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Supplemental information

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Supplementary Information

High-Resolution Imaging of Protein Secretion at Single-Cell Level using Plasmon-enhanced FluoroDOT Assay

AUTHORS
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Supplemental Items

Table S1: Characteristic features of ELISpot/FluoroSPOT and FluoroDOT assays.
Related to Figure 1.

	ELISpot/ FluoroSPOT Assay	**FluoroDOT Assay**
Image Readout	Low resolution analog signal: Spots	High resolution digital signal: Clusters of dots
Signal Amplification Approach	Extended incubation and analyte perfusion	Plasmon-enhanced fluorescence
Spatial information of Secretion (cell shape/directional/polarized secretion)	Lost in 3D membrane	Intact on glass bottom substrate
Information at early time-points	Not available as requires extended duration of incubation	Available and can detect secretion as soon as it starts (within an hour)
Information from doublets/dividing cells	Not available: Doublets counted as single large spot	Available: Can resolve doublets/dividing cells
Sub-cellular information correlation with secretion	Not feasible due to cells getting washed off during the assay	Cells can be fixed, stained, and imaged for revealing sub-cellular information (e.g., nuclear staining, fluorescent protein encoded intracellular bacteria)
Image Readout Device	ELISpot/FluoroSPOT Reader (Less readily available)	Epi-fluorescence microscope (More readily available)
Quantification of Secreting Cells	Possible by counting number of Spots	Possible by counting number of Clusters

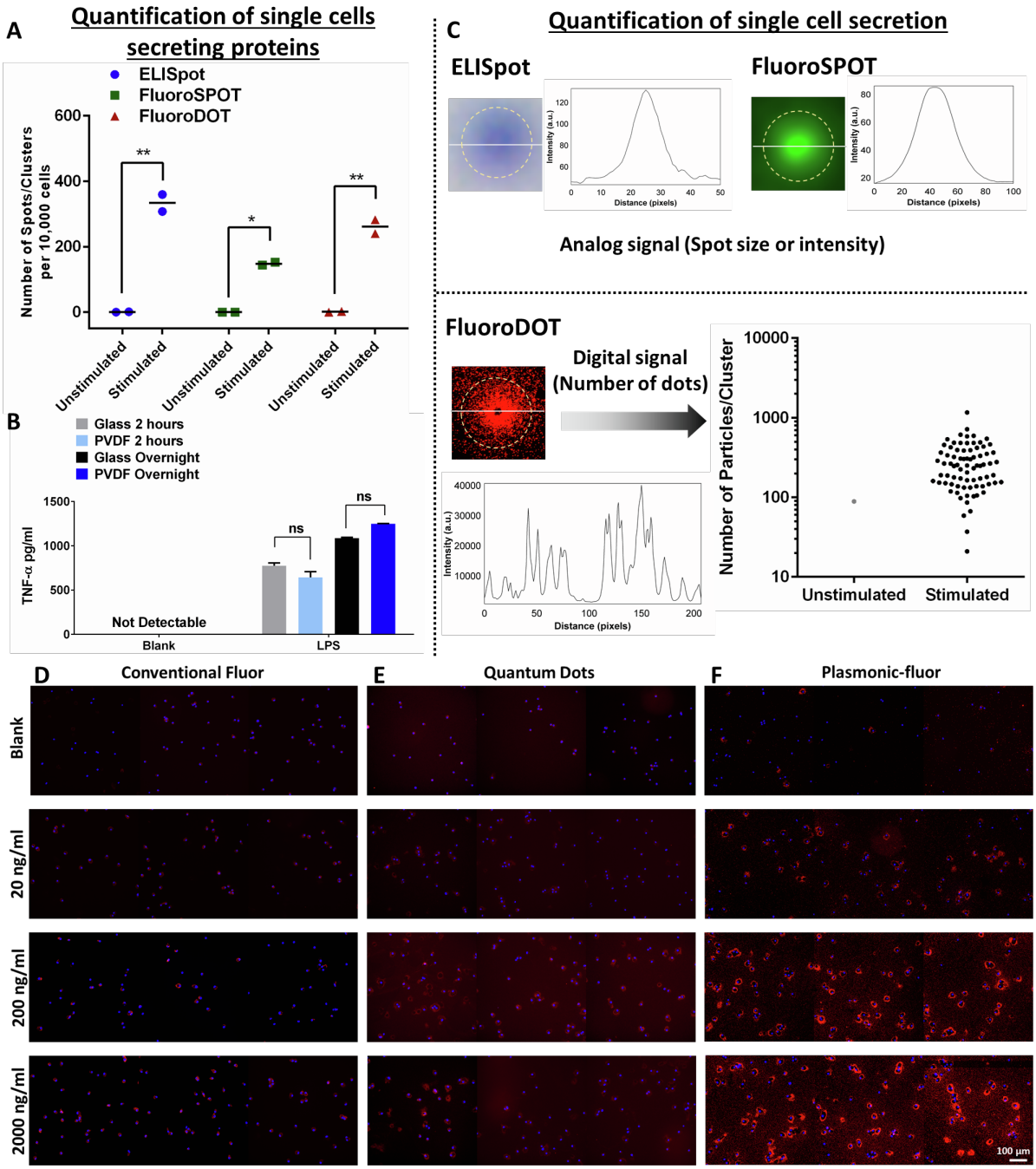

Figure S1: Comparison of FluoroDOT assay with conventional assays and labels. Related to Figure 1. A) Quantification of single cells secreting proteins using ELISpot, FluorSPOT and FluoroDOT assays, 2-way ANOVA with Sidak's multiple comparison test. ELISpot ** p=0.0013, FluoroSPOT *p= 0.0146, FluoroDOT **p=0.0028. B) Comparison of the amount of TNFα secreted on different substrates. C) Demonstration of representative analog signal of ELISpot and FluoroSPOT assays and digital signal of FluoroDOT assay. Yellow dotted line indicates the spot. Line-scan (indicated by white line) was performed to quantify the intensities using ImageJ. JAWS II DCs were treated with 200 and 2000 ng/ml lipopolysachharide (LPS). Cy5/DAPI merged epifluorescence microscopy images of protein secretion using, D) conventional fluor (Cy5), E) quantum dot 655, and F) Cy5-plasmonic-fluor. Each panel contains 3 representative 20x images stitched together. Scale bar: 100 µm.

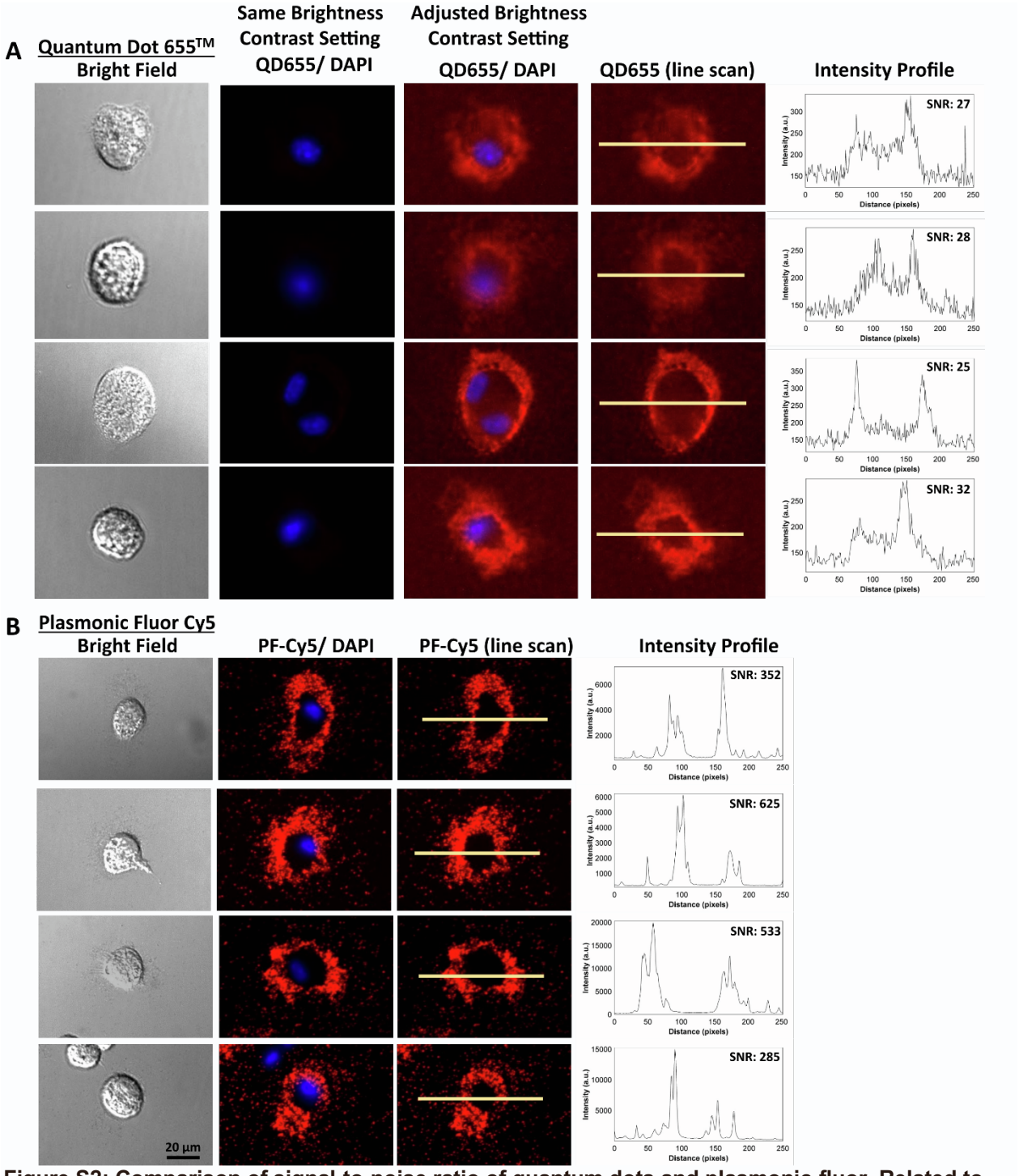

Figure S2: Comparison of signal-to-noise ratio of quantum dots and plasmonic-fluor. Related to Figure 1. JAWS II DCs were treated with 200 ng/ml lipopolysachharide (LPS) and bright field images (left panel), Cy5/DAPI merged images (middle panel) of a single cell secreting TNF-α, representative line-scans and corresponding Signal-to-noise ratio (SNR) (right panel) visualized using A) quantum dot 655 (images with same and adjusted brightness contrast) and B) Cy5-plasmonic-fluor. LED (XCITE 110 LED Lamp) was used a light source. Quantum dot imaging was performed using a Qdot-specific filter cube and plasmonic-fluor was imaged using standard Cy5 filter.

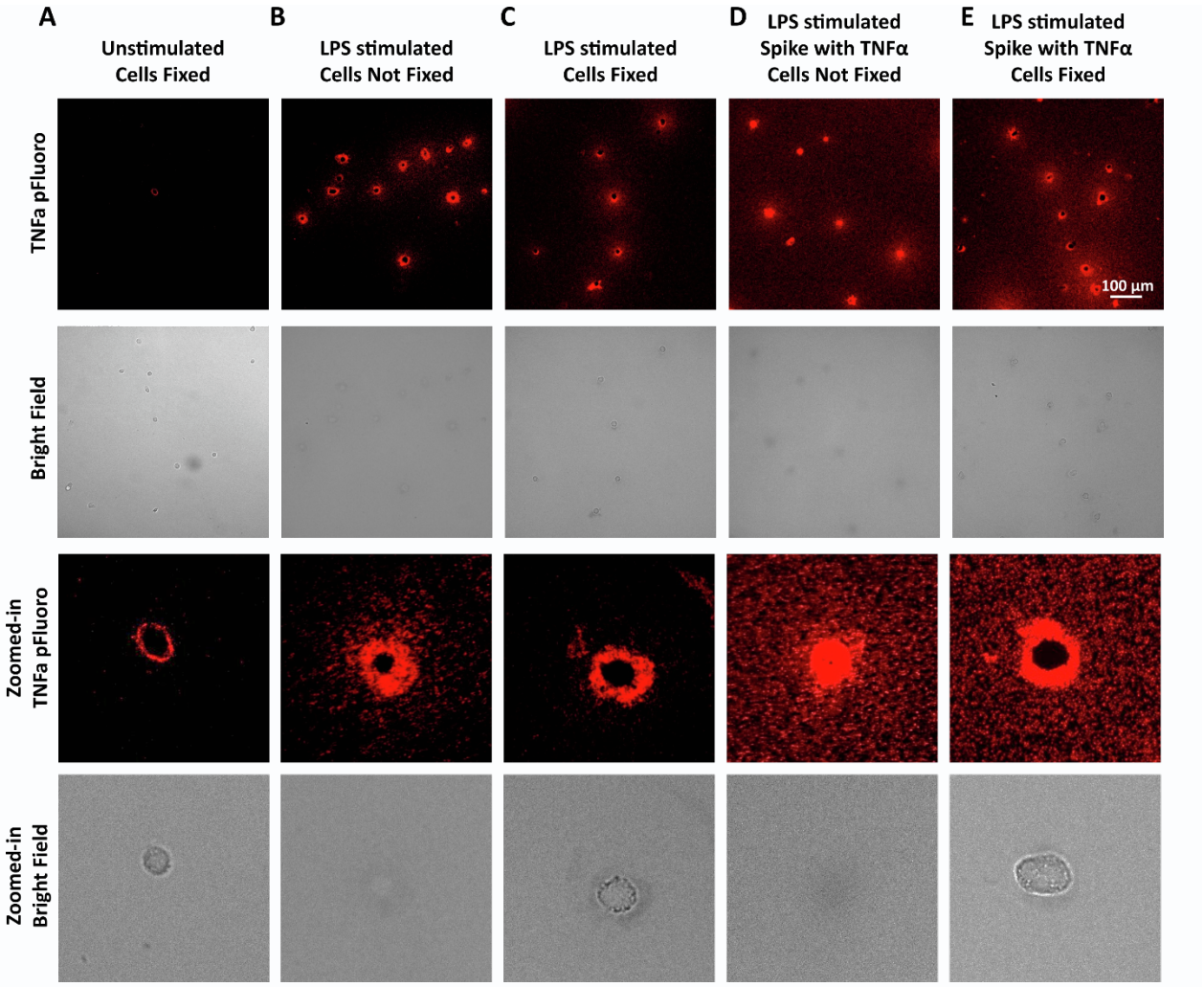

Figure S3: Studying the secretion of TNFα beneath the cell body. Related to Figure 1. JAWS II DCs were treated with 200 ng/ml lipopolysachharide (LPS). FluoroDOT images (top panel) of cells secreting TNFα and the corresponding bright field images (bottom panel).

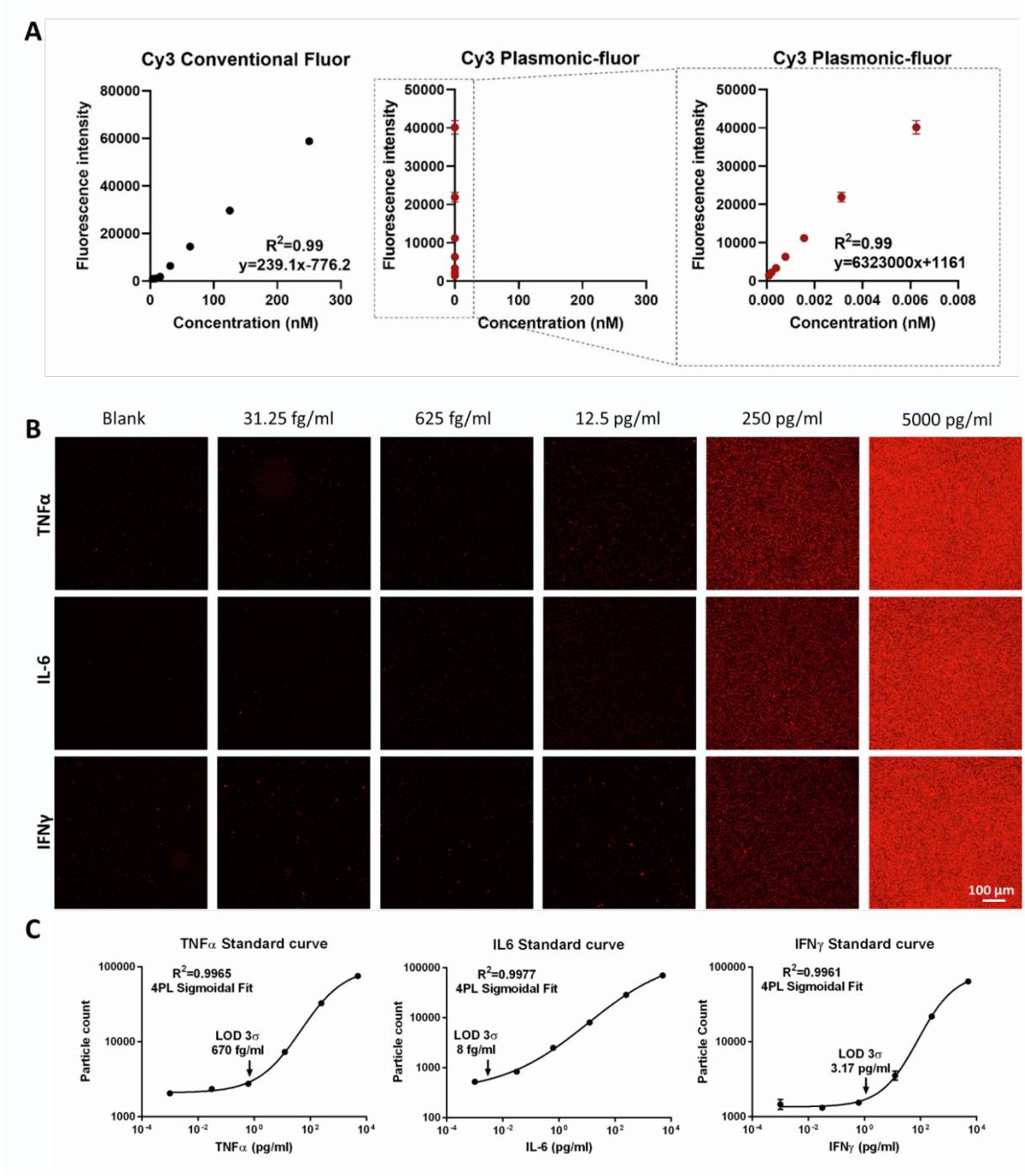

Figure S4: Comparison of brightness of conventional fluorophore and plasmonic-fluor and standard curves using plasmon-enhanced fluorescence-linked immunosorbent assay. Related to Figure 2. A) Fluorescence intensity of Cy3-conventional fluor and Cy3-plasmonic-fluor at their different molar concentrations. Data represented as mean ± s.d (n = 2 repeated tests). B) Density-dose dependence images demonstrating increasing density of plasmonic-fluors with increasing concentration of cytokine TNFα, IL-6 and IFNγ, scale bar: 100 µm and C) standard curves for the plasmon-enhanced fluorescence-linked immunosorbent assay (p-FLISA) for TNFα, IL-6 and IFNγ. The data was fit using 4-parameter logistic (4 PL) sigmoidal curve. Limit of detection (LOD) is defined as (mean+3σ) of the blank.

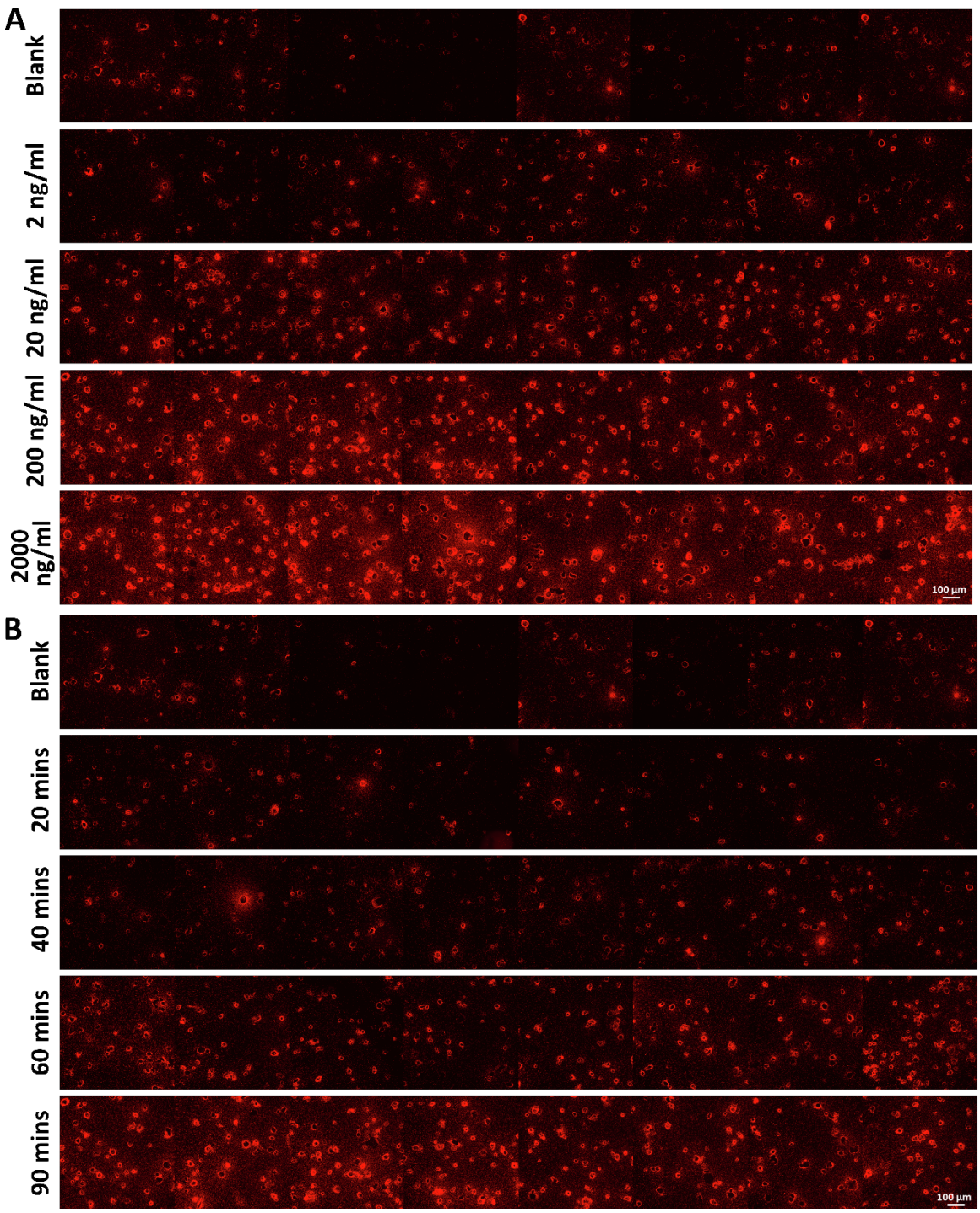

Figure S5: TNF-α FluoroDOT assay performed on JAWS II DCs. Related to Figure 3. JAWS II DCs treated with A) 0, 2, 20, 200, and 2000 ng/ml LPS for 90 minutes and B) 200 ng/ml of LPS treated for 20 minutes, 40 minutes, 60 minutes, and 90 minutes. Cy5 epifluorescence microscopy images of the assay using Cy5-plasmonic-fluor. Each panel contains eight representative 20x images stitched together. Scale bar: 100 µm.

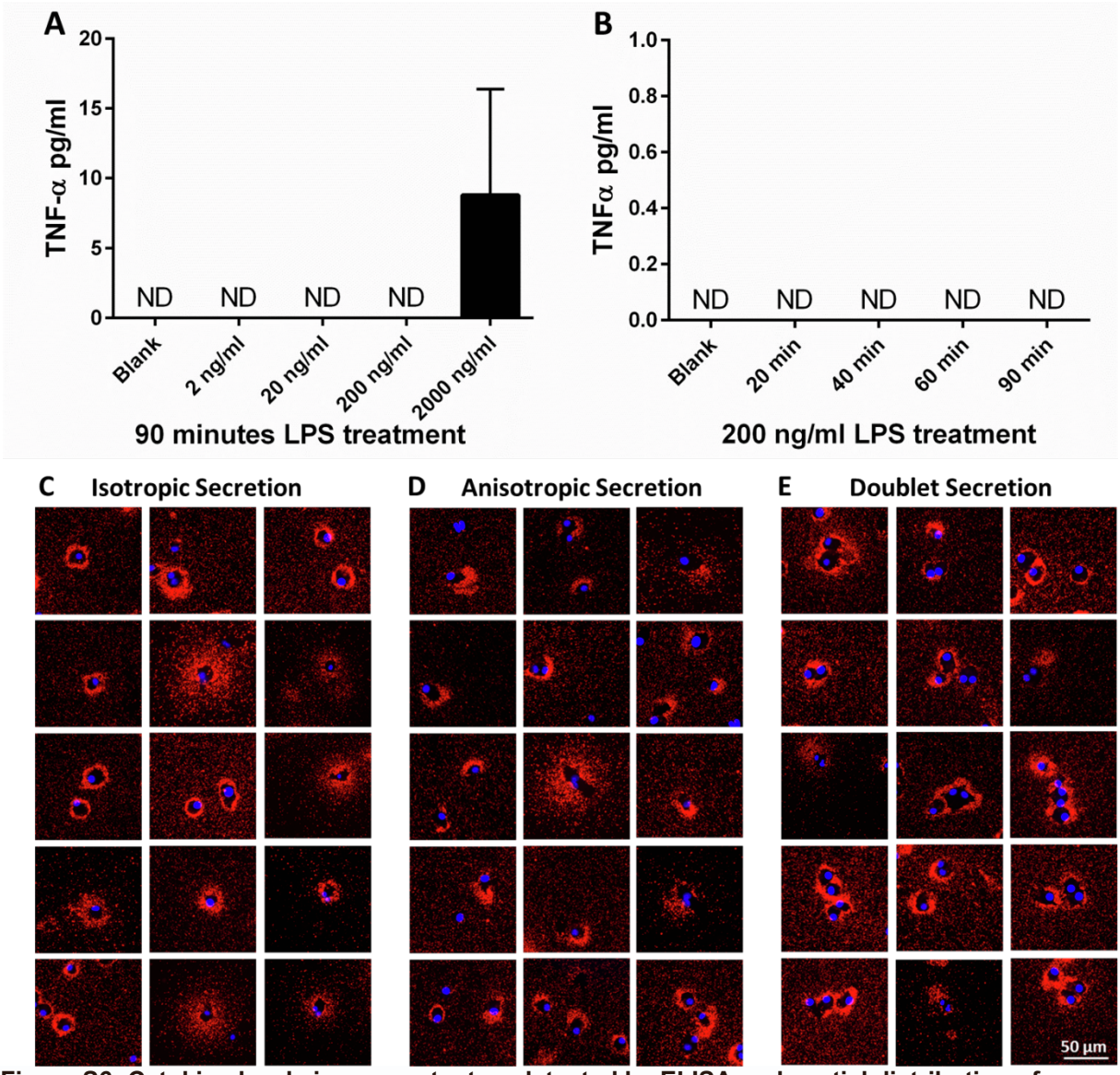

Figure S6: Cytokine levels insupernatant as detected by ELISA and spatial distribution of secretions by FluoroDOT assay. Related to Figure 3. TNF-α secretion analyzed by ELISA of the supernatant of JAWS II DCs after stimulation of LPS for A) 90 minutes with 0, 2, 20, 200 and 2000 ng/ml and B) 20, 40, 60, and 90 minutes with 200 ng/ml, n=2. Representative Cy5 (red) and DAPI (blue) merged images revealing details of TNFα secretion for C) isotropic secretion, D) anisotropic secretion, and E) secretion by two cells in proximity (doublet secretion). Scale bare: 50 µm.

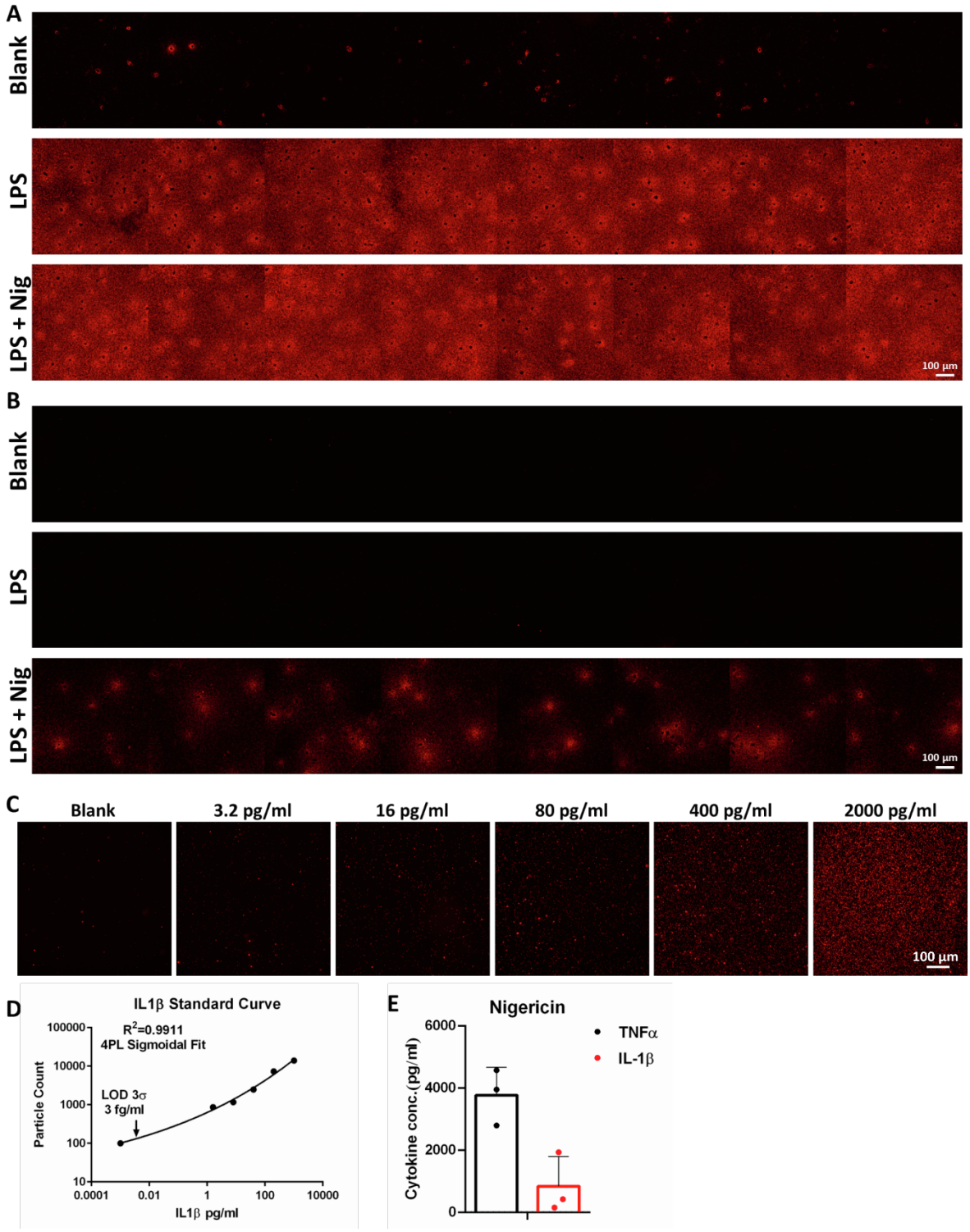

Figure S7: FluoroDOT assay on murine alveolar macrophages. Related to Figure 4. Murine alveolar macrophages treated with 500 ng/ml LPS with and without 20 µM nigericin. A) TNFα secretion and B) IL-1β secretion observed after 4 hours of LPS treatment and 30 minutes of nigericin treatment. Cy5 epifluorescence microscopy images of the assay using Cy5-plasmonic-fluor. Each panel contains eight representative 20x images stitched together. Scale bar: 100 µm. C) Density-dose dependence images demonstrating increasing density of plasmonic-fluors with increasing concentration of IL1β, scale bar: 100

μm and D) standard curve for the plasmon-enhanced fluorescence-linked immunosorbent assay (p-FLISA) for IL1β. E) TNFα and IL1β secretion analyzed by ELISA of the supernatant of alveolar macrophages after stimulation by LPS for 4 hours and nigericin for 30 minutes. The data was fit using a 4-parameter logistic (4 PL) sigmoidal curve. Limit of detection (LOD) is defined as (mean+3 σ) of the blank.

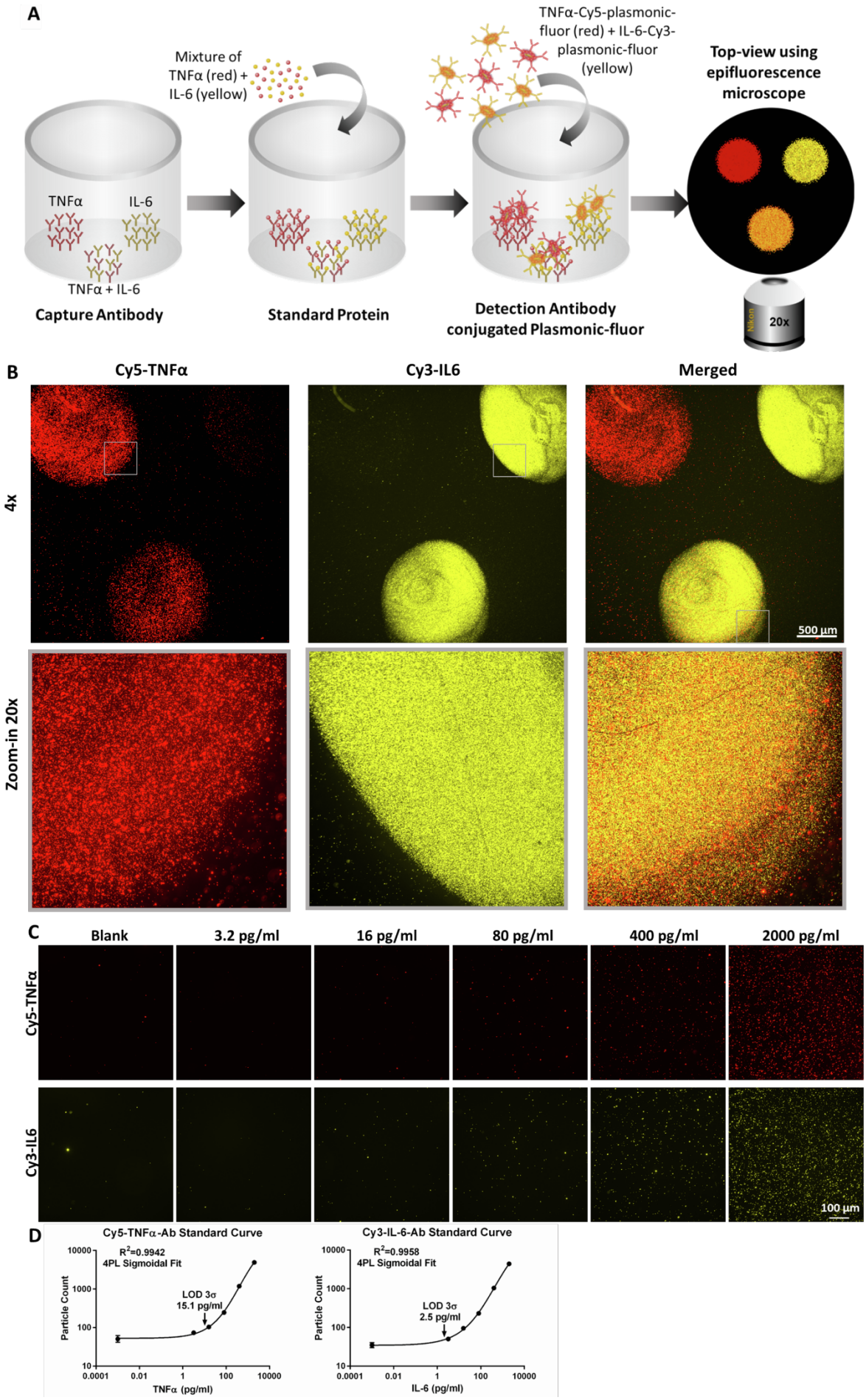
A
Mixture of TNFα (red) + IL-6 (yellow)
TNFα-Cy5-plasmonic-fluor (red) + IL-6-Cy3-plasmonic-fluor (yellow)
Top-view using epifluorescence microscope
TNFα
IL-6
TNFα + IL-6
Capture Antibody
Standard Protein
Detection Antibody conjugated Plasmonic-fluor
20x
B
Cy5-TNFα
Cy3-IL6
Merged
4x
500 µm
Zoom-in 20x
C
Blank
3.2 pg/ml
16 pg/ml
80 pg/ml
400 pg/ml
2000 pg/ml
Cy5-TNFα
Cy3-IL6
100 µm
D
Cy5-TNFα-Ab Standard Curve
R²=0.9942
4PL Sigmoidal Fit
LOD 3σ
15.1 pg/ml
Particle Count
TNFα (pg/ml)
Cy3-IL-6-Ab Standard Curve
R²=0.9958
4PL Sigmoidal Fit
LOD 3σ
2.5 pg/ml
IL-6 (pg/ml)

Figure S8: Multiplexed FluoroDOT assay. Related to Figure 5. A) Schematic illustration depicting the step-by-step method used for validation of specificity of antibody-conjugated plasmonic-fluor by spotting capture antibodies of both cytokines (TNFα and IL-6) in the same well of a microtiter plate. B) Epifluorescence images of antibody-conjugated plasmonic fluors for their respective cytokine: TNFα (top-left) and IL-6 (top-right) and both TNFα and IL-6 (bottom-middle). The top images were collected using a 4x objective, and the grey-boxes shown in 4x images were taken using the 20x objective, as shown below. C) Density-dose dependence images demonstrating increasing density of plasmonic-fluors with increasing concentration of cytokine TNFα, IL-6 in a multiplexed assay, scale bar: 100 μm and D) standard curve for the multiplexed plasmon-enhanced fluorescence-linked immunosorbent assay (p-FLISA) for TNFα and IL-6. The data was fit using a 4-parameter logistic (4 PL) sigmoidal curve. Limit of detection (LOD) is defined as (mean+3 σ) of the blank.

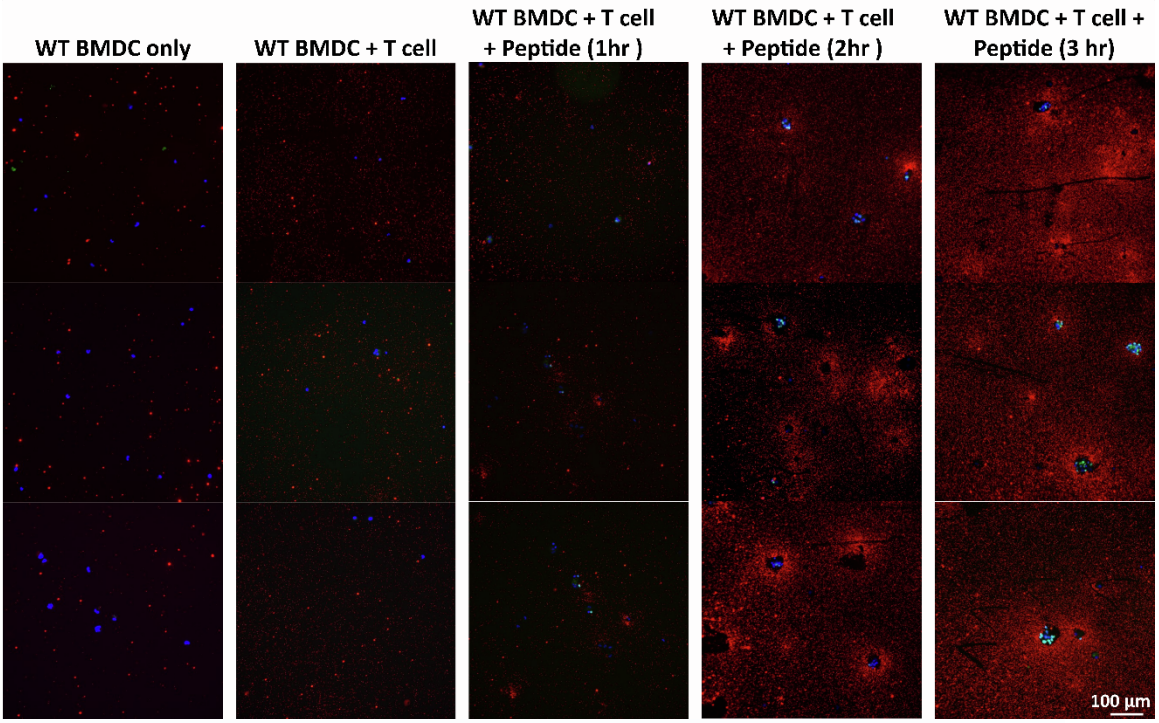

Figure S9: FluoroDOT assay using co-cultures. Related to Figure 7. IFNγ secretion by mycobacterium tuberculosis (*Mtb*) MHCII-peptide (P25)-specific $CD4^+$ T cells when co-cultured with BMDCs for 1 hour, 2 hours, and 3 hours. Cy5 (red) epifluorescence microscopy images of the cells using Cy5-plasmonic-fluor. Each panel contains three representative 20x images stitched together depicting co-cultures of BMDCs and T cells (DAPI staining nuclei) along with GFP expression (green) triggered in activated $CD4^+$ T cells. Scale bar: 100 µm.

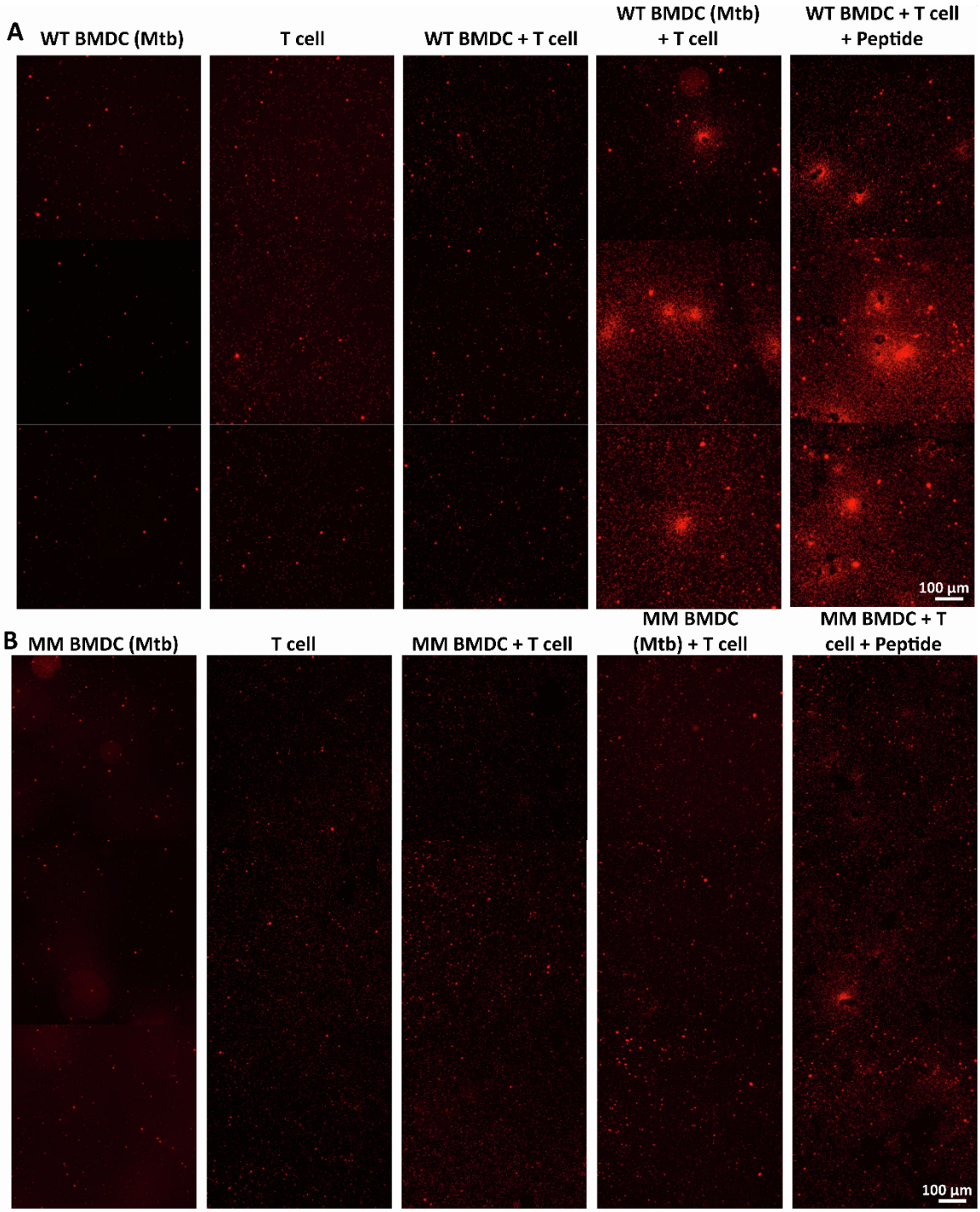

Figure S10: FluoroDOT assay using wild-type and mismatched BMDCs in co-cultures. Related to Figure 7. A) IFNγ secretion by mycobacterium tuberculosis (*Mtb*) MHCI-peptide-specific T cells when co-cultured with a) Wild-type (WT) BMDCs infected with *Mtb* and B) Mismatched (MM) BMDCs infected with *Mtb*. Cy5 epifluorescence microscopy images of the cells using Cy5-plasmonic-fluor. Each panel contains three representative 20x images stitched together. Scale bar: 100 µm.