Supplementary information

for

Real-time single-cell imaging of protein secretion

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Supplementary figures

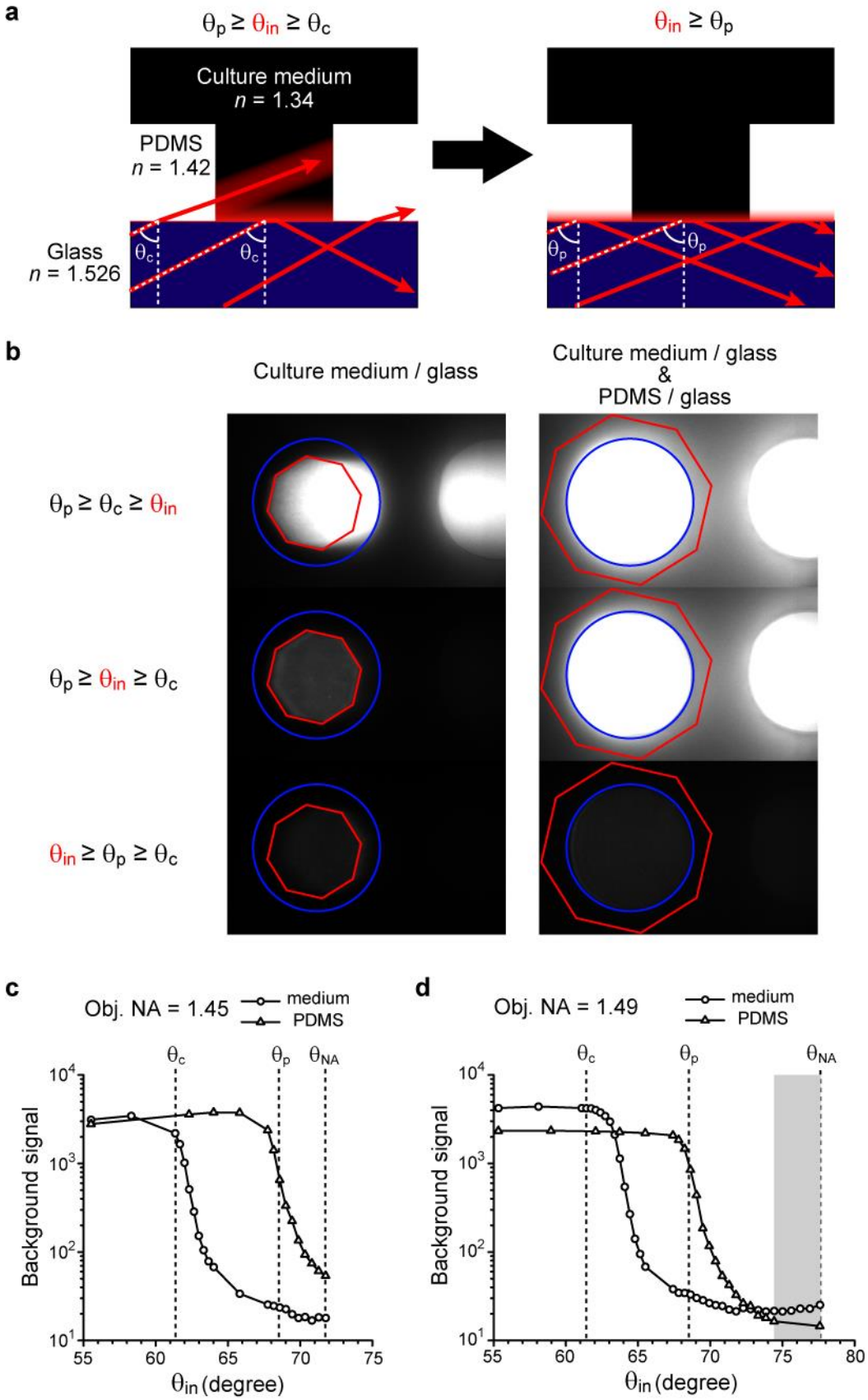


Figure S1: PDMS MWA chip. (a) TIRFM with a PDMS MWA chip. Incident light could not be totally reflected at the interface between glass and PDMS when the incident angle θ_{in} was smaller than the critical angle θ_p for a PDMS-glass interface, even if θ_{in} was larger than the critical angle θ_c for a culture medium-glass interface (left). The stray light will illuminate the upper part of microwells, resulting in a background level that is too high. An objective lens with NA > 1.42 can transmit incident light with a larger angle than θ_p, and allows total reflection at any area (right). (b) TIRFM image of each condition. Blue circle indicated shape of a target well. Red octagonal indicated surface area of laser illumination. When θ_{in} was smaller than θ_c, the laser was transmitted into the medium and illuminated. When θ_{in} was greater than θ_c but smaller than θ_p, the laser was totally reflected at the interface of medium/glass alone. When θ_{in} was increased greater than θ_p with an objective lens with NA = 1.49, a TIRFM image was achieved with a low background level in the entire well. Relationship between the incident angle and background signal with an objective lens with NA = 1.45 (c) and NA = 1.49 (d). Background signal from the medium/glass (○) or PDMS/glass (△) interface decreased when θ_{in} was greater than θ_c or θ_p, respectively. Maximum angle calculated from the NA of the objective lens is shown as θ_{NA}. Although the 1.45-NA objective lens was applicable for TIRFM with a PDMS MWA chip, the 1.49 NA objective lens provided lower background with larger θ_{in} (grey band in (d)).

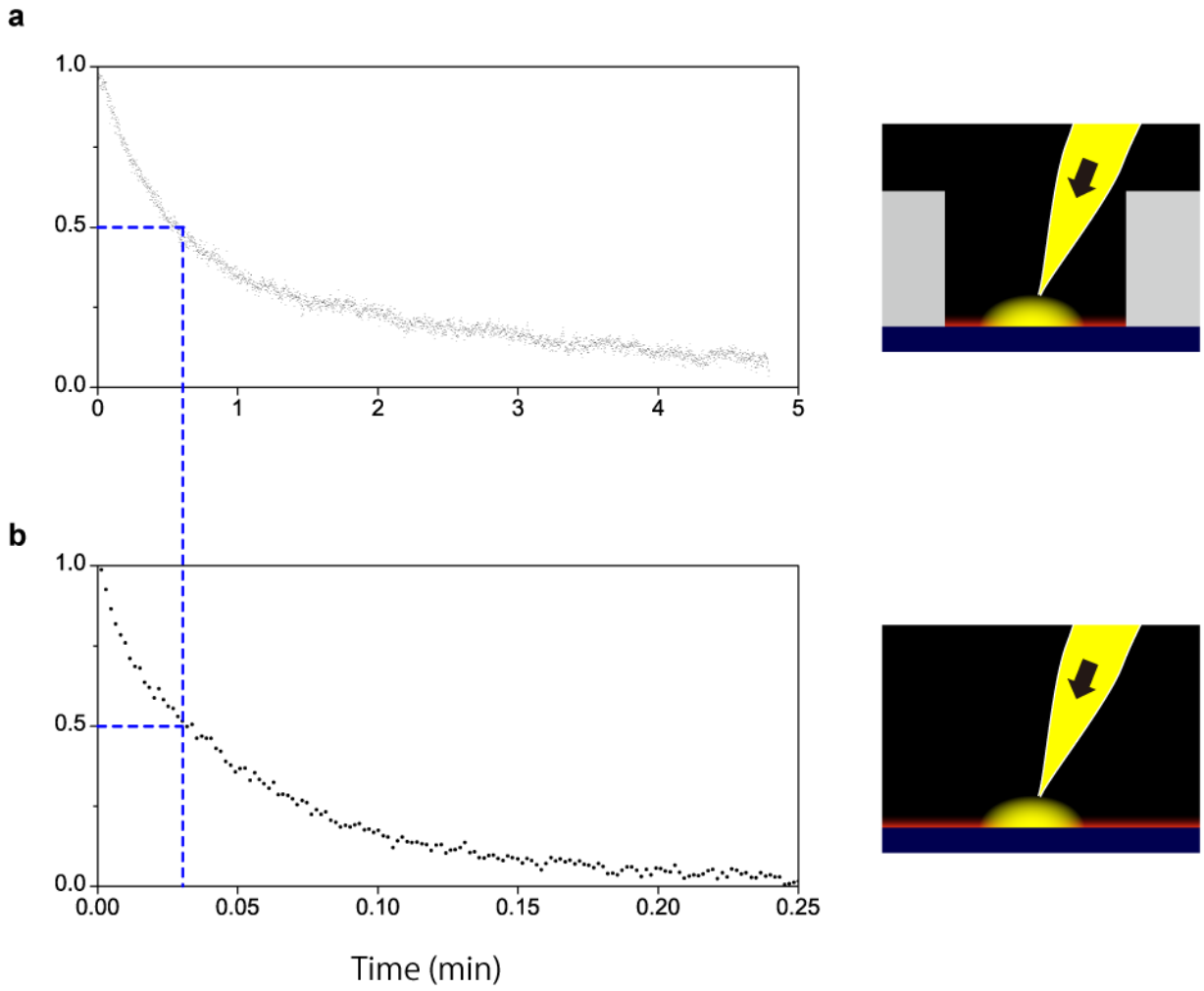


Figure S2: Restriction of the diffusion of fluorescent protein with a microwell. CF660R-labelled antibody was ejected into a microwell (a) or a glass-bottomed dish (b). The decrease in the fluorescence signal was measured at 0.1-s intervals by TIRF imaging. The vertical axis shows normalized intensity of the fluorescence signal under each condition. The diffusion rate of the protein was approximately 20-fold slower in the microwell than in the glass-bottom dish.

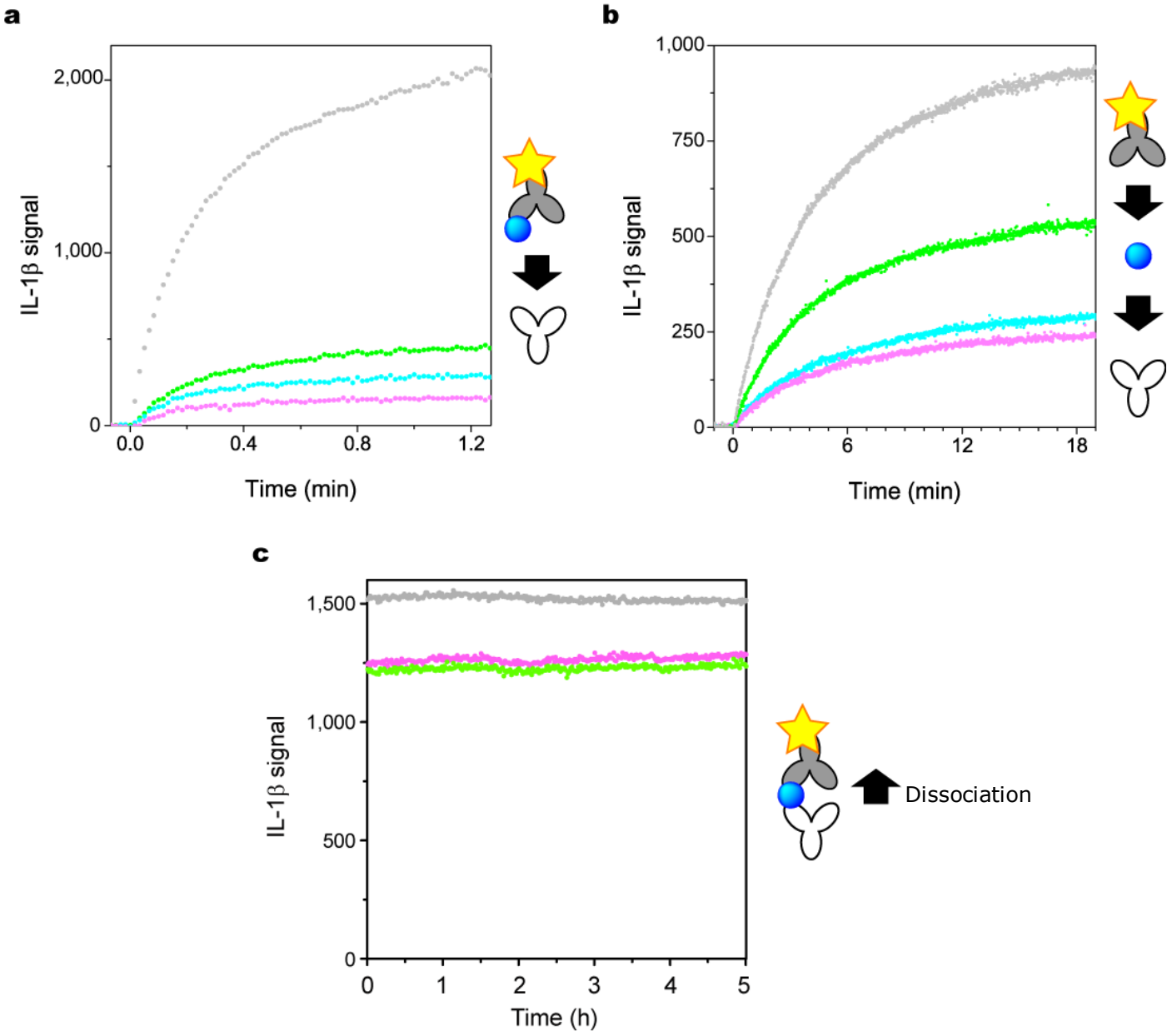


Figure S3: Determination of immunocomplex association and dissociation rates on the microwell bottom. (a) Time course of the IL-1β signal after introducing preformed recombinant IL-1β/detection antibody immunocomplex, which represents the association rate of the capture antibody. (b) Time course of the IL-1β signal after introducing the recombinant IL-1β alone. The increment in (b) was 15-fold slower than that in (a), indicating that slow association of the detection antibody was the rate-limiting step. (c) Time courses of IL-1β immunocomplex dissociation several hours after recombinant IL-1β ejection. The slow decrease in the IL-1β signal was likely observed due to the strong affinity and avidity of the IL-1β immunocomplex.

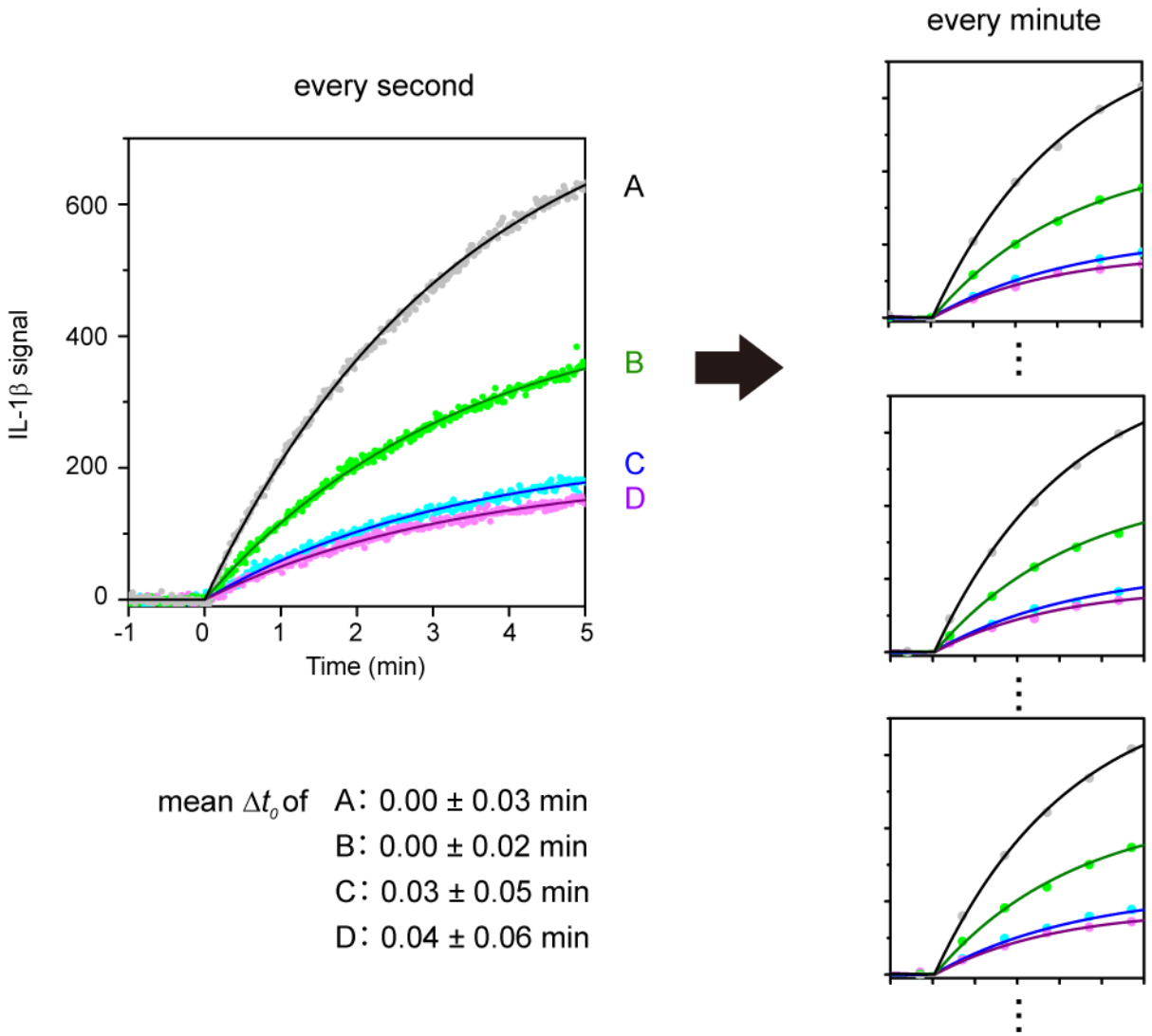


Figure S4: Determination of fitting accuracy. We extracted 60 data set sequences with a time interval of 1 min from data with 1-s time interval (shown in Figure 2c) and fitted them to equation (3) with the defined parameter of $\tau = 3$ to estimate the ejection time of t_o. The ejection time was successfully calculated within 0.1 min of accuracy, although the accuracy tended to be reduced with decreasing signal.

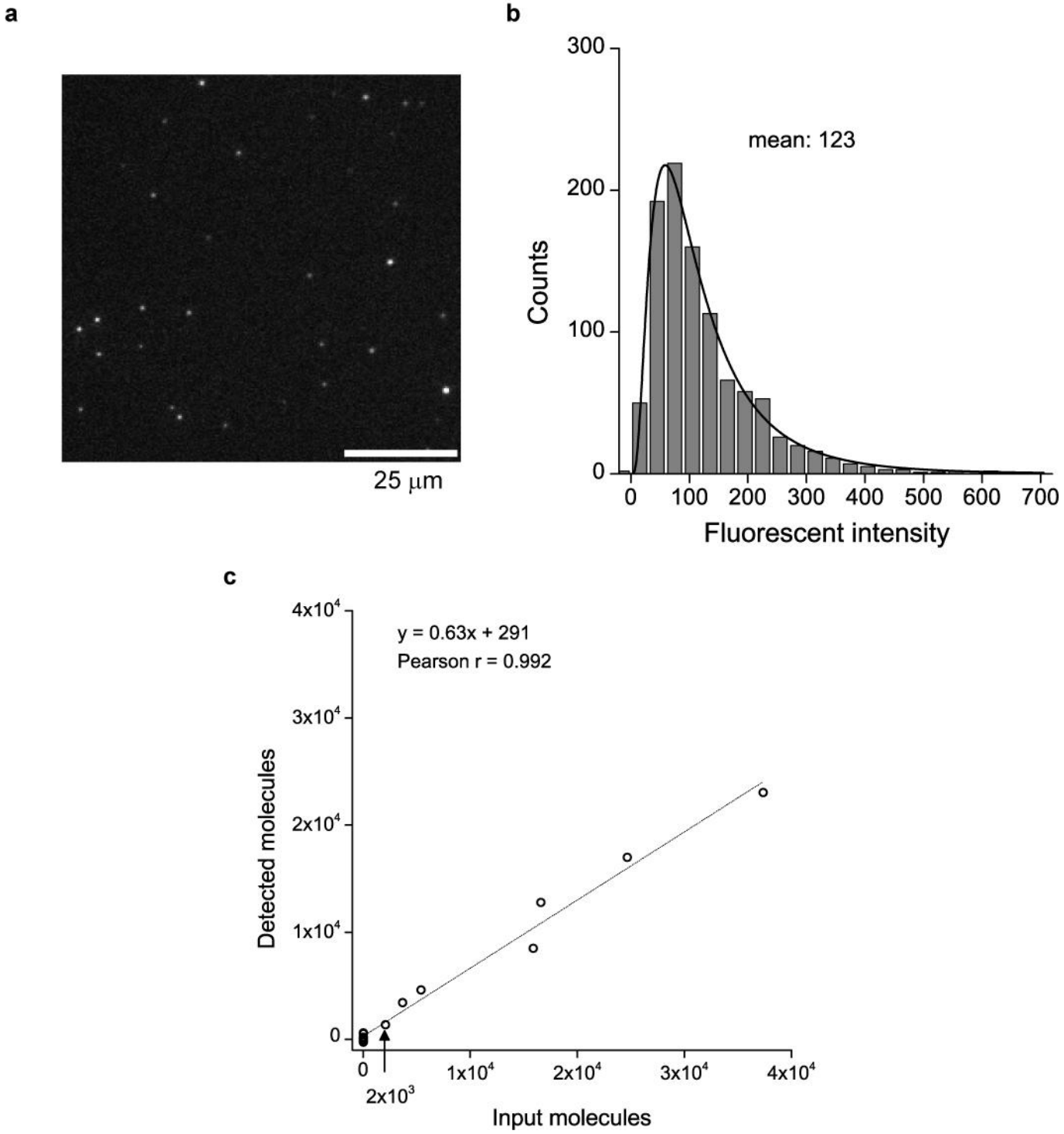


Figure S5: Determination of the detection limit. (a) TIRF image of sparse immunocomplexes after the wash-out of free detection antibody. The image was captured applying 60 ms exposure time and 16 frame averaging. (b) A histogram of the fluorescence intensity of individual immunocomplexes. The curve was fitted by log-normal distribution (adjusted R^2 = 0.990). The mean intensity was calculated as 123. (c) Determination of the detection limit with the help of recombinant IL-1β ejection. We ejected 100 ng/mL of recombinant IL-1β and observed an increase in the signal for 6 min. The ejection position was approximately 5 μm above the well bottom. To calculate the volume ejected, we added a reference dye to the recombinant reagent, and ejected the mixture into the microwells closed with sealing oil. The ejected amount of recombinant IL-1β is shown as the number of input molecules. The signal increase was converted into the number of molecules with mean intensity, as shown in (b). The detected amount linearly correlated with the input amount. We were able to detect 2,000 input molecules with a significant difference (>3 σ from the average of non-ejected controls).

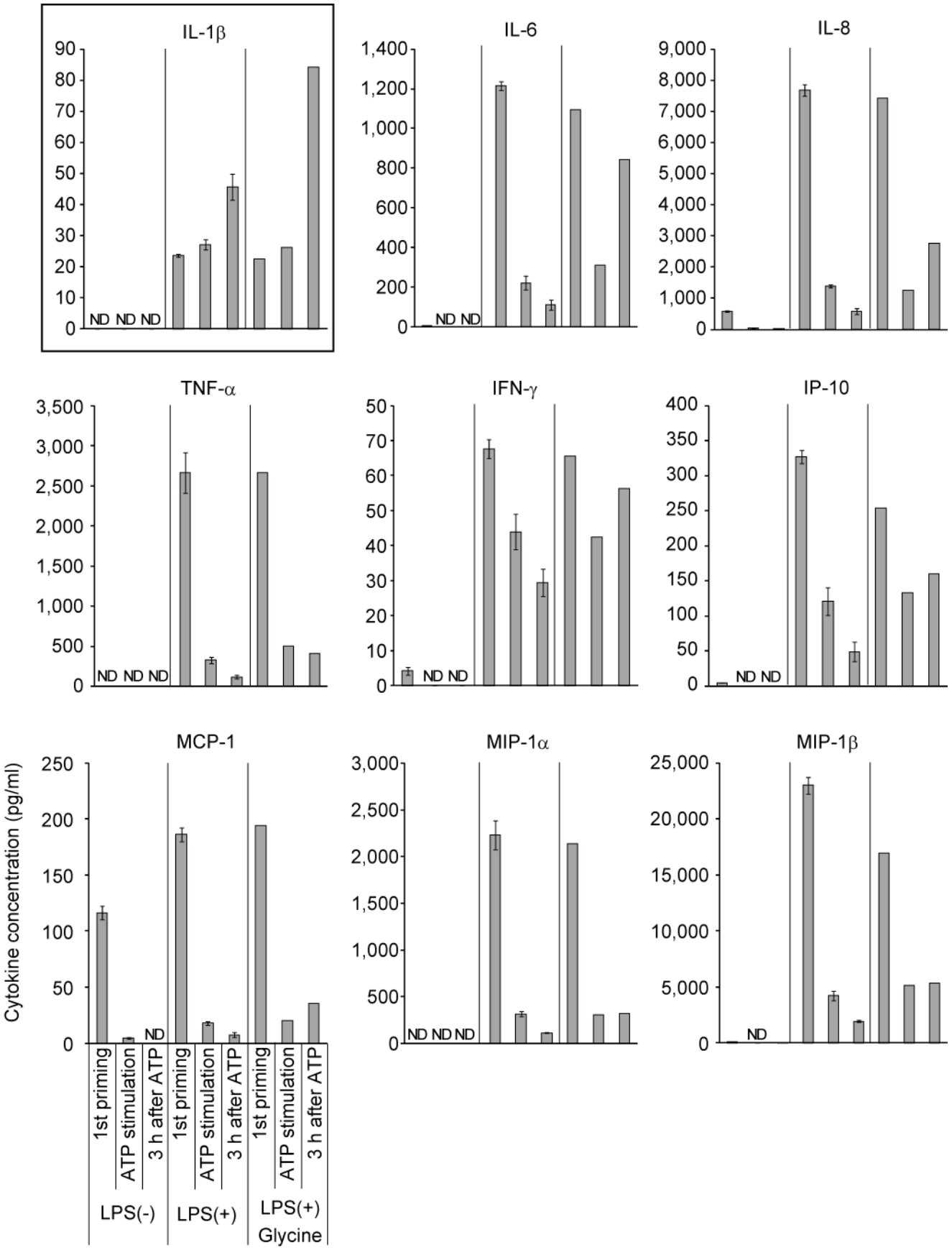

IL-1β
IL-6
IL-8
TNF-α
IFN-γ
IP-10
MCP-1
MIP-1α
MIP-1β
Cytokine concentration (pg/ml)
ND
1st priming
ATP stimulation
3 h after ATP
LPS(-)
LPS(+)
LPS(+)
Glycine

Figure S6: Bulk measurement of humoral factors in the culture supernatant of a population of monocytes. Bar charts depict the quantities of the nine most highly secreted humoral factors out of the 27 measured factors. Vertical scale displays the amount of each humoral factor (in pg/mL) during the indicated time period. All experiments, except for those with glycine, were performed in duplicate, and average values are shown. Error bars indicate minimum and maximum measurements. Three hours of priming in the presence or absence of 1 μg/mL LPS (1st priming), brief stimulation with 5 mM ATP for 10 min (ATP stimulation), and additional incubation for 3 h after ATP removal (3 h after ATP) were sequentially performed on the same samples. Cells were maintained at a density of 5×10^5 cells/mL. The addition of glycine (5 mM), known to protect cells from cytolysis, increased secretion of several humoral factors during the 3-h incubation period after ATP stimulation. Under our experimental conditions, brief ATP stimulation enhanced IL-1β release, whereas single LPS stimulation was sufficient to induce other cytokines besides IL-1β. The glycine in the media prevented decreases in the secretion of several cytokines other than IL-1β after ATP stimulation, implying that monocytes were protected from cell death after cytolysis in the presence of glycine.

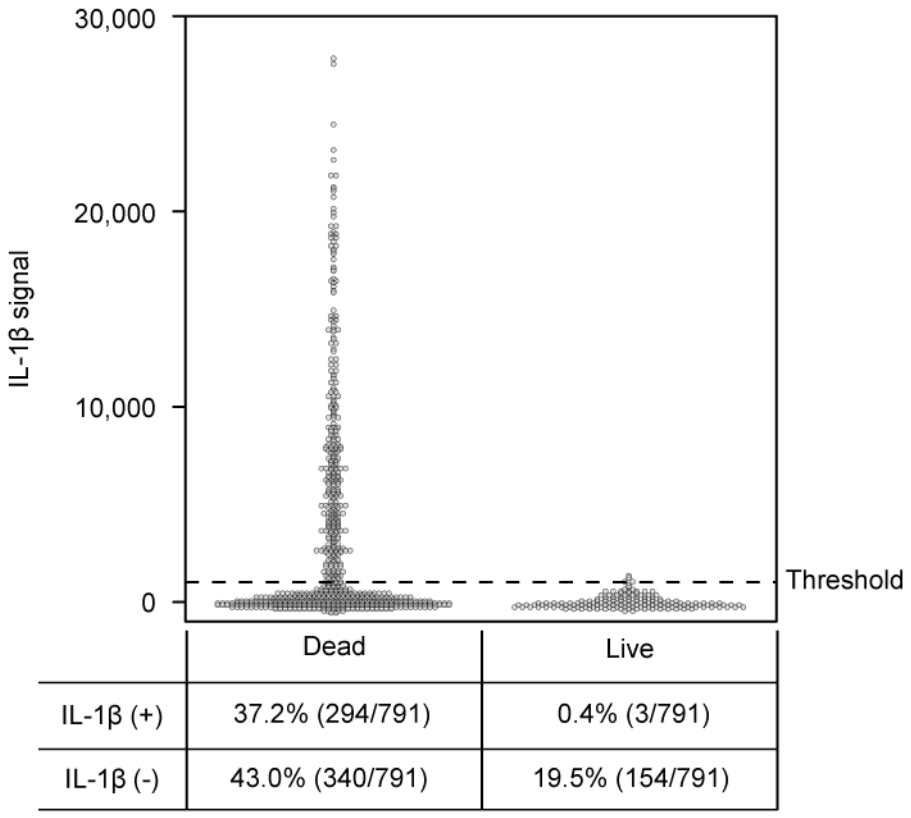
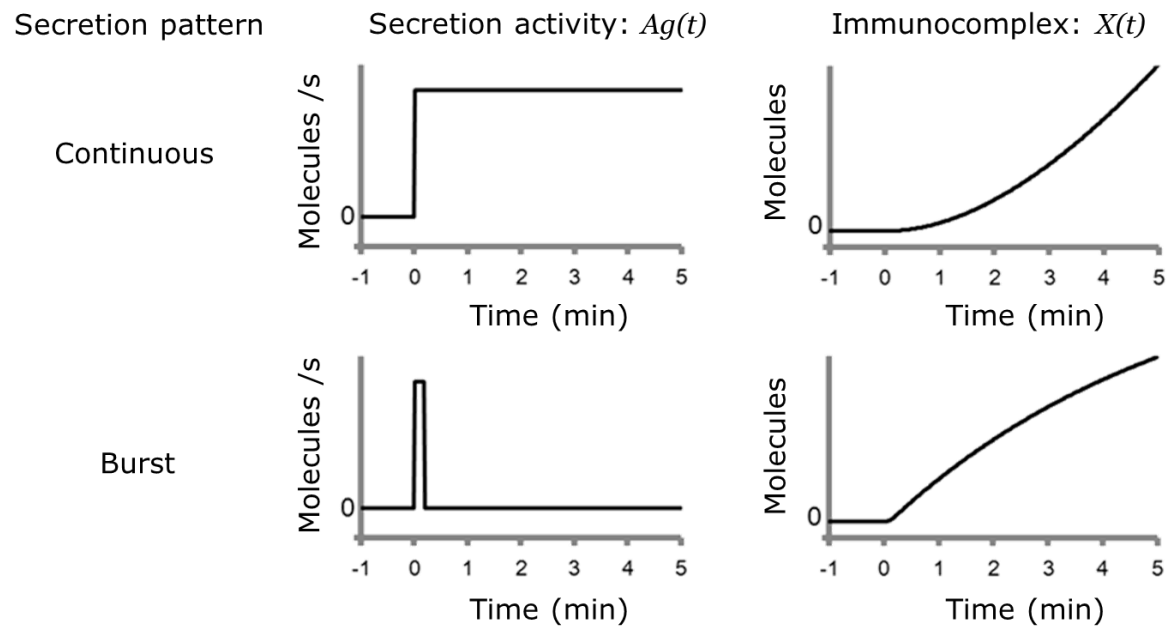


Figure S7: Snapshot measurement of IL-1β release from LPS-primed and ATP-stimulated monocytes. Dot graph showing the secretion level of IL-1β from individual dead [calcein (-)/SYTOX (+)] or live [calcein (+)/SYTOX (-)] cells. Vertical scale corresponds to the relative intensity of the IL-1β signal. IL-1β (+) cells were defined by their signals above the detection limit (dotted line), and IL-1β (-) cells were defined by their signals below the detection limit; 38% of cells released detectable quantities of IL-1β, and 99% of the IL-1β (+) cells were dead.



$$\frac{dX(t)}{dt} = k_{on}\, Ab_{free}\, Ag_{free} - k_{off} X(t)$$

$$Ag_{free} = \int Ag(t)\, dt - X(t)$$

$$Ab_{free} = Const.$$

k_{on}: Association rate constant, $10^5\ M^{-1} \cdot s^{-1}$
k_{off}: Dissociation rate constant, $10^{-4}\ s^{-1}$
Ab_{free}: $Detection\ antibody, 30\ nM$

Figure S8: Numerical simulation of immunocomplex formation. To understand the secretion pattern of IL-1β, we simulated the time-course of imunocomplex formation upon the two types of secretion patterns. We used three postulates that simplified the numerical simulations based on our model experiments: (1) A percentage of secreted cytokine was immediately captured and the remaining cytokine was dissipated. (2) The concentration of detection antibody (Ab_{free}) was regarded as constant in the open-end microwells because the detection antibody was rapidly provided from mass volume of the detection medium. (3) The formation of immunocomplex followed the law of mass action. We used typical k_{on} and k_{off} values for the antibody. The results indicated that IL-1β release occurred in bursts rather than a continuous release because the rising curve of IL-1β secretion from LPS/ATP-stimulated monocytes showed a concave-down function.

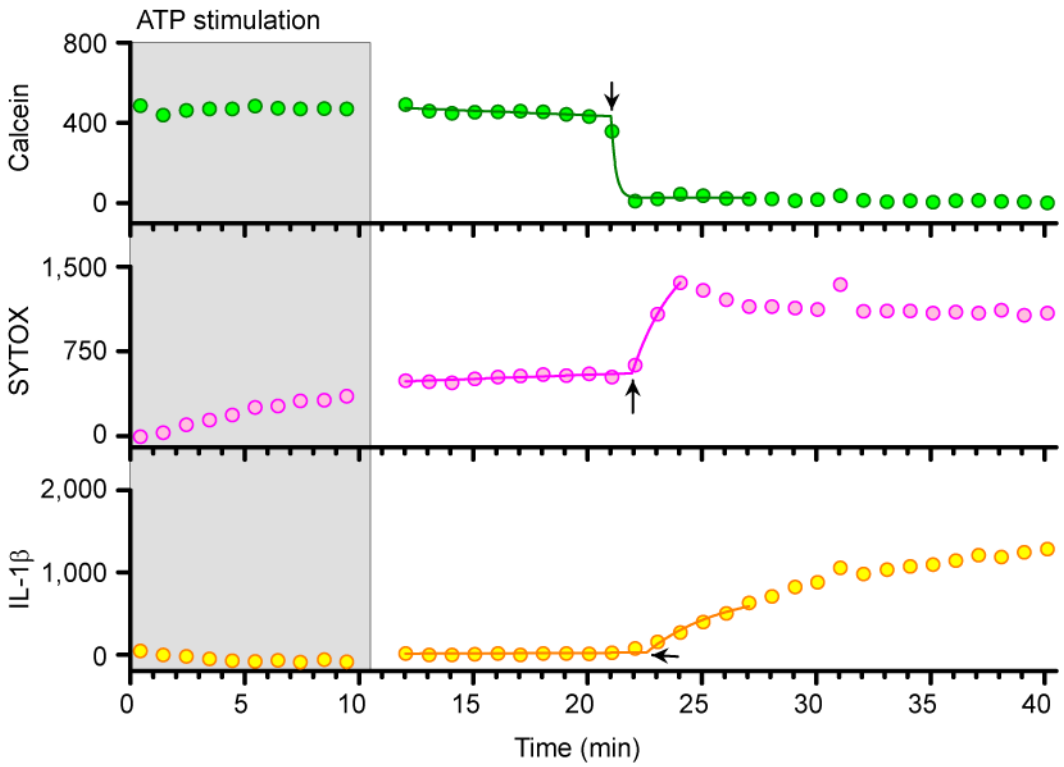


Figure S9: Illustration of the method for detecting the transition time. Time-course graphs are shown, expanded around the transition time of calcein disappearance (cropped from Figure 3c). The signal transition time t_0 was calculated by curve-fitting with equations (1) to (3) (displayed in the Methods section). The arrows show the t_0 for each signal. To estimate the onset of secretion, the IL-1β signal was fitted with the determined time constant τ_i (3 min) for 5 min from transition.

Table S1: Dose effect of LPS on ATP-induced IL-1β release based on snapshot measurements[1)]

LPS stimulation	Dead	IL-1β (+)	Mean secretion signal of IL-1β (+) cells
10 ng/mL	78%	9%	1.7×10^3
1 μg/mL	80%	38%	8.1×10^3

1) Experimental conditions were identical to those given in Figure S7, except for LPS concentrations.

Movie 1: IL-6 secretion from an LPS-stimulated human monocyte. The images were taken at 1-min intervals. The video plays at 900× fast speed. 'DIA' denotes the diascopic illumination image; 'SYTOX blue' denotes the epifluorescence image of SYTOX blue; 'calcein' denotes the epifluorescence image of calcein; 'CF660R IL-6' denotes the TIRFM image of the IL-6 immunocomplex labelled with CF660R; 'All channel' is a merged image; and 'Fluo. merged' denotes the merging of the three fluorescence images. The wells had a diameter of 70 μm. Initially, the monocyte floated in the well. After 1 h 23 min, the cell adhered and began migration. Because the cell deformed into a thin shape, its contours were ambiguous in the DIA image. After 90 min, the IL-6 signal began to appear around the cell.

Movie 2: IL-1β release from an LPS/ATP-stimulated human monocyte. Images were collected at 1-min intervals. The movie plays at 900× fast speed. 'DIA' denotes the diascopic illumination image; 'SYTOX blue' denotes the epifluorescence image of SYTOX blue; 'calcein' denotes the epifluorescence image of calcein; and 'CF660R IL-1β' denotes the TIRF image of IL-1β immunocomplex labelled with CF660R. 'All channel' has been merged from all four images, and 'Fluo. merged' has been merged from the three fluorescence images. The wells had a diameter of 70 μm. The monocyte was pre-primed with 1 μg/mL LPS and introduced into the MWA chip. Monocytes were briefly stimulated with ATP from 23 to 32 min. After ATP stimulation, the detection medium was replaced with fresh medium. At 45 min, calcein dissipated from the cell, followed by SYTOX influx and IL-1β release.

Supplementary methods

Bulk measurement of humoral factors from cell population culture

Isolated human monocytes were suspended in 200 μL of culture medium in a 96-well culture plate at a density of 5×10^5 cells/mL and cultured overnight. The cells were primed in the presence or absence of 1 μg/mL LPS for 3 h. The cell cultures were recovered into 1.5-mL sample tubes and centrifuged at 300 × *g* for 3 min. The supernatants were stored as '1st priming' fractions. Culture medium with 5 mM ATP was added to the cell pellets and incubated for 10 min; supernatants were collected and stored as 'ATP 10 min' fractions. Then, culture medium with or without LPS was added to the cell pellets and incubated for 3 h, after which the supernatants were collected and stored as '3 h after ATP' fractions. Twenty-seven humoral factors were measured using the Bio-Plex suspension array system with Bio-Plex Pro™ human cytokine, chemokine, and growth factor assays (M50-0KCAF0Y).

Feasibility demonstration of TIRFM on the platform

To determine the relationship between incident angles and the signal/noise ratio of the images acquired, a PDMS well chip was filled with 20 μg/mL of CF660R-labeled streptavidin and was imaged at various angles of incident illumination. To compare the objectives with different NA, we measured fluorescence intensities resulting from illumination by stray light from the medium-glass interface or PDMS-glass interface. Actual angles of incident light were calculated from the shift length of the centre of the laser light passing through a block with uniform refractive index (n = 1.52) after exiting the objective lens.

Evaluation of the platform with model experiments using a microinjector

To evaluate the molecular kinetics on the platform, IL-1β release from a single cell was mimicked by flash release through a femtolitre injector (Femtojet; Eppendorf, Hamburg, Germany). For comparison of the diffusion rates from experiments with or without microwell structure, 30 nM of CF660R dye-labelled detection antibody was flash-released and the decrease in fluorescence intensity was monitored by TIRFM at 0.1-s intervals. For determination of the rate of association between IL-1β molecules and each antibody, 100 ng/mL of recombinant human IL-1β (201-LB, R&D systems) alone or as a preformed complex with containing fluorescent detection antibody was injected at different pressures for 0.1 s into a microwell of a PDMS MWA chip pre-filled with detection medium, and the signal increase under each condition was monitored by TIRFM at 1-s intervals. To estimate the lower limit of detection, test reagent containing 100 ng/mL of IL-1β and concentration reference dye was injected for different durations into the microwells sealed with silicon oil. Six minutes after injection, the intensities of IL-1β signal and reference dye were measured. The amount of injected molecules was calculated using the intensity of the reference dye. The lower limit of detection was theoretically calculated as three times the standard deviation (σ) above he averaged background intensity measured in microwells without IL-1β introduction. The detected intensity of the IL-1β signal was converted into the number of molecules using the average intensity of an immunocomplex, determined as follows: recombinant IL-1β (5 nM)-fluorescent detection antibody (500 nM) complex was preformed, diluted 1,000-fold, and introduced into a capture antibody-coated flow cell. Following this, the flow cell was flushed with fresh medium without fluorescent antibody and observed with TIRFM under the same experimental setup as that used for the estimation of the lower limit of detection. Integrated intensity of each fluorescent spot was measured after background subtraction. To ensure the position of each fluorescent spot, high-contrast images were acquired applying 1-s exposure and 30 frame averages. We performed a distributional analysis to ensure that the spots actually corresponded to a single immunocomplex (based on the single peak histogram).

Snapshot measurement of cytokine secretion from stimulated monocytes

Monocytes in the MWA chip were measured after stimulation by LPS or LPS/ATP at 625 positions, corresponding to 2,500 microwells, by multichannel microscopy, i.e. DIA images, epifluorescence images for calcein and SYTOX blue, and TIRF images for CF660R fluorescence dye. Imaging analysis was performed using the NIS Elements imaging software. The number of cells and cellular states in each microwell were examined visually on the DIA, calcein, and SYTOX blue images. The MFI of CF660R dye was measured in each microwell. Background intensities were determined as the mean intensity of the darkest pixel in the entire excited area for each point and subtracted from the MFI of CF660R. Mean (μ) and standard (σ) deviation of the MFI of empty microwells

were calculated. All MFIs were offset with μ. MFIs of single cells are shown as dot plots. The percentages of IL-1β signal–positive or IL-1β signal–negative and live (calcein [+], SYTOX [-]) or dead (calcein [-], SYTOX [+]) cells were calculated based on the detection limit of 3σ.