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Supplementary Information

Pushing the limits of detection for proteins secreted from single cells using quantum dots

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Methods

Image processing and fluorescent spot analysis. Matlab (MathWorks, MA) was used to further analyze images based on individual fluorescent spots. Images were first converted to binary using a built-in command (graythresh). Discrete, contiguous fluorescent spots were then identified, and the area and mean intensity of each spot was quantified. Processed images were created to depict the size of each QD spot in black and the average intensity by color-coded outlines based on a heat map. The intensity versus area values were then plotted for control samples to establish a gate for single QDs. For positive TNF-α conditions, spots that deviated from the control distribution in terms of larger size or diminished intensity were considered to be either quenched single QDs or large QD clusters.

Bulk ELISA experiments. U-937 cells were seeded at densities of 1,000, 2,000, or 5,000 cells per well in 96-well plates and differentiated with 50 ng/ml PMA for 48 h. Following a 24 h resting period in culture media, cells were stimulated with 100 ng/ml LPS and cell supernatant was collected after 3 or 6 h LPS incubation. ELISA experiments were performed on cell supernatants to quantify TNF-α secretion, as described in the main text. Briefly, monoclonal human anti-TNF-α capture antibody (5 μg/ml in 0.1 M $NaHCO_3$, pH 9.2) was incubated in flat, non-tissue culture treated 96-well plates (Thermo Fisher Scientific, IL) for 2 h. Wells were then blocked with StartingBlock (Thermo Fisher Scientific, MA) for 15 min, followed by incubation with 50 μL of cell supernatant for 2 h at room temperature. After washing with PBS+, biotinylated anti-TNF-α detection antibody was incubated (5 μg/ml for 30 min in PBS+), followed by Neutravidin-HRP (1:500 dilution in PBS+) for 30 min. Finally, wells were washed and 100 μL 1-step Ultra TMB substrate (Thermo Fisher Scientific, IL) was added for 15 min before quenching with an equal volume of 2 M H_2SO_4. TMB absorbance at 450 nm was quantified using an Infinite 200 PRO Multimode Reader (TECAN, Switzerland). Concentration was calibrated using the purified human TNF-α results and linear regression.

Results and Discussion

Analysis of QD fluorescent spots

The complex relationship intensity profile that was observed for the ChemAmp technique would make it difficult to quantify concentration, particularly at high values. To address this issue, we processed images using Matlab to identify all contiguous fluorescent spots, which were then enumerated and assessed for intensity and area. Representative processed images are shown for select TNF-α concentrations in Fig. S4, along with the corresponding raw and grayscale images. Spot number per image increased progressively until reaching a plateau around 10 pg/ml, then fell sharply at 16 pg/ml before rising again (Fig. S4B). We then normalized the intensity by spot number, and the results are presented in Fig. S4C. We found that the intensity per spot was consistent at low TNF-α concentrations, decreased for the region spanning 0.3 to 30 pg/ml, and increased markedly at high concentrations. Since this data was likely confounded by two factors, homoquenching that decreases intensity and clustering that decreased spot number, we directly plotted intensity versus area. Density plots are shown for all TNF-α concentrations in Fig. S5A. Based on controls, which we believe primarily contained single QDs, we observed that intensity and area were well-correlated, with only a small number of outliers that may have been related to QD blinking. Next we defined a gate characteristic to single QDs, indicated by the polygons in Fig. S5A. As TNF-α concentration increased, spot distributions shifted to both lower intensity and area (region **a**) or generally larger area (region **b**). We interpreted the shifts into region **a** as corresponding to quenched single QDs and possibly small clusters, and region **b** corresponding to large clusters with varying degrees of quenching. We acknowledge that this is a crude way to assess these effects, and it is possible that some large, highly quenched clusters could be located within the single QD gate or region **a**. However, we found that the percentage of spots within regions **a** (quenched QDs) and **b** (clusters) successfully illustrated key features (Fig. S5B). For example, quenching was significant even at low TNF-α concentrations, reaching a maximum at 0.1 pg/ml. Clustering was also present at low TNF-α concentrations but rose more slowly, and peaked at 16 pg/ml. Both

quenching and clustering decreased at high TNF-α concentrations, although this was likely due to difficulty distinguishing single QDs at such high surface density. The above analysis was repeated for the QD IC, and results are presented in Figs. S6 and S7. As expected, quenching was minimal, as indicated by a relatively flat intensity per spot response curve (Fig. S6B) and very low percentages of both quenched and clustered spots (Fig. S7B). Taken together, we believe that the image processing data could make it possible to calibrate TNF-α concentration for the ChemAmp technique across the fully dynamic range of interest. Specifically, the combination of average intensity, intensity/spot, and ratio of abnormal spot (quenched and clustered) data could be used in a "look-up" table approach (Table S1). This would start with a general determination of the concentration range. The low range extends up to ~0.3 pg/ml, and is characterized by intensity/spot values slightly >1 and % quenched values that roughly scale with intensity. The moderate range from 0.3 to 30 pg/ml is dominated by quenching, and as such is characterized by intensity/spot values <1 and generally high numbers of quenching and clustered spots (>10% each). The high range >30 pg/ml has substantially greater intensity and intensity/spot values and very low numbers of quenched spots. Within the low and high ranges, we believe that intensity provides an accurate calibration of TNF-α concentration. The moderate range would be most complex, requiring the combination of intensity, % quenched, and % clustered data to approximate TNF-α concentration. We acknowledge that more detailed investigation will be required to confirm these results and improve robustness so that the ChemAmp technique can be deployed for various applications, including single cell secretion studies.

Bulk ELISA experiments to assess cell secretion

To assess potential paracrine effects that would affect bulk populations, but not single cells, we performed experiments in which PMA-differentiated, LPS-activated U937 cell density was varied from 1,000 to 5,000 cells per well. ELISAs were performed on supernatants after 3 and 6 hours incubations with LPS, and results were calibrated to TNF-α concentration per well

(Fig. S9A) and converted to number of molecules secreted per cell (Fig. S9B). TNF-α concentration increased with both time and cell density, as expected. Interestingly, the number of TNF-α molecules secreted per cell also increased in a dose dependent manner with cell density, particularly for the 3 hour data. We could not asses whether this trend extended to lower densities because ELISA signal was undetectable above background. Notably, the average secretion rate for the 1,000 cell/well and 3 hour incubation time condition was >100,000 molecules/cell. This was orders of magnitude greater than single cell studies for both TMR and QD IC assays, which had average secretion rates of approximately 230 and 50 molecules/cell, respectively. These average secretion numbers include the zero-count population. These data add to a growing body of literature suggesting the importance of paracrine signaling in the activation of macrophage populations.

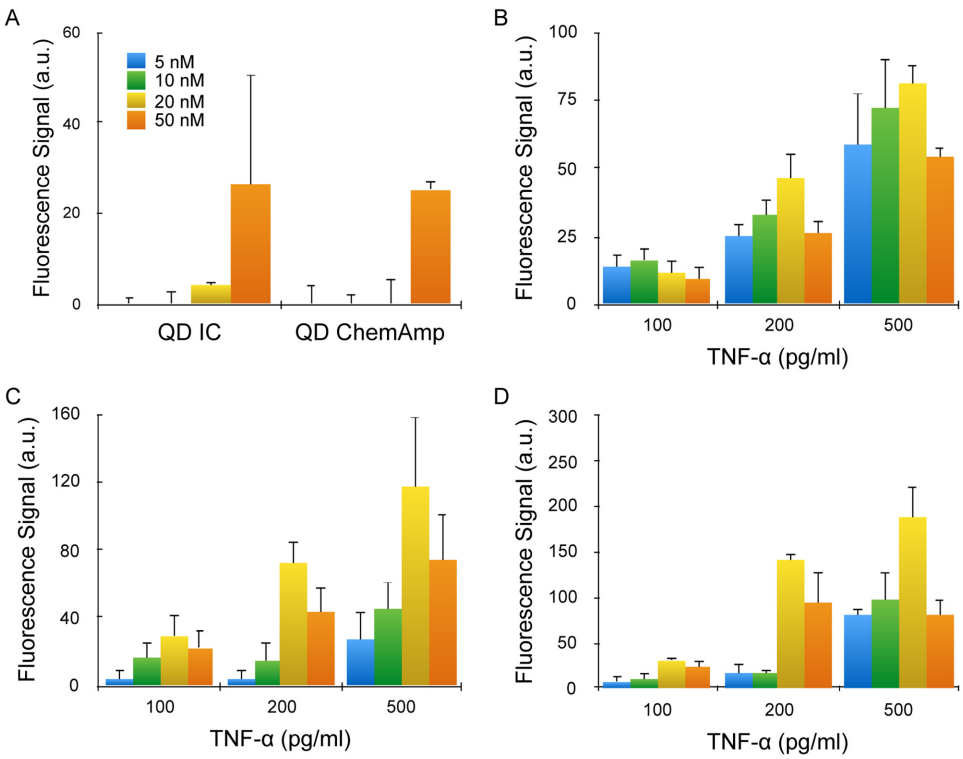

Fig. S1. TNF-α detection using QDs in microtiter plates. (A) Background for the quantum dot (QD) immunoconjugate (IC) and tetrazine-modified QD at 5, 10, 20, and 50 nM concentrations. Background was defined as signal coming from nonspecific QD in the absence of TNF-α. (B-D) Specific signal after background subtraction for (B) QD IC and the ChemAmp technique using (C) TCO and (D) PEG-TCO modified detection antibody. Results were similar at 100, 200, and 500 pg/ml TNF-α concentrations. Error bars represent the standard error from at least three independent experiments.

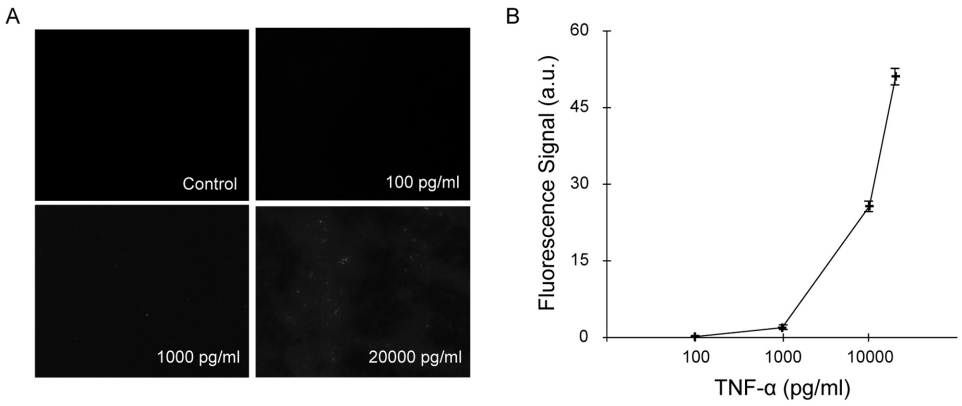

Fig. S2. TNF-α detection by imaging rhodamine. (A) Representative images at select TNF-α concentrations for tetramethylrhodamine (TMR)-modified detection antibody. (B) Fluorescence intensity standard curve obtained using different TNF-α concentrations, after background subtraction. Detection threshold was ~1 ng/ml (60 pM). Error bars represent the standard error from at least three independent experiments.

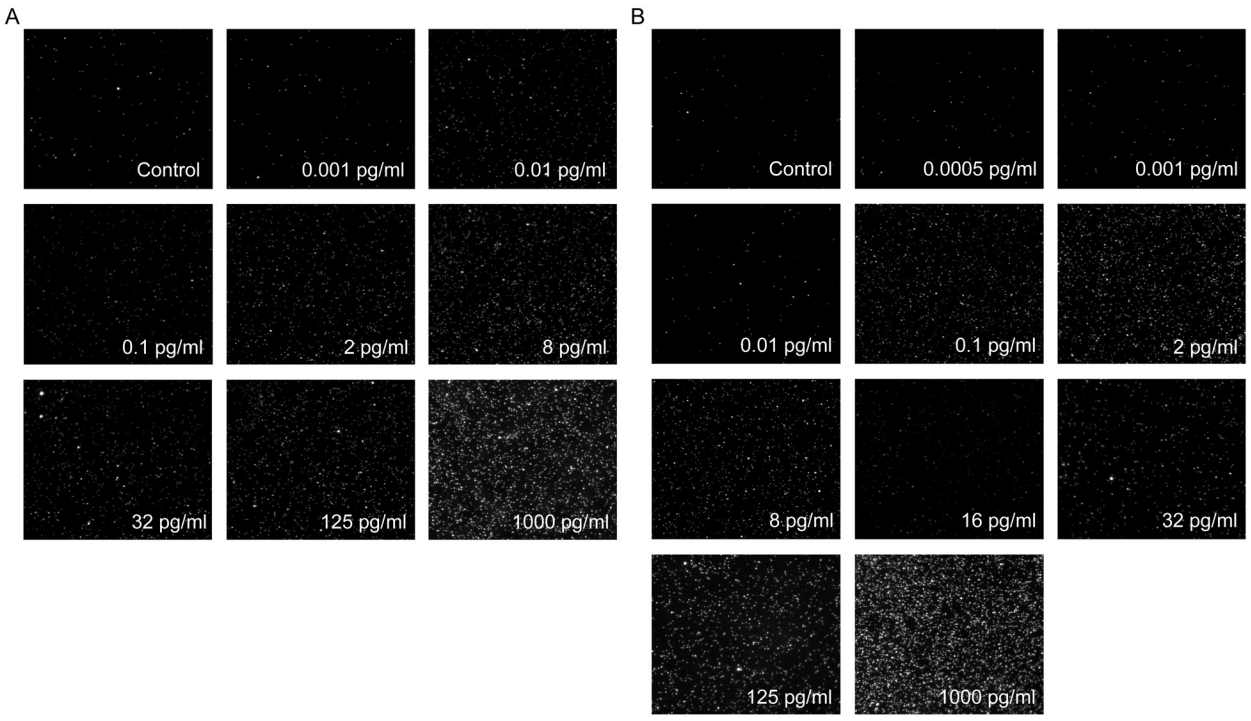

Figure S3. Representative QD images at all TNF-α concentrations. Results shown are for (A) QD IC and (B) ChemAmp cases, and TNF-α concentration is indicated in each image.

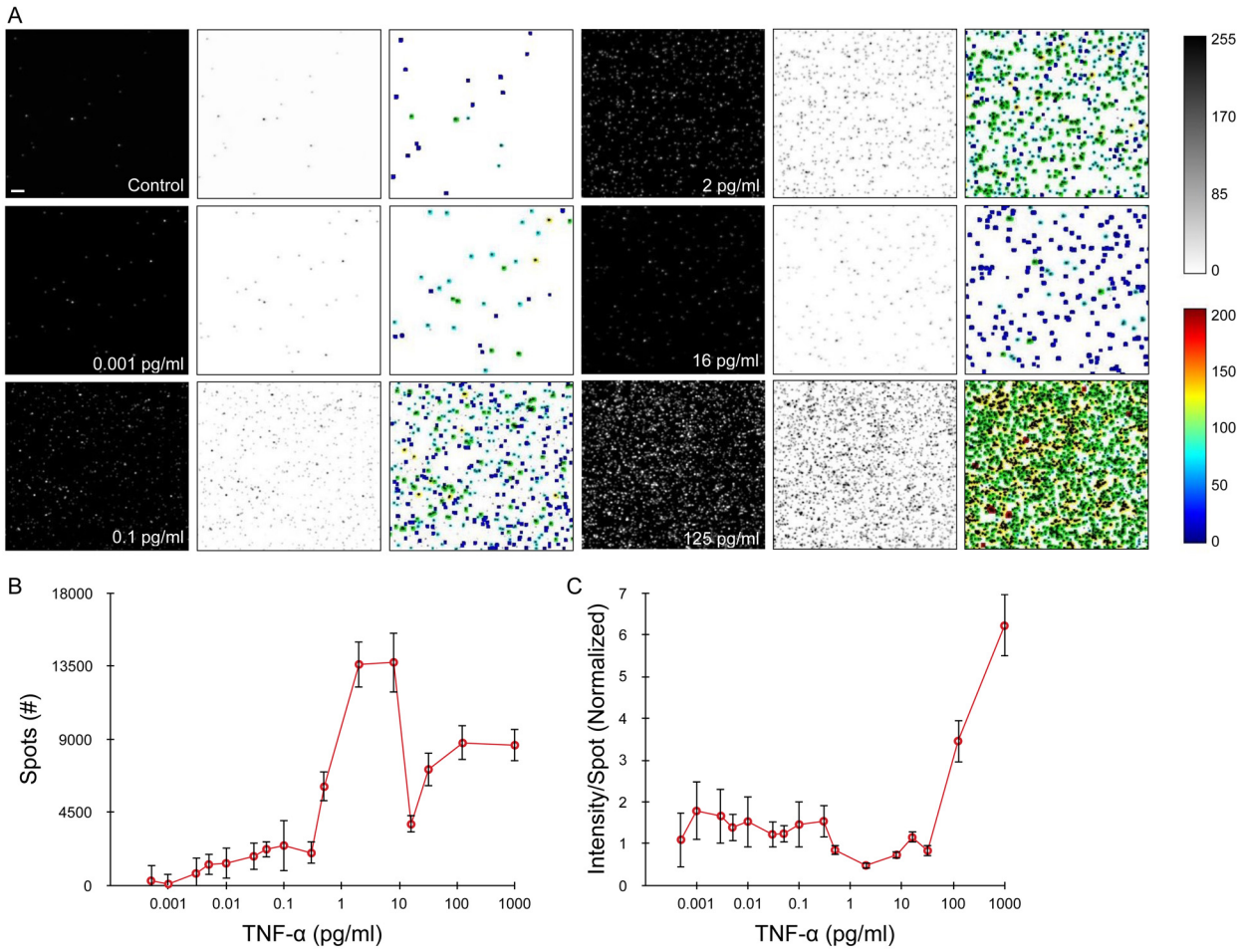

Figure S4. Image processing of fluorescent spots obtained with the ChemAmp technique. (A) Representative processed images at select TNF-α concentrations, shown side-by-side with raw fluorescence and grayscale images for comparison. Intensity scalings are indicated by the legends in the lower right hand corner (white to black for grayscale, heat map for processed). (B) Spot number at different TNF-α concentrations. Control value was subtracted. (C) Plot of intensity per spot, normalized to the control (value = 1). Results were close to the control up to 0.3 pg/ml, then decreased below 1 due to quenching. Above 30 pg/ml, QDs were too close together to distinguish. Scale bar is 100 μm. Error bars represent the standard error from at least three independent experiments.

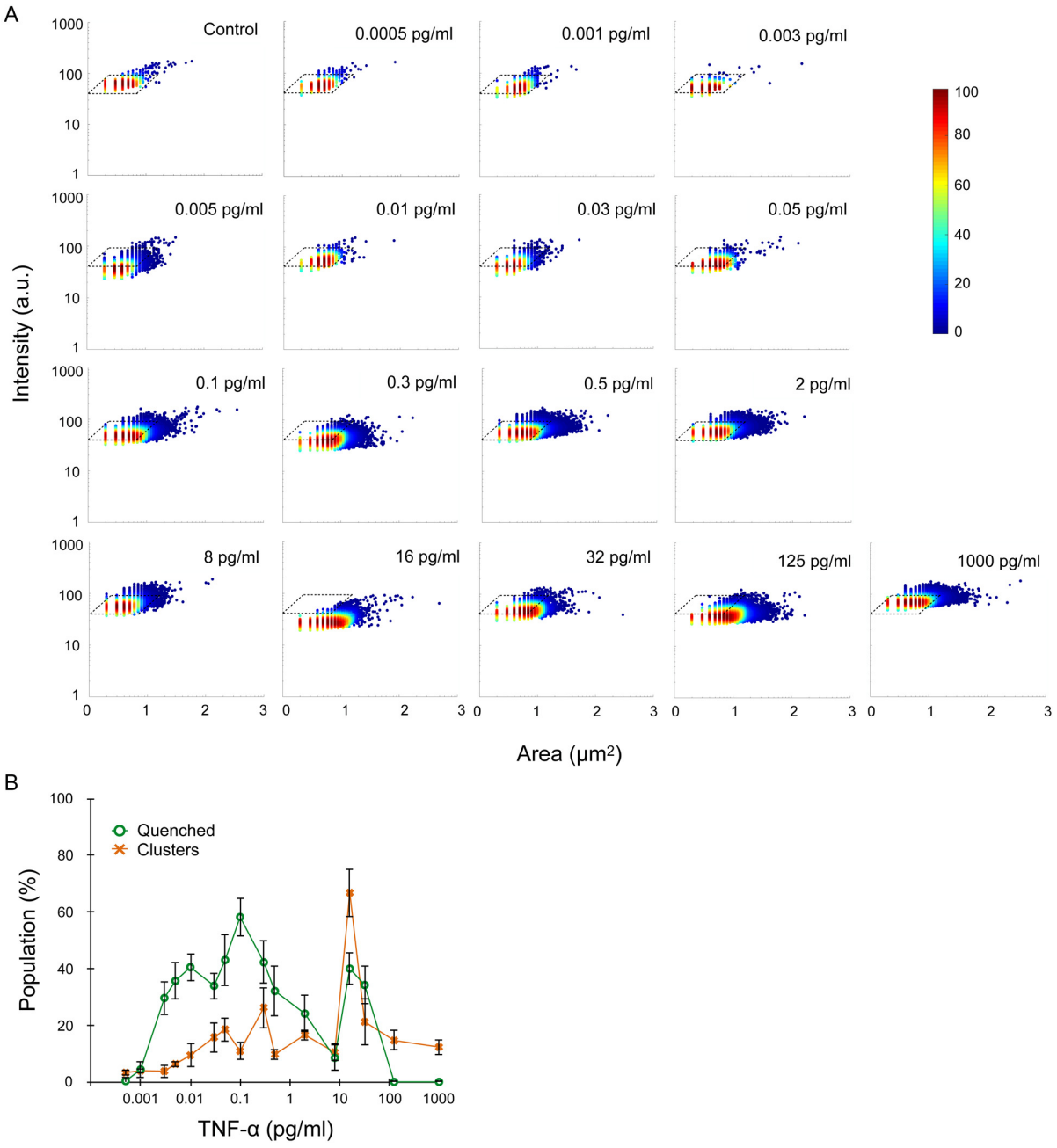

Figure S5. Analysis of intensity versus area plots for the ChemAmp technique. (A) Representative density plots of spot intensity vs area for all TNF-α concentrations. (B) Total percentage of quenched and clustered spots plotted versus TNF-α concentration. Error bars represent the standard error from at least three independent experiments.

Table S1. Reference table for the QD ChemAmp technique. The combination of all metrics could be used as a unique identifier for TNF-α concentration.

TNF-α (pg/ml)	Intensity (Normalized)	Intensity/Spot (Normalized)	Quenched (%)	Clusters (%)
0	1.0	1.0	2.4 ± 0.2	3.0 ± 0.6
0.0005	1.3 ± 0.5	1.1 ± 0.7	2.7 ± 1.3	6.3 ± 1.2
0.001	1.9 ± 0.4	1.8 ± 0.7	6.8 ± 2.9	7.0 ± 1.0
0.003	2.4 ± 0.2	1.7 ± 0.6	32.1 ± 5.9	6.8 ± 2.4
0.005	2.4 ± 0.3	1.4 ± 0.3	38.1 ± 6.6	9.4 ± 1.1
0.01	2.7 ± 0.7	1.5 ± 0.6	43.0 ± 4.8	12.4 ± 4.2
0.03	2.5 ± 0.3	1.2 ± 0.3	36.4 ± 4.8	18.8 ± 5.3
0.05	2.8 ± 0.3	1.2 ± 0.2	45.5 ± 9.1	21.6 ± 4.2
0.1	3.5 ± 0.4	1.5 ± 0.6	60.7 ± 6.8	13.9 ± 3.1
0.3	3.3 ± 0.5	1.5 ± 0.4	44.7 ± 7.7	29.3 ± 7.3
0.5	3.8 ± 0.4	0.8 ± 0.1	34.5 ± 9.0	12.8 ± 2.0
2	4.2 ± 0.5	0.5 ± 0.1	26.6 ± 6.6	19.6 ± 1.9
8	6.4 ± 0.4	0.7 ± 0.1	11.0 ± 4.6	13.5 ± 3.3
16	3.6 ± 0.2	1.1 ± 0.1	42.4 ± 5.9	69.9 ± 8.6
32	4.2 ± 0.4	0.8 ± 0.1	36.7 ± 6.7	24.2 ± 8.2
125	20.6 ± 2.2	3.5 ± 0.5	0.3 ± 0.4	17.7 ± 3.7
1000	36.6 ± 2.6	6.2 ± 0.7	0.1 ± 0.1	15.4 ± 2.6

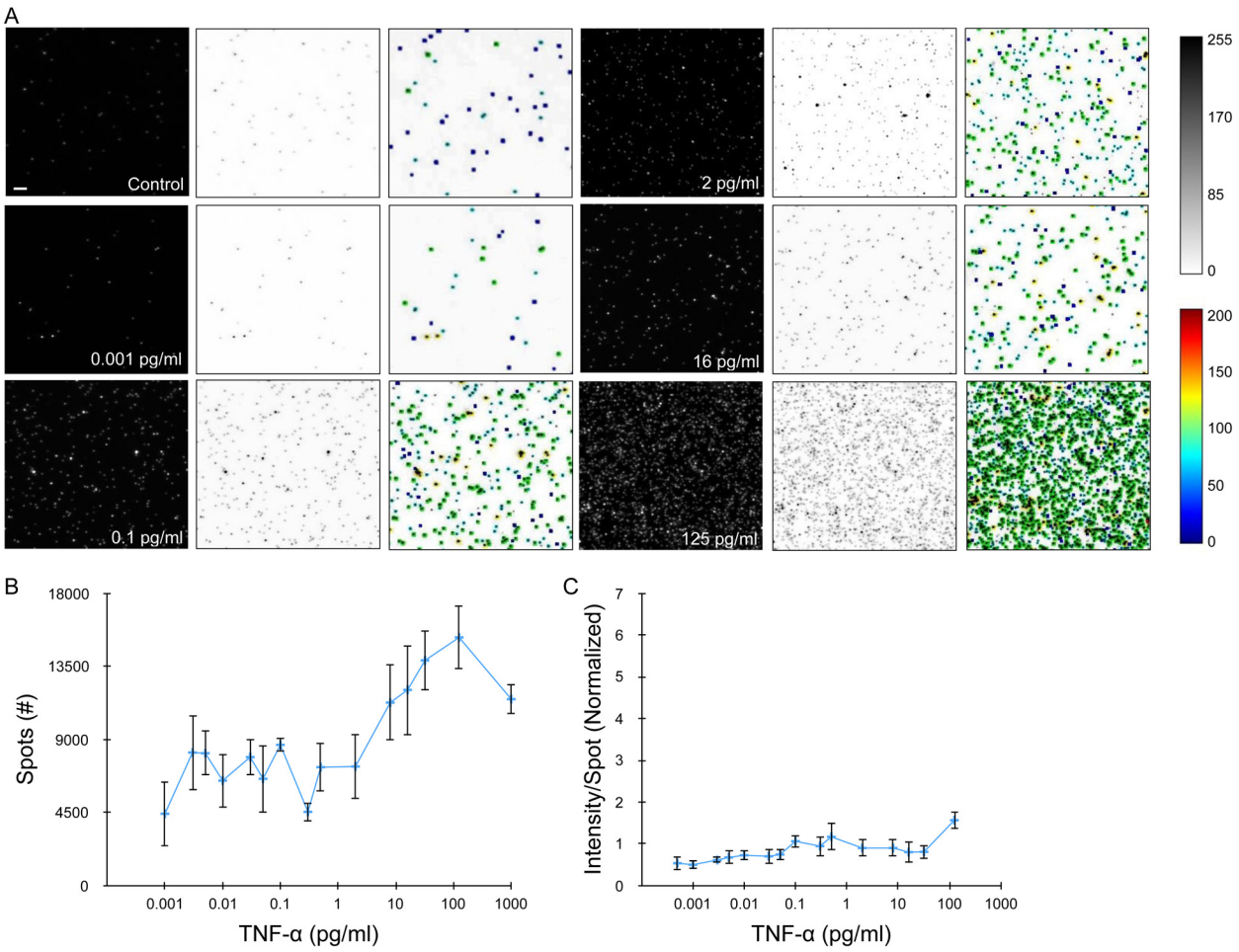

Figure S6. Image processing of fluorescent spots obtained with the QD IC. (A) Representative processed images at select TNF-α concentrations, shown side-by-side with raw fluorescence and grayscale images for comparison. Intensity scalings are indicated by the legends in the lower right hand corner (white to black for grayscale, heat map for processed). (B) Spot number at different TNF-α concentrations. Control value was subtracted. (C) Plot of intensity per spot, normalized to the control (value = 1). Results were close to the control up to 0.3 pg/ml, then decreased below 1 due to quenching. Above 30 pg/ml, QDs were too close together to distinguish. Scale bar is 100 μm. Error bars represent the standard error from at least three independent experiments.

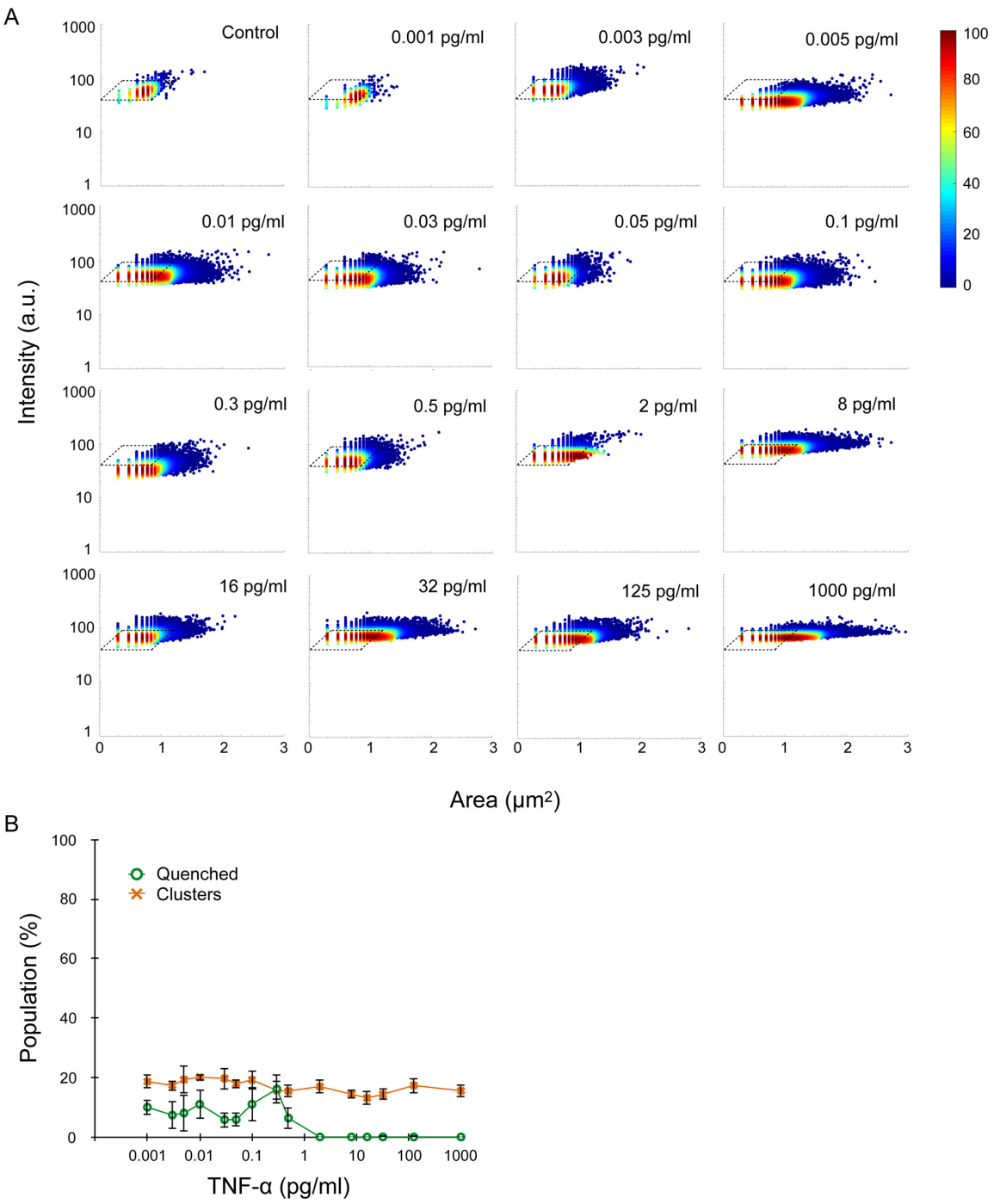

Figure S7. Analysis of intensity versus area plots for the QD IC. (A) Representative density plots of spot intensity vs area for all TNF-α concentrations. (B) Total percentage of quenched and clustered spots plotted versus TNF-α concentration. Error bars represent the standard error from at least three independent experiments.

Table S2. Reference table for the QD IC.

TNF-α (pg/ml)	Intensity (Normalized)	Intensity/Spot (Normalized)	Quenched (%)	Clusters (%)
0	1.0	1.0	4.1 ± 0.5	2.4 ± 0.6
0.001	1.1 ± 0.2	0.5 ± 0.2	14.1 ± 2.4	21.0 ± 2.4
0.003	1.6 ± 0.2	0.5 ± 0.1	11.5 ± 4.6	19.7 ± 1.6
0.005	1.9 ± 0.1	0.6 ± 0.1	12.1 ± 6.2	21.7 ± 4.7
0.01	1.8 ± 0.3	0.7 ± 0.2	15.1 ± 5.0	22.4 ± 1.0
0.03	2.2 ± 0.3	0.7 ± 0.1	9.9 ± 2.5	22.0 ± 3.7
0.05	1.9 ± 0.3	0.7 ± 0.2	9.9 ± 2.4	20.3 ± 1.6
0.1	2.4 ± 0.4	0.7 ± 0.1	15.1 ± 5.8	21.6 ± 3.1
0.3	2.3 ± 0.3	1.1 ± 0.2	20.2 ± 5.1	18.0 ± 3.1
0.5	2.7 ± 0.6	0.9 ± 0.2	10.4 ± 3.7	17.8 ± 2.3
2	3.4 ± 0.7	1.2 ± 0.3	0.3 ± 0.2	19.3 ± 2.5
8	3.5 ± 0.6	0.9 ± 0.2	0.5 ± 0.3	16.8 ± 1.5
16	3.7 ± 0.6	0.9 ± 0.2	0.3 ± 0.3	15.5 ± 2.3
32	3.7 ± 1.1	0.8 ± 0.3	0.0	16.7 ± 1.9
125	4.0 ± 0.8	0.8 ± 0.2	0.0	19.7 ± 2.6
1000	6.2 ± 0.7	1.6 ± 0.2	0.2 ± 0.2	17.9 ± 2.1

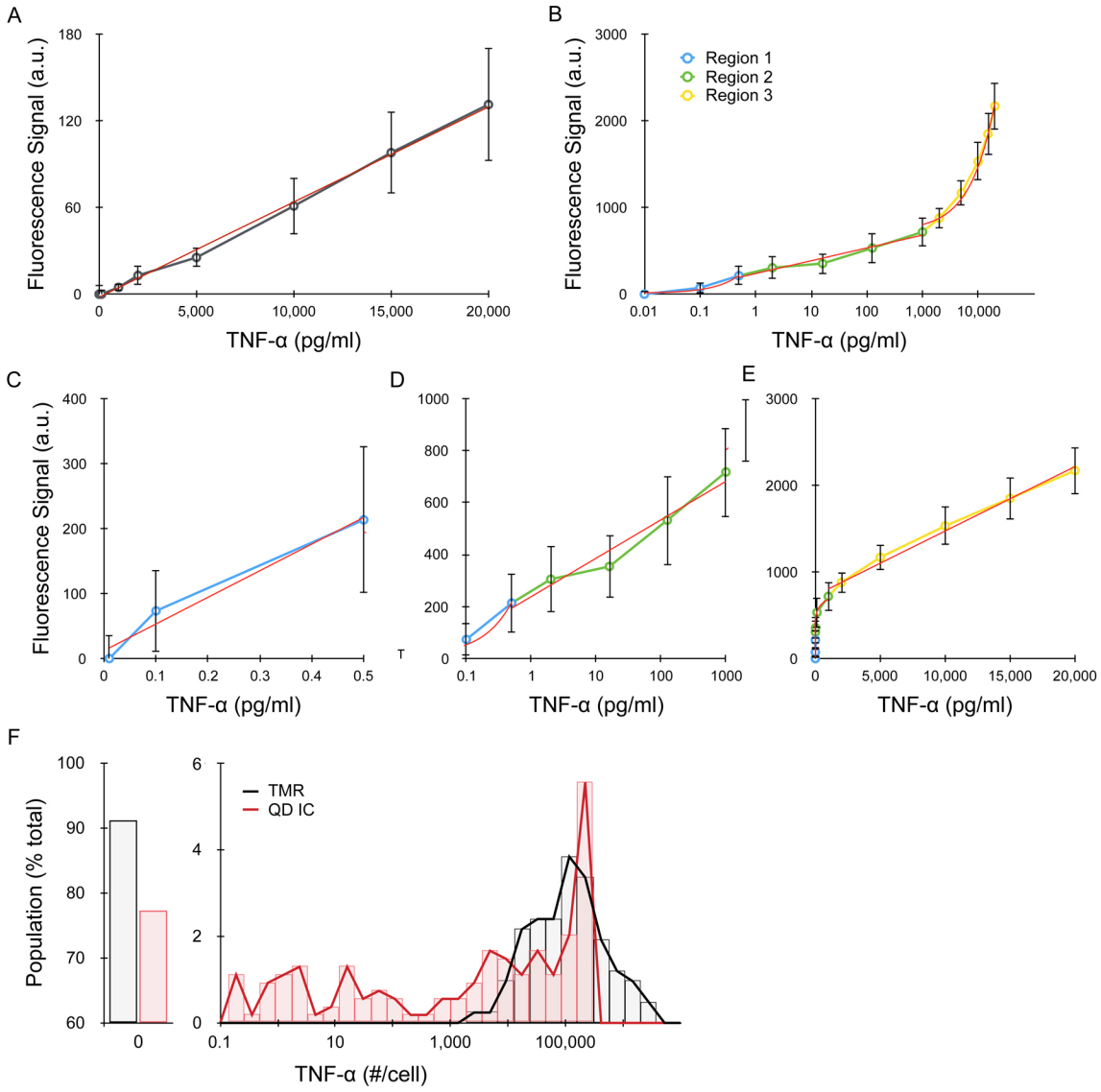

Figure S8. Calibration of TNF-α for single cell secretion studies. (A,B) Standard curves obtained for (A) TMR and (B) QD IC. The fits used to calibrate concentration in single cell secretion studies are shown in red. (C-E) Three separate fittings were used to encompass the full dynamic range of the QD IC, including (C) low, (D) moderate, and (E) high TNF-α ranges. (F) Histogram of single cell secretion after calibrating TNF-α concentration and number per cell. This data includes false-positives (3% of total population) that were removed from Fig. 4E in the main text. Error bars represent the standard error from at least three independent experiments.

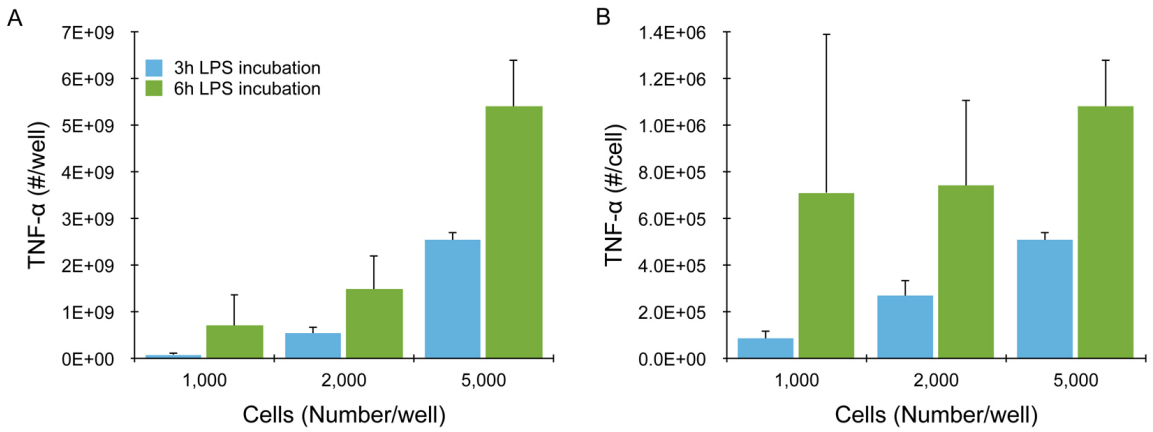

Figure S9. Bulk TNF-α secretion experiments at different cell densities. U937 cells were plated at 1,000, 2,000, or 5,000 cells/well, differentiated with 50 ng/ml PMA, and stimulated with 100 ng/ml LPS for 3 or 6 hours. TNF-α was quantified by ELISA, and results are presented as TNF-α (A) concentration and (B) molecule number per cell. TNF-α secretion increased on a per cell basis with time and seeding density. Error bars represent the standard error from at least three independent experiments.