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Supplementary information

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Tracking endocytosis and intracellular distribution of spherical nucleic acids with correlative single-cell imaging

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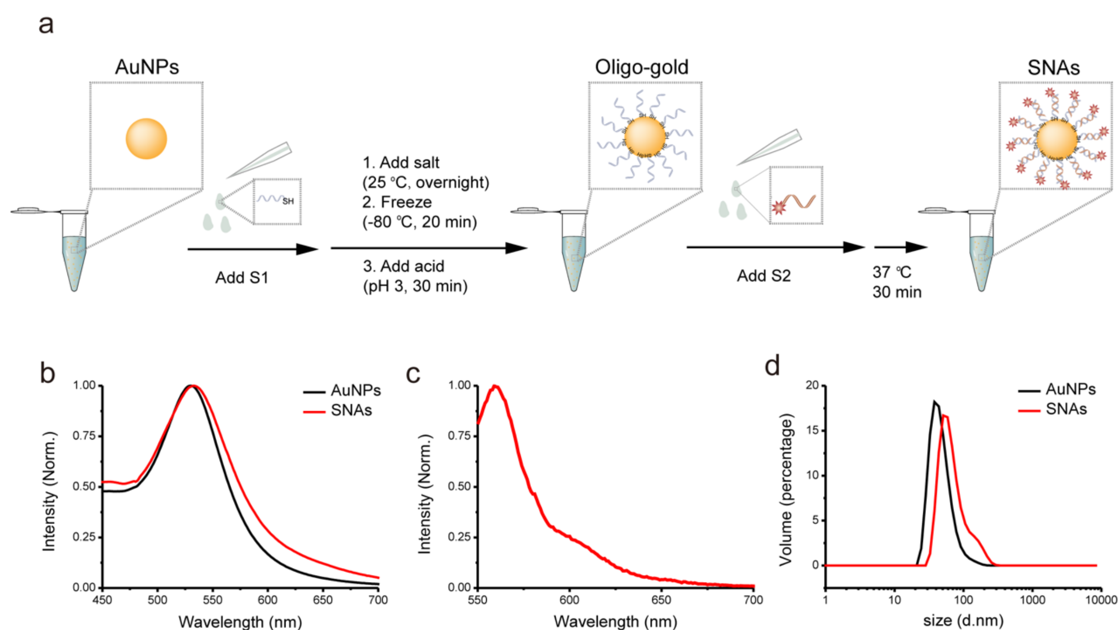
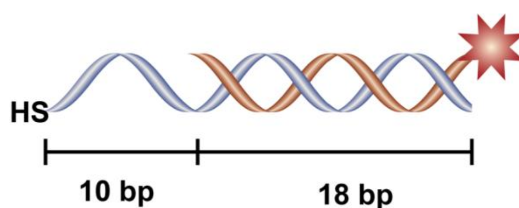
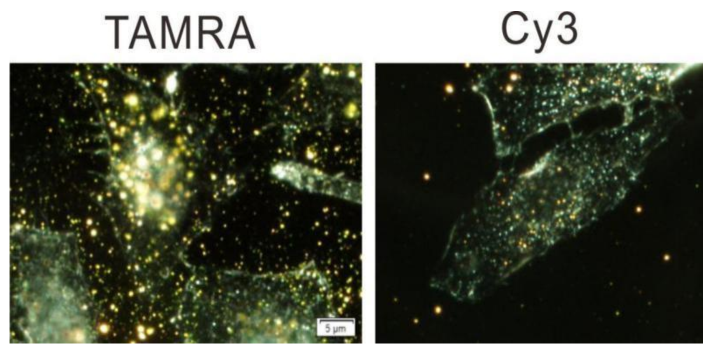
SUPPLEMENTARY FIGURES



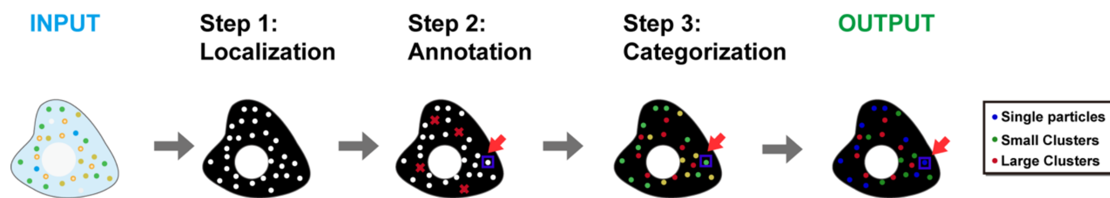
Supplementary Fig. 1 The process for preparation of SNAs. a) Schematic illustration of procedure for SNAs fabrication. b) Uv-vis spectra of bare AuNPs (black line) and SNAs (red line), showing that dsDNA modification of AuNPs results a slight (~ 2-3 nm) red shift. c) Fluorescence emission spectra of SNAs (excitation wavelength 530 nm). d) the hydrodynamic diameter of AuNPs (black line) and SNAs (red line), showing a increasement of size (~15-20 nm).



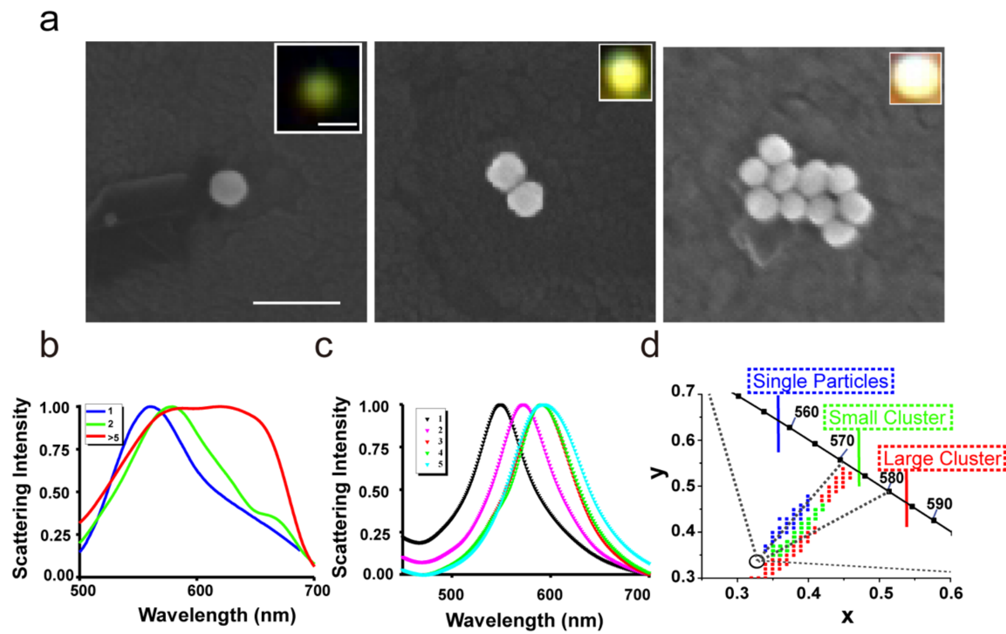
Supplementary Fig. 2 Schematic illustration of the 28-nt thiolate ssDNA (blue) attached on AuNP cores, and CY3 modified 18-nt ssDNA (orange) complementary to it.



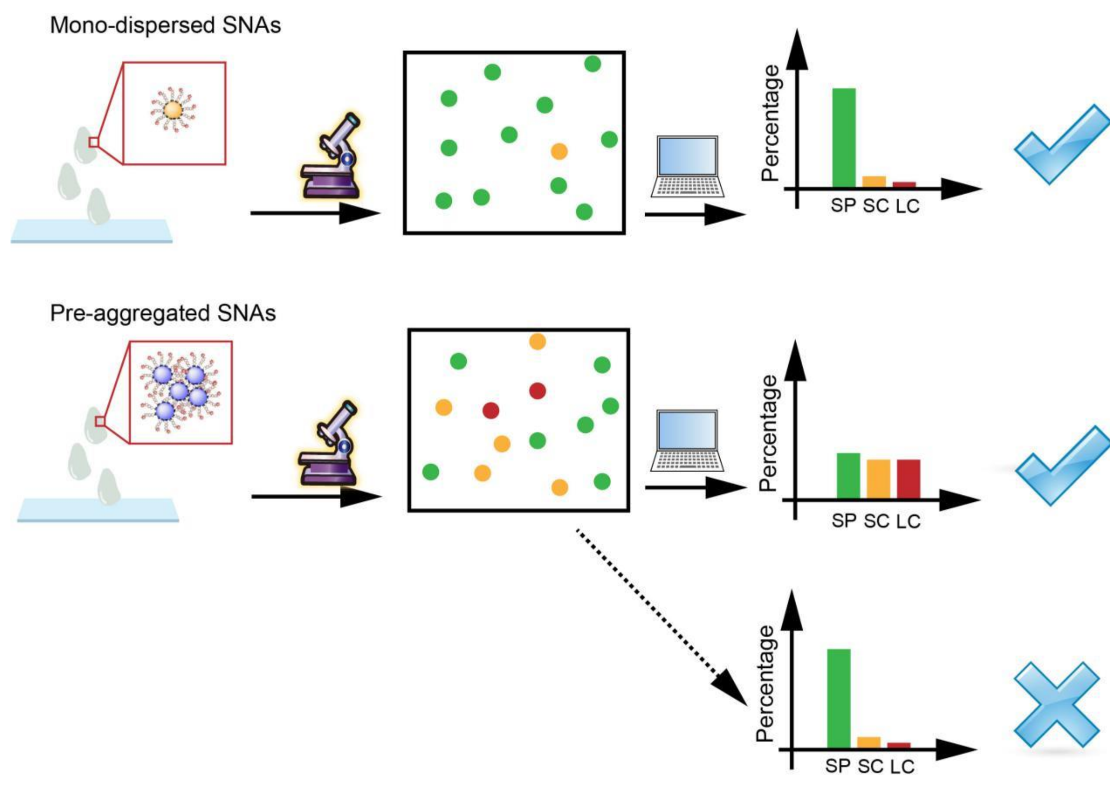
Supplementary Fig. 3 Representative DFM images of Hela cells incubated with SNAs employing TAMRA and Cy3 as the fluorophore, respectively. Incubation time: 4 h. In the case of TAMRA, large amounts of bright yellow spots were observed out of cells, indicating self-aggregation of SNAs occurred.



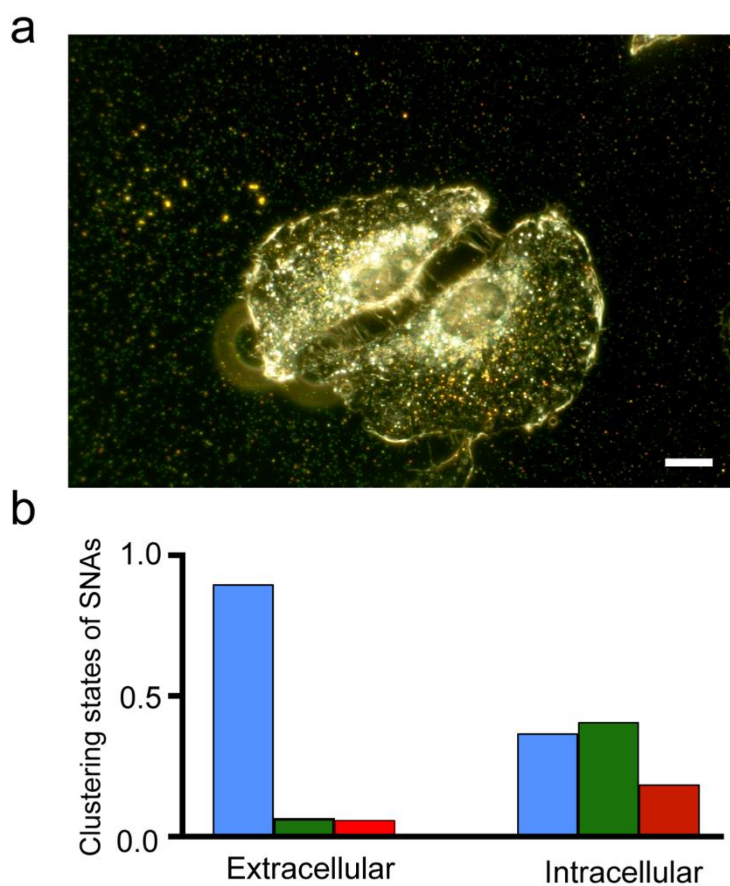
Supplementary Fig. 4 A brief schematic illustration of the colorimetry-based algorithm.



Supplementary Fig. 5 Correlation of plasmonic colors, scattering spectra, aggregations states and CIE coordinates of 50 nm SNAs. (a) Representative SEM images for single particles, small clusters and larger clusters and the corresponding in-situ DFM images. Scale bar: 200 nm in SEM images; 500 nm in the inserted DFM images. (b) The normalized scattering spectra of the three SNAs clusters in (a). (c) The FDTD simulation result of SNAs clusters containing 1-5 single particles. (d) CIE-map distribution of the three SNAs clusters in (a). Figure reproduced with permission from ref. 3, Copyright© 2017, Springer Nature and ref. 7, Copyright© 2020, American Chemical Society.



Supplementary Fig. 6 Schematic illustration of how to evaluate the algorithm by pre-analysis of SNAs samples of different clustering states. If the classification results generated by the algorithm can not match the observation under DFM imaging, parameters in the algorithm should be further optimized.



Supplementary Fig. 7 Statistic analysis of clustering states of intracellular SNAs based on DFM imaging. (a) A representative DFM image of extracellular and intracellular SNA clusters. Cell were incubated with SNAs probes for 2 h. Scale bar: 10 μm. (b) Clustering state analysis results of 1000 extracellular and intracellular SNAs by colorimetry-based algorithm. Blue, single particles; green, small clusters; red, large clusters. Figure reproduced with permission from ref. 7, Copyright© 2020, American Chemical Society.

Supplementary Table 1. The sequences of oligonucleotide used in this protocol

S1- Thiol-modified DNA oligonucleotides	5'-SH-AAAAAAAAAA GAG CTG CAC GCT GCC GTC-3'
S2- Fluorophore-labeled DNA oligonucleotides	5'-Cy3-GAC GGC AGC GTG CAG CTC-3'

Supplementary Table 2. The parameters used for intracellular SNA analysis with colorimetry-based classification algorithm in our lab.

Parameter	Value	Detailed description*
Lower Brightness Threshold (T_L)	80	Fig. H5
Upper Brightness Threshold (T_U)	160	Fig. H5
Lower Segment Volume Threshold (V_L)	1 pixel	Fig. H6
Upper Segment Volume Threshold (V_U)	20 pixels	Fig. H6
Hue Value Threshold (H)	120	Fig. H7&H8
Intensity Threshold (I)	240	Fig. H9
Saturation Threshold (S)	0.33	Fig. H10
Domain Boundary for Single Particles	530-570	Fig. H11
Domain Boundary for Small Clusters	570-580	Fig. H11
Domain Boundary for Large Clusters	580-620	Fig. H11

* All the figures and corresponding text description are listed in the pdf file '*How to use the algorithm*', which is included in Supplementary Data.

Note the algorithm parameters listed in this work were optimized for SNAs, microscope set-up and imaging conditions in our lab, and may not be appropriate for direct use (**Supplementary Table 2**). To adapt this algorithm to properly analyze DFM image data of other plasmonic nanoprobes in the automatic manner, optimization of algorithm parameters is critical. Here we provide a general procedure for optimization of algorithm parameters, taking SNAs as the example (**Supplementary Note 1**).

Supplementary Methods

Preparation of SNAs ● Timing ~ 3 d, Hands-on time ~ 3 h

CRITICAL Methodologies to prepare fluorescently labelled plasmonic nanoparticles have been well established in literature, via attachment of numerous fluorophores to plasmonic cores of different components and morphology[1]. In our case, SNA probes are fabricated by assembly CY3 labelled DNA with 50-nm spherical AuNPs core (**Supplementary Fig.1**).

! CRITICAL Synthesis of SNAs has be to performed under strict and precise reaction conditions, to achieve satisfactory reproducibility.

1. Synthesize citrate-stabilized 50-nm AuNPs according to previous literature[2] and characterize the product solution by UV-Vis and TEM. Alternatively, commercially available 50-nm AuNPs can be purchased and directly used after quality confirmation by UV-Vis and TEM.

CRITICAL STEP The concentration of AuNPs solution are determined by the absorbance at 520 nm (https://www.tedpella.com/gold_html/gold-tec.htm#anchor803003).

2. Centrifuge the tubes containing oligonucleotide S1 and S2 (See **Supplementary Fig. 2** and **Supplementary Table 1** for the thiol-modified DNA oligonucleotides and fluorophore-labeled DNA oligonucleotides sequences used in this protocol)14000 g for 2 min at room temperature (20-25°C). S1 represents ssDNA attached on the surface of AuNPs, with SH at 5' terminal, and S2 represents ssDNA with fluorescent unit at 5' terminal which could hybridize with S1[3].

CRITICAL STEP Do not open the tubes before centrifugation to avoid loss of DNA powder.

3. Dissolve Oligonucleotide S1 and S2 in an appropriate volume of milli-Q water, and dilute each sample to 100 µM concentration according to their molar mass. Divide each sample into 10 µL aliquots, label each tube and store them at -20 °C.

PAUSE POINT The oligonucleotide solution can be stored at -20 °C for no more than 6 months.

4. Modify the AuNPs with the oligonucleotide using your preferred method. In this protocol we use the "salt-aging method" [4], which is recommended for non-experienced users. First, prepare a solution of 100 µL AuNPs (1 nM) in a tube.

CRITICAL STEP Alternative methods for modifying AuNPs surface have been described in the literature, such as the "pH-assisted method" [5]and "freeze-thaw method"[6].

CRITICAL STEP The concentration of the AuNPs solution are determined by measuring the absorbance at 520 nm.

5. Pre-treat the S1 Oligonucleotide with TCEP solution (30 mM, pH 8) for 1 h at 25 °C.

! CAUTION Thiolated oligonucleotide S1 solution is slowly oxidized at -20 °C. It is necessary to reduce the stock solution of thiolated oligonucleotide S1 by TCEP before further usage.

6. Add 3 µL (100 µM) prepared S1 oligonucleotide solution into AuNPs solution (1nM, molar ratio of DNA to AuNPs 3000:1), and incubate this oligo-gold mixture solution at room temperature for 8 hours.

7. Add 11 µL phosphate buffer (0.1 M; pH 7.4) to the solution.

8. Increase the concentration of NaCl to 0.025 M by adding 1.25 µL NaCl (2 M) to the solution. Incubate the solution at room temperature for 30 min. Repeat this process until a concentration of 0.1 M NaCl is reached.

9. Centrifuge the oligo-gold solution at 4700 g, 4 °C for 3 minutes. Remove the supernatant containing the free oligonucleotide, leaving the AuNPs at the tube bottom. Resuspend the AuNPs in 100 µL phosphate buffer (10 mM; pH 7.4). Repeat this washing process three times. In the last washing step, resuspend the AuNPs in washing buffer (100 mM; pH 7.4), because a high salt concentration is needed for forming duplex DNA.

? TROUBLESHOOTING

10. Add 3 µL (100 µM) S2 oligonucleotide solution to the washed oligo-gold solution (100 µL), and incubate this hybrid-oligo-solution for at least 30 minutes at 37 °C.

11. Wash the solution for three times as described in Step 9. In the last washing step, resuspend the AuNPs in washing buffer (100 mM; pH 7.4). The final solution contains the SNAs (1 nM), which can serve as a dually emissive fluorescent and plasmonic probe.

CRITICAL STEP It is important to make sure that no free DNA-fluorophore residue is present in solution because free DNA-fluorophore may interfere the fluorescence imaging results of SNAs.

PAUSE POINT The SNA solution can be stored at 4 °C for no more than 2 weeks.

Quality control tests (Supplementary Fig.1b, c, d)

CRITICAL The optical properties of SNAs have to be carefully examined and evaluated before imaging experiments.

12. Before using SNAs for further investigation, ensure that they are of sufficient quality. Quality control tests include UV-visible spectroscopy (option A), fluorescence spectroscopy (option B), TEM imaging (option C) and dynamic light scattering measure (option D).

(A) Uv-visible spectra measurement ● **Timing 30 min**

(i) Record Uv-vis spectra of the SNAs sample solution between 400-800nm. Well-formed mono-dispersed SNA particles should exhibit a sharp absorption peak at ~535 nm. In addition, compared to bare AuNPs, a red shift of 2-3 nm should be observed.

(ii) To measure the modification efficiency of AuNPs, first add 5 μL DTT solution (1.0 M in 180 mM PB solution) to 100 μL of the SNAs solution overnight to displace the DNA from the AuNPs surface overnight.

(iii) Centrifuge the resultant solution at 4700 g at room temperature for 3 minutes to remove AuNPs precipitates.

iv) Record Uv-vis spectra of supernatant between 200-340 nm, and use the absorbance (A) at 260 nm to determine the DNA concentration (C_DNA) in supernatant following Beer's Law. Calculate the modification efficiency according to the following equation:

$$Q_{modification} = \frac{C_{DNA}}{C_{AuNPs}}$$

where C_{DNA} represents the measured concentration of DNA in supernatant, C_{AuNPs} represents the concentration of AuNPs in initial mixture solution, while $Q_{modification}$ represents the modification efficiency on the surface of AuNPs.

(B) Fluorescence emission spectra measurement ● Timing 30 min

(i) Measure the fluorescence emission spectra of the SNAs using a fluorescent spectrophotometer. In our case, we use Cy3 as fluorescence label, thus we set the excitation wavelength at 530 nm, and record the emission profile between 550-700 nm. The fluorescence emission spectra of SNAs should be similar to fluorescence label.

(ii) Measure the modification efficiency of AuNPs by measuring the fluorescence emission spectra of a series of SNA dilutions. We recommend using concentrations of 1 nM, 5 nM, 10 nM, 100 nM, 1000 nM to make a standard curve.

(iii) Add 5 μL DTT solution (1.0 M in 0.18M PB solution) to SNAs solution to displace the DNA from the AuNPs surface for overnight. Centrifuge the resultant solution at 4700 g at room temperature for 3 minutes to remove AuNPs precipitates. Measure the supernatant by fluorescence spectrometer to determine the concentration of DNA originally modified on AuNPs surface. Calculate the modification efficiency according to equation:

$$Q_{modification} = \frac{C_{fluo-DNA}}{C_{AuNPs}}$$

where $C_{fluo\text{-}DNA}$ represents the measured concentration of fluorescent ssDNA in supernatant, C_{AuNPs} represents the concentration of AuNPs in intimal mixture solution, while $Q_{modification}$ represent the modification efficiency on the surface of AuNP.

(C) Preparation of TEM imaging sample ● Timing 45 min

i. Dilute samples of AuNPs, ssDNA-AuNPs, and SNAs solution to 0.1 nM in milliQ water, and drop them on a copper mesh, respectively.

ii. Incubate for 30 min at 37 °C and analyze the samples by TEM imaging.

(D) Dynamic light scattering (DLS) measurement ● Timing **30 min**

i. Measure the hydrodynamic radius (Rh) distribution of the SNAs sample solution using DLS. A sharp peak should be observed with a red shift of ~15 nm compared to bare AuNPs solution.

Supplementary Note 1

Adaption of colorimetry-based classification algorithm:

To optimize algorithm parameters, at first a set of reference values of parameters should be obtained by analyzing representative image spots in DFM images. Then to check if these parameters work properly, a pre-analysis should be performed by analyzing the clustering states of mono-dispersed and pre-aggregated SNA samples. The algorithm parameters are ready for use if the clustering states of these pre-aggregated samples can be efficiently recognized. Otherwise, further optimization has to be conducted.

Extract reference values of parameters

1. Select a DFM image. It is recommended to apply the best DFM image you have acquired to do the following steps.

2. Select a green, yellow and bright yellow/red spot, respectively, with “square selection” and count the pixel numbers of the spot in x and y direction using ImageJ. These values provide selection reference for “V_U” in **Supplementary Table 2**.

3. Extract the RGB value of the bright yellow/red spot, and calculate intensity of each pixel according to Equation 1[7, 8]. The maximum intensity of this spot provides a selection reference for “I” threshold in **Supplementary Table 2**. Meanwhile, the intensity of boundary pixels in the bright yellow/red spot provides a selection reference for “T_U” threshold in **Supplementary Table 2**.

$$I = R * 0.114 + G * 0.5876 + B * 0.299 \qquad \text{Equation 1}$$

4. Extract the RGB value of the green spot, and calculate intensity of each pixel according to Equation 1. The maximum intensity of this spot provides a selection reference for “T_L” threshold in step in **Supplementary Table 2**.

5. Select a noise spot (e.g. intracellular organelle), transfer the RGB value of each pixel into HSB value, the maximum H value and average S value provide a selection reference for “H” threshold and “S” threshold in **Supplementary Table 2**.

6. Extract the RGB value of the green, yellow and bright yellow/red spots in DFM images, and calculate the corresponding (x,y) coordinates distribution in the CIE map of each pixel according to

Equation 2. These values provide selection reference for "domain boundary" in **Supplementary Table 2**.

$$\begin{bmatrix} X \\ Y \\ Z \end{bmatrix} = \begin{bmatrix} 0.49 & 0.31 & 0.20 \\ 0.12 & 0.81 & 0.01 \\ 0.00 & 0.01 & 0.99 \end{bmatrix} \begin{bmatrix} R \\ G \\ B \end{bmatrix} \qquad \textit{Equation 2}$$

$$x = \frac{X}{X+Y+Z}$$

$$y = \frac{Y}{X+Y+Z}$$

$$z = \frac{Z}{X+Y+Z}$$

Evaluate the reference values of parameters

1. Drop mono-dispersed and pre-aggregated SNAs solution on a glass slide, respectively.

2. After 10 minutes, wash these two sample slides with milli-Q water and dry them with nitrogen gas.

3. Record DFM images of these two samples and analyze these DFM images with the algorithm.

For the mono-dispersed SNAs sample, most of DFM image spots should be classified as single particles. While for the pre-aggregated SNAs sample, the amounts of clusters should be distinctly different (See **Supplementary Fig. 6** for schematic illustration).

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