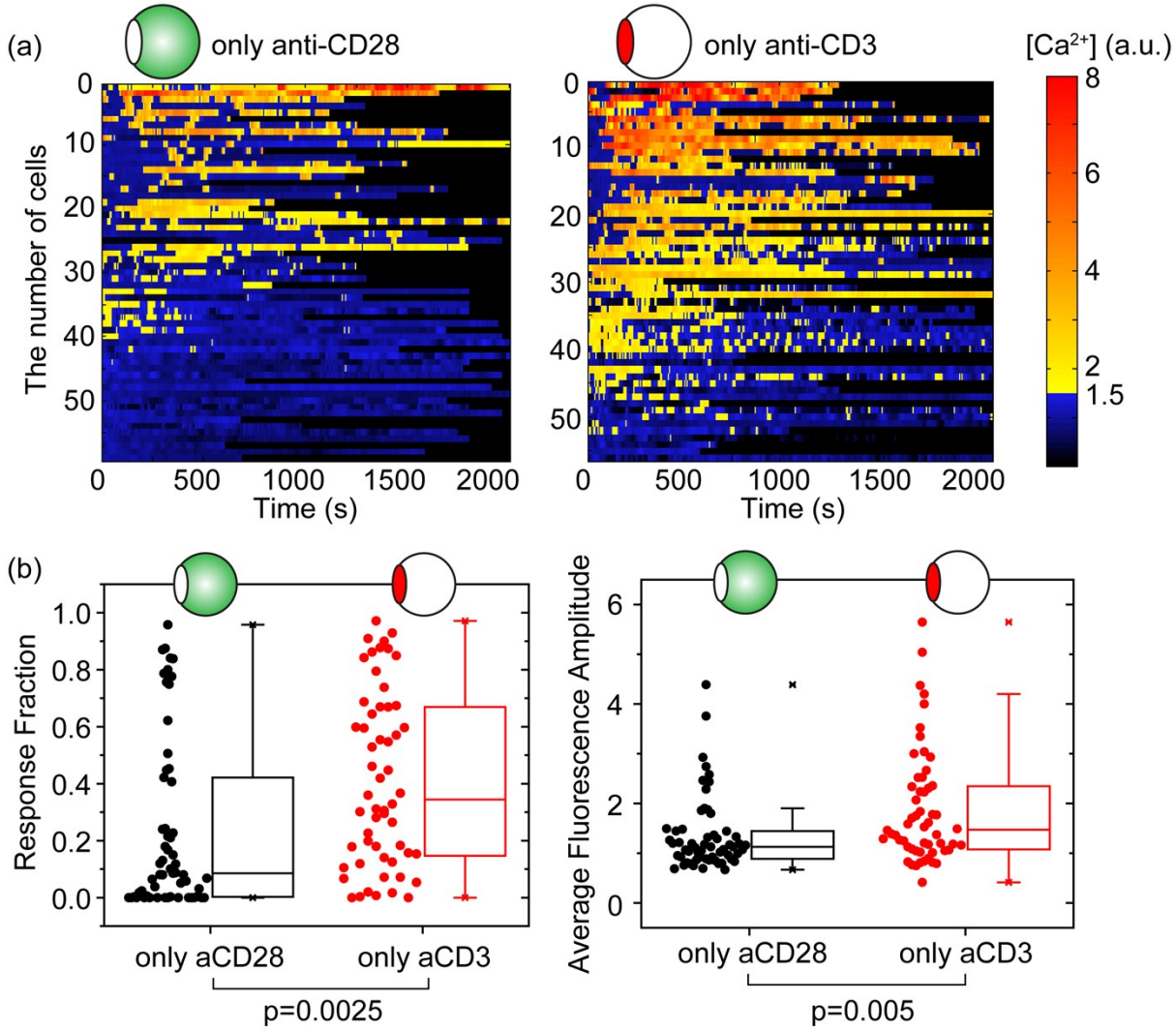


Figure S1. (a) Color-coded plots show calcium response of T cells activated by Janus particles that were functionalized with only anti-CD3 (shown in red) or anti-CD28 (shown in green). Each horizontal line indicates the calcium response of a single T cell as a function of time. The calcium response, measured as the fluorescence intensity of the calcium indicator Fluo-4, was normalized and color-coded on the same scale. (b) Quantification of T cell activation. Response fraction, defined as the fraction of time when the normalized fluorescence intensity of a T cell is greater than the threshold, quantifies the persistence of a calcium response. Average fluorescence amplitude, defined as the time-average of normalized fluorescence intensities on the entire calcium plot for each T cell, measures the average intensity of a calcium response. Both parameters extracted from results shown in (a) are plotted in a scatter plot together with a box plot. Each box plot indicates the median and the interquartile range from 25% to 75% of the corresponding data set. Statistical significance is highlighted by p-values (student's t test) as indicated.

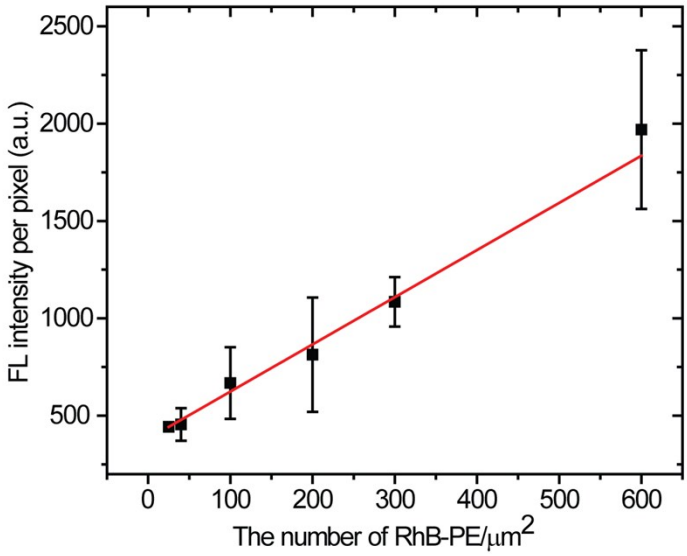
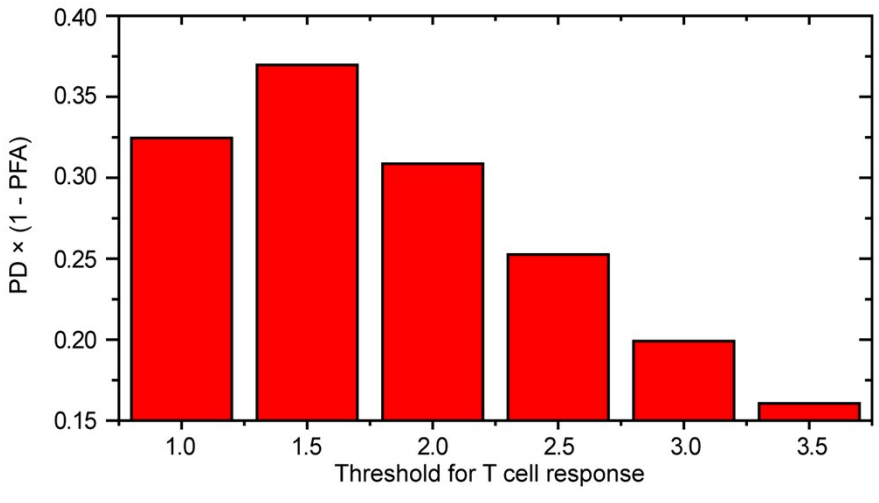


Figure S2. Calibration curve to determine the surface density of fluorescently labeled antibodies on particles. Average fluorescence intensity per pixel (a.u.) of lipid bilayer-coated silica particles is plotted against the surface density of RhB-PE. Each error bar represents the standard deviation of at least three sample sets. The data were fitted to a linear equation: $y = 2.42x+381$.

(a)



(b)

Threshold	1	1.5	2.0	2.5	3.0	3.5
PD	0.6470	0.4506	0.3564	0.2811	0.2157	0.1704
PFA	0.4984	0.1795	0.1342	0.1014	0.0768	0.0572
PD × (1 - PFA)	0.3246	0.3697	0.3086	0.2526	0.1991	0.1606

Figure S3. Data showing the determination of threshold of T cell calcium response. A proper threshold should minimize the false detection (probability of false alarm, PFA) while retaining the true activation signal (probability of detection, PD), resulting in a maximum value of PD × (1-PFA). (a) PD × (1-PFA) calculated from six different thresholds. (b) A table showing data for PD, PFA, and PD × (1-PFA), calculated for each threshold. The threshold was determined to be 1.5.

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