Supporting Information for "Super-Resolution Imaging Reveals a Difference between SERS and Luminescence Centroids"

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SERS Solution Preparation

Silver nanoparticles are synthesized via the method developed by Lee and Meisel.[1] SERS samples are prepared using 2 mL of the aqueous colloids to which is added 200 μL of 21 nM Rhodamine 6G (R6G) dye in methanol and 2 mL of 20 mM aqueous sodium bromide. The solution is vortexed for 60 seconds and then incubated for one hour.

Substrate Preparation

As previously reported, aluminum metal is deposited through an alphanumeric TEM grid (Ted Pella, Inc.) which acts as a shadow mask on the surface of an indium tin oxide-coated glass coverslip (15-30 nm ITO on 0.17 mm borosilicate glass, SPI Supplies)[2]. After deposition, the coverslips are placed in an Oxford RF plasma cleaner and exposed to atmosphere for 10 minutes, which increases the hydrophilicity of the surface, allowing more uniform sample drying.

SERS Sample Deposition

After the SERS sample has incubated for one hour, 4 μL are dropcast onto the gridded area of the ITO coverslip and dried under a stream of nitrogen. Once dry, the coverslip is then washed with nanopure water to remove any crystals of sodium bromide on the surface and is dried again under nitrogen. Finally, 1 μL of 500x diluted Spherotech Blue Sky fluorescent polystyrene beads (0.5 μm diameter) is dropcast and dried on the coverslip. These beads are used to correct for stage drift in CCD movies prior to super resolution analysis and are also used to match angular position when the sample is transferred between the optical and electron microscopes.

SEM Images of Aggregates in Article and Associated Intensity Time Traces

The following figure contains the original SEM images collected for all aggregates in the Article. These are provided so the junction regions and particle surface can be viewed entirely without the spatial intensity overlays. The associated intensity time traces and selected spectral data are shown so that the on/off behavior of the SERS can be confirmed. Intensity thresholds are determined using a complete series of spectral data, as described in the main text.

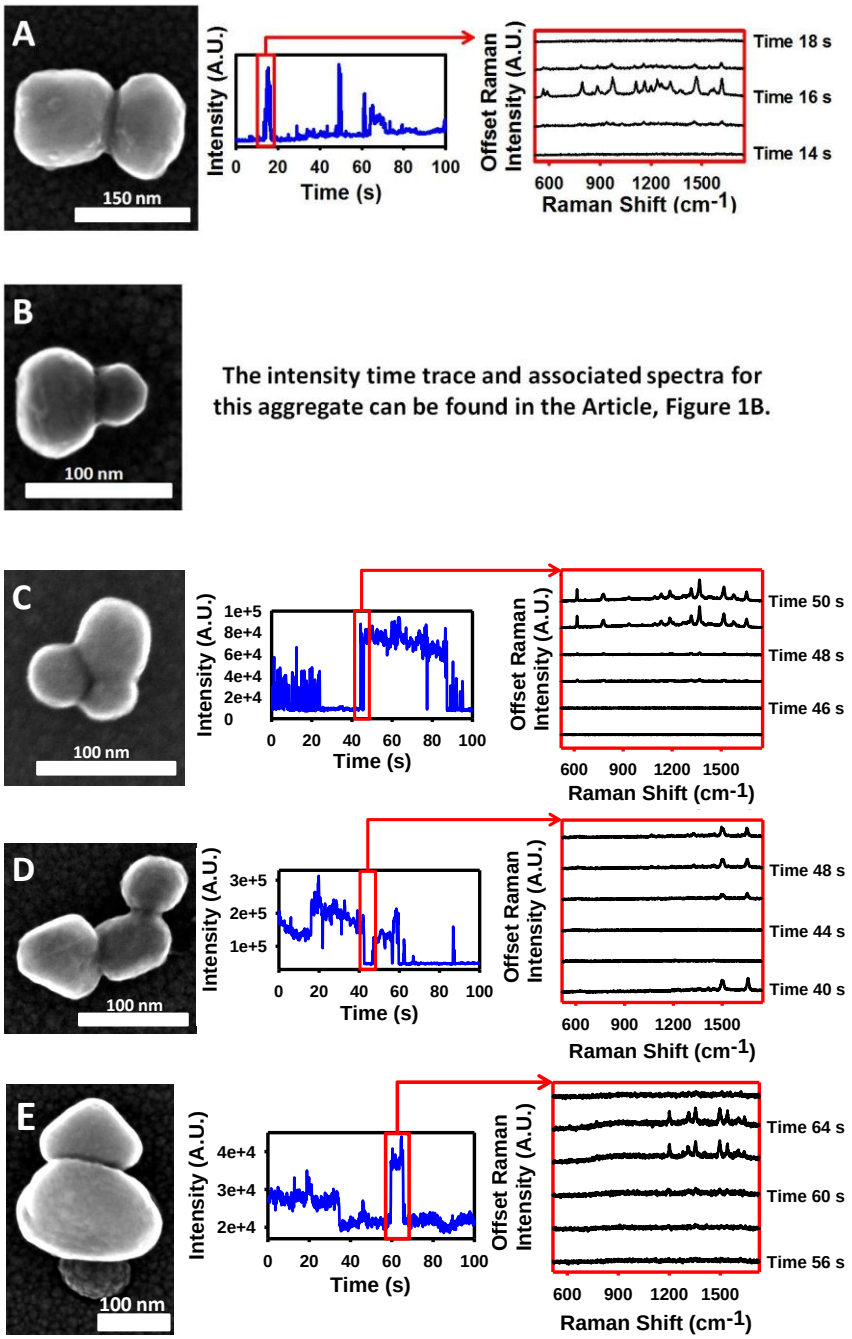

Figure S-1: SEM images and associated intensity time traces and selected spectra data corresponding to the following figures from the main manuscript: A) Figure 2B, B) Figure 3B, C) Figure 5B, D) Figure 6A, E) Figure 6B.

Super Resolution Imaging Through Point Spread Function Fitting

As described previously, point spread function fitting (PSF) is used to isolate and track the position of the SERS and luminescence signals[3]. To clarify, we will walk through the entire process for the dimer shown in Figure 2 in the Article. Spectra are collected using a 1200 groove/mm grating in the PI ACTON SpectraPro 2500i spectrograph. This data is used to confirm which simultaneously collected CCD images will represent R6G SERS and luminescence emission or simply luminescence emission. For a specific region of interest (ROI) in the CCD images, we integrate the total intensity and plot it as a function of time (Figure S-2A). We then set a threshold for the data where every frame whose intensity is below the threshold will be designated as nanoparticle luminescence and everything above will be luminescence and R6G SERS.

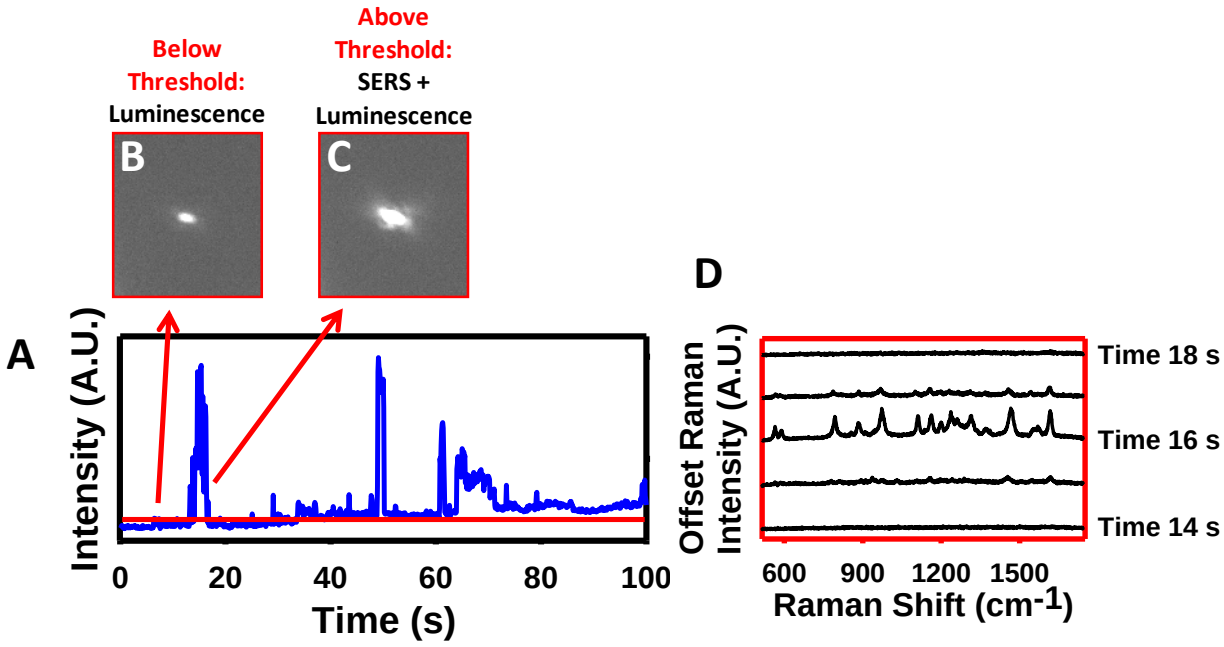

Figure S-2: A) Intensity Time Trace for Square Dimer. B) Diffraction-limited CCD image of luminescent aggregate. C) CCD image of same luminescent aggregate with SERS emission from one or more R6G molecules. D) Correlated spectra for CCD images shown in B and C.

This threshold allows us to independently track the emission from the metal nanoparticles (luminescence) and the emission from R6G molecules probing an aggregate's surface. The threshold is set as close as possible to the background signal to prevent any SERS from biasing the position of the luminescence (fitting process to be described later). This is confirmed by comparing the intensity time trace with the spectral data to confirm that no SERS peaks are evident for frames whose intensity is below the threshold. Figure S-2B was collected at approximately 14 seconds when no R6G peaks were evident in the spectral data and is

designated as luminescence emission. Figure S-2C was collected at approximately 16 seconds when one or more R6G molecules were emitting and is designated as luminescence with SERS emission. Note that the optical images shown have a slight ellipticity; this is due, in part, to the beamsplitter used to split the signal between the spectrometer and the imaging CCD, as well as to the highly polarized emission which originates from a nanoparticle dimer, as described in detail elsewhere.[4]

After the threshold has been drawn, the emission pattern for each frame whose intensity falls below the threshold is fit to a single 2-dimensional Gaussian as defined:

$$I(x,y) = z_0 + I_0 e^{\left[-\frac{1}{2}\left[\left(\frac{x-x_0}{s_x}\right)^2+\left(\frac{y-y_0}{s_y}\right)^2\right]\right]} \qquad \text{(S-1)}$$

where I is the intensity for a given position (x,y) in space, z_0 is the background intensity, I_0 is the intensity at the center of the fit, $s_{x,y}$ are the standard deviations for x and y, respectively, and x_0 and y_0 are the centroid of the fit. We have six adjustable parameters in our fits—z_0, I_0, x_0, y_0, s_x, and s_y—and we fit each diffraction limited spot to equation S-1 using nonlinear least squares by minimizing by the standard error of the fit using homewritten Matlab code. This fitting process will produce a collection of emission centroid positions for all frames designated as "luminescence emission." This data can be plotted to determine the average position for the luminescence centroid throughout the course of an experiment as shown in Figure S-3A.

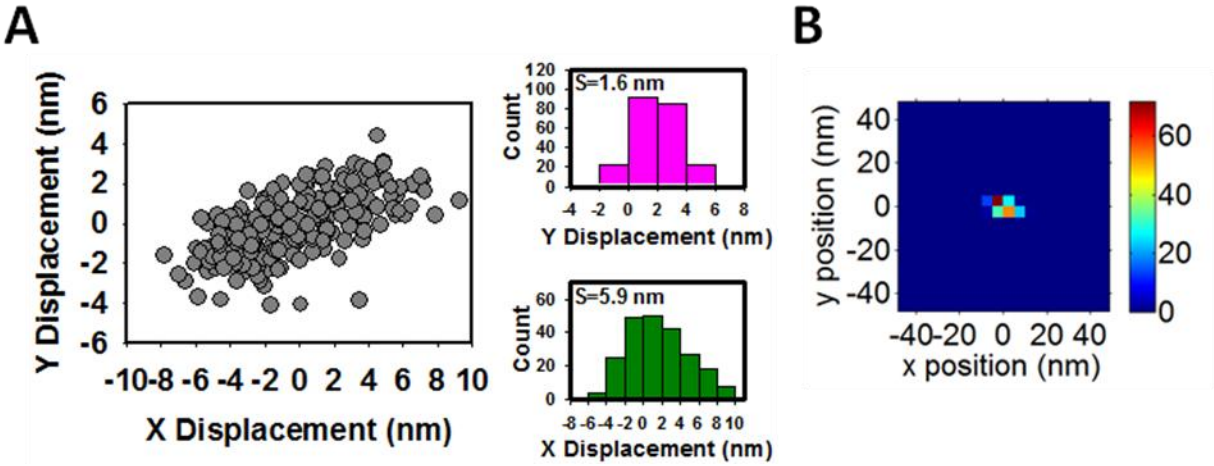

Figure S-3: (A, left) Scatter plot showing the luminescence centroid positions for the square dimer aggregate from Figure 2B. (0,0) represents the mean position of all luminescence centroids. (A, right) Histograms showing the centroid displacement from the mean in both the x (green) and y (pink) directions. (B) Two-dimensional histogram, showing that the frequency with which a particular x_0, y_0 location is fit. (This histogram contains a vertical reflection of the data in the scatter plot) Note that the elliptical shape observed in the scatter plot is due to low probability events and the bulk of the emission originates from a well-defined region.

Histograms for the scatter plot data are shown in Figure S-3A (right) and S-3B, and show that most of the centroid data are clustered around some mean position, arbitrarily defined as (0, 0). The slight ellipticity is most likely due to low single SERS events that bias the centroid towards the origin of the SERS, as we have previously reported.[2] Once the average position of the

luminescence is determined, the point spread function of the luminescence contribution can be subtracted from the raw image data for all frames above our intensity threshold, which is the superposition of the luminescence and SERS emission. The remaining signal will be only due to SERS emission and can also be fit to the same Gaussian equation listed previously (Equation S-1). The centroid data extracted from the fit can be plotted as a scatter plot as shown in Figure S-4.

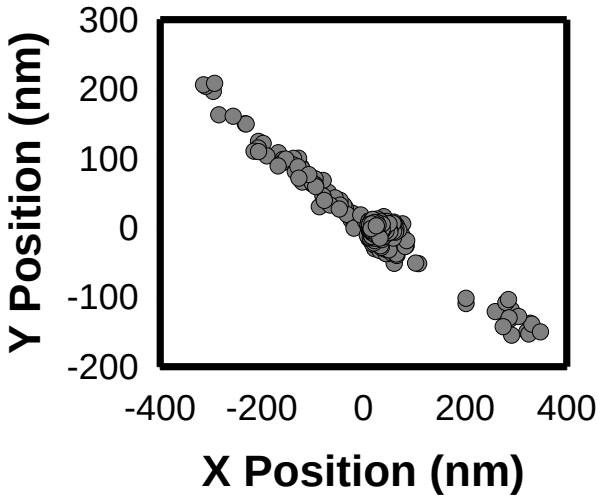

Figure S-4: SERS centroid data distributed about luminescence centroid mean (0,0).

Although this can give us some information, the centroid overlap makes it difficult to know where most of the events are occurring and their relative intensity. To counter this issue, we can bin the data in 4.6 nm bins to calculate the frequency with which a particular centroid position is fit (as in Figure S-3B). From there, we calculate the mean intensity of all points that fall within a given bin to construct the spatial intensity maps shown in the manuscript. (Note: only Gaussian fits that have an R^2 value greater than or equal to 0.8 are included in this binned data.) The spatial intensity map for the data in Figure S-4 can be found in the Article in Figure 2A.

One other important detail to note is that the point spread functions of at least 3 or more fluorescent marker beads are also fit to two-dimensional Gaussians to extract their centroid positions. These centroid positions are calculated for all 1000 frames of a CCD movie and are used to correct for drift in the microscope stage. Both the luminescence and SERS centroid positions are corrected using this fluorescent bead data. This point spread function fitting technique allows us to track various emission signals as a function of time and space to an accuracy of +/- 5 nm, a significant improvement over the optical resolution of ~250 nm.

The following figure contains the luminescence centroid scatter plots and histograms with associated standard deviation for all the aggregates found in the Article.

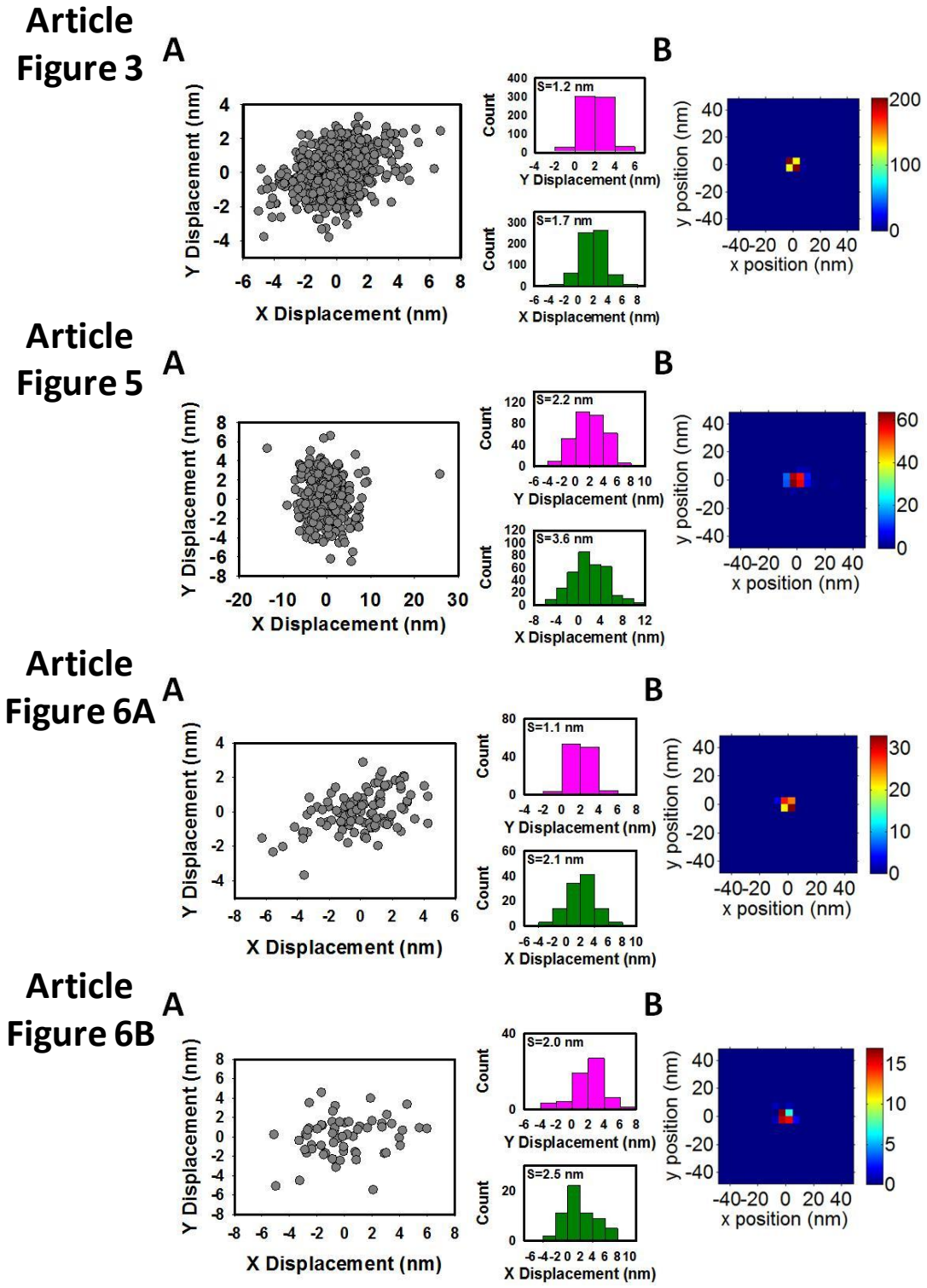

Figure S- 5: (A, left) Scatter plots showing the luminescence centroid positions for the aggregates in the Article. (0,0) represents the mean position of all luminescence centroids. (A, right) Histograms showing the centroid displacement from the mean in both the x (green) and y (pink) directions. (B) Two-dimensional histogram, showing that the frequency with which a particular x_0, y_0 location is fit. (This histogram contains a vertical reflection of the data in the scatter plot).

Calculating the Resolution of the Luminescence and SERS Signals

The accuracy of determining the position of a diffraction-limited emitter through point spread function fitting can be calculated using the following expression[5,6]:

$$\sigma_{u_i} = \sqrt{\left(\frac{s_i^2}{N} + \frac{a^2/12}{N} + \frac{8\pi s_i^4 b^2}{a^2 N^2} \right)} \qquad \text{(S-2)}$$

In this equation, the standard error of the mean of the point spread function, σ_μ, is dependent upon: the standard deviation of the Gaussian fit (s_x and s_y from equation S-1), s_i; the pixel size of the CCD camera in nm, a; the standard deviation of the background in photons, b; and the number of collected photons, N. As an example, we calculate the theoretical accuracy of the fit for the nanoparticle aggregate shown in Figure 2B and Figure S-3. For the luminescence data, we use the average number of emitted photons for N, while in the SERS data, we choose the frame corresponding to the *lowest* SERS signal. For the latter case, this provides an upper limit of the calculated accuracy, since all events with higher signal will have a lower calculated accuracy.

Table S-1: Standard error of the mean for the SERS and luminescence centroids

Parameter	Luminescence	SERS (worst case)
***s* (pixels, pixel)**	4.03	4.26
***a* (nm per pixel)**	46	46
***s* (nm)**	185.38	195.96
***b* (photons)**	62.9	62.9
***N* (photons)**	3.3×10^5	5.25×10^4
σ_μ (nm)	**0.78**	**5.09**

Spatial Intensity Map of Figure 5A in Article

All spatial intensity maps shown in the Article were prepared using the fitting algorithm described above, with the exception of Figure 5A. The intensity time trace for this aggregate showed significant "flicker noise" which makes it difficult to draw a clean threshold using the associated spectral data. For this map, instead of the normal single threshold, two thresholds were defined.

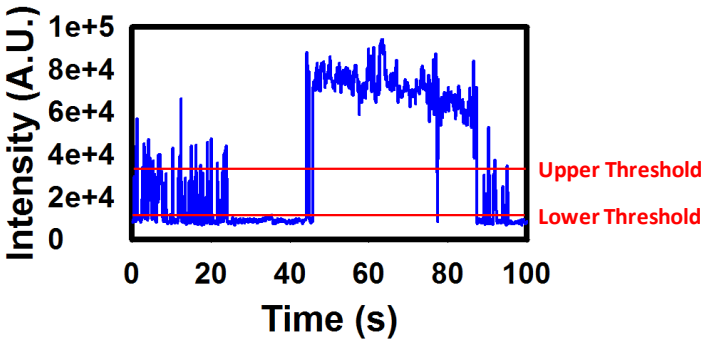

Figure S-6: Intensity time trace threshold levels for Article Figure 5A

The lower threshold was drawn just above the luminescence background and the upper threshold was drawn towards the top of the flicker noise. This parameter and the fact that $R^2 \geq 0.8$ prevents most of those flicker on events from being included in the spatial intensity map. The flicker events leads to poor fitting resolution, which extends the spatial intensity map over a larger area than is possible based on the size of the nanoparticle (although the shape and intensity gradient behavior is preserved). The majority of data appearing in that spatial intensity map will be fit from the strong on event from ~50 to 90 seconds. Other than this change in threshold levels, the Gaussian fitting procedure was the same for the aggregate of Figure 5 in the Article.

DDA Results for Figure 6B

The dielectric function of silver chosen to parametrize our DDA calculations was taken from Palik.[7] All numerical electrodynamics studies involved an interdipole spacing of 1 nm except for the calculations underlying Fig. 4B, which were performed using a 2 nm interdipole spacing.

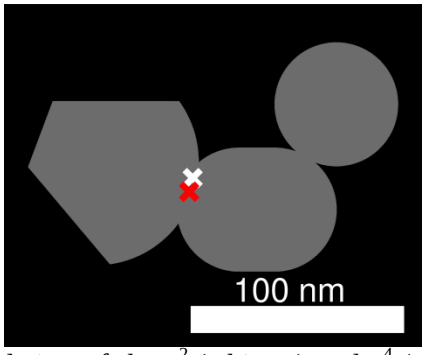

Figure S-7: Rough DDA calculation of the ε^2 (white x) and ε^4 (red x) centroid positions for the modeled trimer

References

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(7) *Handbook of Optical Constants of Solids*; Palik, E. D., Ed.; Academic Press: New York, 1985, pp 275-367.