Supporting Information for

Tempo-Spatially Resolved Scattering Correlation Spectroscopy under Dark-Field Illumination and Its Application to Investigate Dynamic Behaviors of Gold Nanoparticles in Live Cells

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THEORY

General Formalism

According to the stationary of the system at equilibrium, $G(t)$ depends on the interval t but not the absolute time t' when the average is taken.[1] As a result, $G(t)$ (equation 4 in the article) can be rewritten as

$$G(t)=\frac{1}{\bar{n}^2}\left\langle \delta n(0)\delta n(t)\right\rangle \tag{S1}$$

Substituting equation 2 into equation S1 yields

$$G(t)=\frac{(\Delta t)^2}{\bar{n}^2}\iint d^3\vec{r}d^3\vec{r}'I(\vec{r})I(\vec{r}')\sum_{j,l}Q_jQ_l\left\langle \delta C_j(\vec{r},0)\delta C_l(\vec{r}',t)\right\rangle \tag{S2}$$

Now $G(t)$ is expressed as the convolution of the auto- and cross-correlation functions of the concentration fluctuations with the illumination intensity. Here the shot noise and noises from other extraneous sources are neglected for simplicity as both of them are uncorrelated.

In consideration the statistics of solute molecules are independent for ideal solution, we omit the correlations between particles of different species. Similarly the spatial correlation length of concentration fluctuations of a particular species must be very small. Therefore,

$$\left\langle \delta C_j(\vec{r},0)\delta C_k(\vec{r}'',0)\right\rangle=\bar{C}_j\delta_{jk}\delta(\vec{r}-\vec{r}'') \tag{S3}$$

where $\bar{C}_j$ denotes the mean-square fluctuations of $C_j(\vec{r}',t)$ of particles in a defined volume, which is equal to its average $\left\langle C_j(\vec{r}',t)\right\rangle$ for Poisson statistics, δ_{jk} is the Dirac delta function.[2]

Then, in order to establish the relationship between $\delta C_j(\vec{r}',t)$ and the initial condition $\delta C_j(\vec{r}',0)$, we introduce the concentration relaxation equation. As the concentration deviation around the ensemble average is small, the linearized rate equation is hold here[3]

$$\frac{\partial\delta C_j(\vec{r},t)}{\partial t}=D_j\nabla^2\delta C_j(\vec{r},t)+\sum_{k=1}^{m}K_{jk}\delta C_k(\vec{r},t) \tag{S4}$$

where the former term on the right side is attributed to diffusion, and the latter one accounts for chemical reaction, D_j denotes the diffusion coefficient of component j and coefficients K_{jk} are composed of chemical rate constants and the equilibrium concentrations of the species.

Equation S4 can be solved subject to the appropriate boundary and initial conditions, and in turn $G(t)$ can be expressed in terms of D and K. As a frequently used method to solve the partial differential equation, a Fourier transform is applied to equation S4

$$\frac{d\tilde{C}_l(\vec{q},t)}{dt}=\sum_{k=1}^{m}M_{lk}\tilde{C}_k(\vec{q},t) \tag{S5}$$

where $M_{lk}=K_{lk}-D_lq^2\delta_{lk}$, $\tilde{C}_l(\vec{q},t)=\int d^3\vec{r}e^{-i\vec{q}\vec{r}}\delta C_l(\vec{r},t)$ is a Fourier transform of $\delta C_l(\vec{r},t)$, and the inverse Fourier transform is $\delta C_l(\vec{r},t)=(2\pi)^{-3}\int d^3\vec{q}e^{i\vec{q}\vec{r}}\tilde{C}_l(\vec{q},t)$.

Equation S5 constitutes a system of linear first-order differential equations with constant coefficients for the Fourier components of the concentration fluctuations. It can be solved through standard methods[4]

$$\tilde{C}_l(\vec{q},t)=\sum_{s=1}^{m}X_l^{(s)}h_s\exp\left(\lambda^{(s)}t\right) \tag{S6}$$

where $X_l^{(s)}$ are the components of the right eigenvectors corresponding to the eigenvalues $\lambda^{(s)}$ of the matric M, and $h_s=\sum_{k=1}^{m}\left(X^{-1}\right)_k^{(s)}\tilde{C}_k(\vec{q},0)$. Let the inverse matrix of eigenvectors be X^{-1}. Hence,

$$\tilde{C}_l(\vec{q},t)=\sum_{s=1}^{m}X_l^{(s)}\exp\left(\lambda^{(s)}t\right)\sum_{k=1}^{m}\left(X^{-1}\right)_k^{(s)}\tilde{C}_k(\vec{q},0) \tag{S7}$$

Considering both Fourier transform and ensemble averaging are independent linear operations so they are in line with the commutative law. Combining equation S7 and equation S3, we obtain

$$
\begin{aligned}
&\left\langle \delta C_j(\vec{r},0)\delta C_l(\vec{r}',t)\right\rangle \\
&=(2\pi)^{-3}\int d^3\vec{q}e^{i\vec{q}\vec{r}'}\left\langle \delta C_j(\vec{r},0)\delta \tilde{C}_l(\vec{q},t)\right\rangle \\
&=(2\pi)^{-3}\int d^3\vec{q}e^{i\vec{q}\vec{r}'}\sum_{s=1}^{m}X_l^{(s)}\exp\left(\lambda^{(s)}t\right) \\
&\qquad \sum_{k=1}^{m}\left(X^{-1}\right)_k^{(s)}\left\langle \delta C_j(\vec{r},0)\delta \tilde{C}_k(\vec{q},0)\right\rangle \\
&=(2\pi)^{-3}\int d^3\vec{q}e^{i\vec{q}\vec{r}'}\sum_{s=1}^{m}X_l^{(s)}\exp\left(\lambda^{(s)}t\right) \\
&\qquad \sum_{k=1}^{m}\left(X^{-1}\right)_k^{(s)}\times\int d^3\vec{r}''e^{-i\vec{q}\vec{r}''}\left\langle \delta C_j(\vec{r},0)\delta C_l(\vec{r}'',0)\right\rangle \\
&=(2\pi)^{-3}\int d^3\vec{q}e^{i\vec{q}\vec{r}'}\sum_{s=1}^{m}X_l^{(s)}\exp\left(\lambda^{(s)}t\right) \\
&\qquad \sum_{k=1}^{m}\left(X^{-1}\right)_k^{(s)}\times\int d^3\vec{r}''e^{-i\vec{q}\vec{r}''}\bar{C}_j\delta_{jk}\delta(\vec{r}-\vec{r}'') \\
&=(2\pi)^{-3}\bar{C}_j\int d^3\vec{q}e^{i\vec{q}(\vec{r}-\vec{r}')}\sum_{s=1}^{m}X_l^{(s)}\exp\left(\lambda^{(s)}t\right)\left(X^{-1}\right)_j^{(s)}
\end{aligned}
\tag{S8}
$$

At last, after substituting equation S8 into equation S2 and execution of the integrals over $\vec{r}$ and $\vec{r}'$, we arrive at the result

$$
G(t)=\frac{(2\pi)^{-3}(\Delta t)^2}{\bar{n}^2}
\int d^3\vec{q}\left|\tilde{I}(\vec{q})\right|^2\sum_{j,l}Q_jQ_l\bar{C}_j\sum_{s=1}^{m}X_l^{(s)}\exp\left(\lambda^{(s)}t\right)\left(X^{-1}\right)_j^{(s)}
\tag{S9}
$$

where $\tilde{I}(\vec{q})=\int d^3\vec{r}e^{-i\vec{q}\vec{r}}I(\vec{r})$ is the Fourier transform of $I(\vec{r})$, and $\bar{n}$ is the average number of detected photons $\bar{n}=\Delta t\int d^3\vec{r}I(\vec{r})\sum_{i=1}^{m}Q_i\bar{C}_i=\tilde{I}(0)\Delta t\sum_{i=1}^{m}Q_i\bar{C}_i$.

METHODS AND EXPERIMENTS

Synthesis of GNPs

Briefly, 5.0 *mL* of HAuCl4 (0.2%, *w*/*w*) was mixed with 90.0 *mL* of water and the solution was heated in a constant-temperature oil bath maintained at 120°C with vigorous stirring. Then, sodium citrate (1%, *w*/*w*) was added rapidly to the boiling solution and the volume added depended on the requirement size of GNPs. Heating was continued for 20 min after the solution turned pink. At last, the GNPs solution was cooled to room temperature and then stored at 4°C for further use. The size of GNPs was observed from a JEM-2100HR transmission electron microscope (JEOL Ltd., Japan).

Modification of GNPs

In short, 0.3 *mL* of 2.0 *mg*/*mL* NHS-PEG disulfide was added to 0.3 *mL* of 4.0 *mg*/*mL* Herceptin in 2.4 *mL* of 0.02 *M* PBS (pH=7.4) and the mixed solution was incubated for 18 h at 25°C. Excess NHS-PEG disulfide was removed from the Herceptin-PEG complex by three rounds of centrifugation and resuspension in 0.02 *M* PBS using ultrafiltration membrane (Micoron YM-100-100000 NMWL, Millipore Ltd., USA). Then, 0.1 *mL* of 0.1 *M* NaOH solution was added to 4.0 *mL* of GNPs solution to adjust pH, followed by mixture of 0.4 *mL* of 0.1 *mg*/*mL* Herceptin-PEG solution. After reacting at 4°C overnight, 2.0 *mL* of 3.0 *mg*/*mL* BSA solution was added as a blocking agent and the reaction continued for another 2 *h* at room temperature. GNPs-PEG-Herceptin conjugates were cleaned up by centrifugation at 10,000 *rpm* for 30 min and resuspension in 4.0 *mL* of 0.02 *M* PBS buffer before use. Control conjugation reactions were conducted to confirm whether labeling was successful, which included incubation with BSA instead of Herceptin at the same concentration. The zeta potential the particles and conjugates was measured by a Malvern Zetasizer Nano ZS90 (Malvern Ltd., U.K.).

Cell culture and GNPs-PEG-Herceptin Uptake

Cervical cancer SiHa cells were maintained in Dulbecco's Modified Eagle's medium supplemented with 10% (*v*/*v*) fetal bovine serum (Gibco Ltd., USA). Cells were grown at 37°C in a humidified air atmosphere with 5% CO2. After the cells reached 70% confluence in a 35- *mm* tissue culture dish (Corning Ltd., USA), they were incubated with 2.0 *mL* GNPs-PEG-Herceptin conjugates solution prepared as described above at 37°C for 1 *h* and washed with PBS three times prior to the dark field imaging. Some cells were stained with a DNA-specific fluorescent probe DAPI (4', 6-diamidino-2-phenylindole) and then imaged by a fluorescence microscope. A certain cell sample incubated with GNPs-PEG-Herceptin conjugates were embedded and made into TEM specimens, and then observed via a TEM (JEOL JEM-1230, JEOL Ltd., Japan) at 100 *kV*.

RESULTS AND DISCUSSION

Calibrations

In traditional confocal FCS, calibrations typically rely on the straightforward measurements of the characteristic diffusion time of a dye molecule with known concentration or diffusion coefficient, i.e., Rhodamine 6G.[5] Unfortunately, because the illumination profile in the detection volume of the confocal FCS is heterogeneous, small changes in the illumination scheme can significantly influence the detection volume. For example, optical aberrations due to incorrect coverslip thickness, refractive index mismatch, or astigmatism, can easily lead to a 50% error in diffusion and even higher errors in concentration measurements.[6] So regular calibrations are necessary for confocal FCS. Since DFSCS is a camera based method, the radial size of the volume (length = width = $2a$) is depend on the pixel size of the camera.[7] Thus, only the height, h, is needed to be calibrated. From $D=a^2/\tau_D$ and $\bar{C}=\bar{N}/8ach$, we can preliminarily concluded that concentration is more sensitive to h than diffusion coefficient. Therefore, we employed a set of commercial GNPs solutions with known concentrations for calibration. It must be mentioned that according to the sample preparation method described above, the gap between two coverslips greatly affect the height of the detection volume in DFSCS. Since we prepare the sample manually, the experimental operations have to be very careful to maintain the parallelism of the height.

Sensitivity of DFSCS System

In order to study the sensitivity of DFSCS system, the dependence of $G(t)$ upon the concentration and D of GNPs and other factors were investigated both in theory and in experiments. As the derivative measures how much one quantity is changing in response to changes in another quantity,[8] we tentatively put forward the first partial derivative

of $G(t)$, dG, respect to a certain factor as the index of the sensitivity of $G(t)$ to this factor. The absolute value of dG took the place of dG if dG was negative. With respect to the identical factor, the bigger amplitude of dG signified the higher sensitivity. The derivative was deduced analytically from the theoretical model. The model of confocal FCS adopted here was the classical expression of single-component diffusion.[9] For comparison, numerical value was calculated based on parameters set as the following: the delay time range from 1 μs to 1000 s in the quasi-logarithmic time scale, N =2(or 20, 200), D = 0.8 $\mu m^2/s$, h or ω_z = 2.557 μm (axial dimension, size of beam waist in the direction of the propagation of light), a or ω_{xy} = 0.4 μm (radial dimension, size of beam waist in the perpendicular direction of the propagation of light).

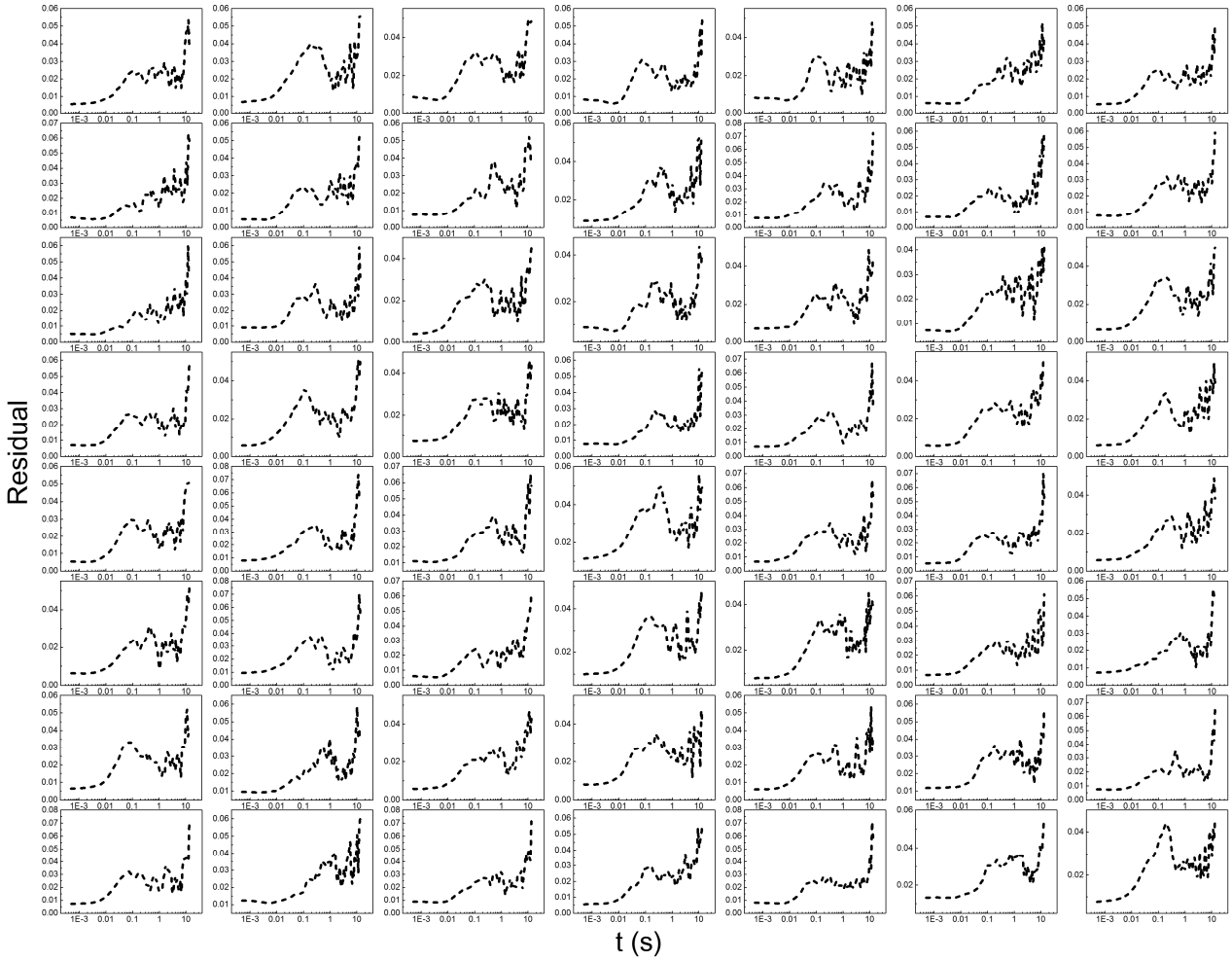

Figure S1. The fitting residuals of all 56 autocorrelation curves in Figure 3.

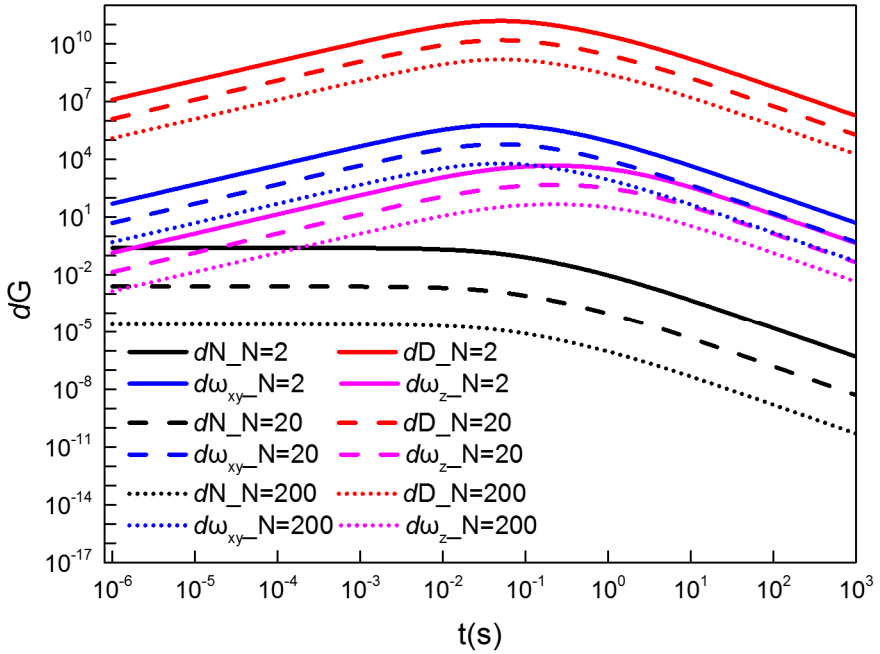

Figure S2. The first partial derivatives of $G(t)$, dG, in confocal FCS respect to each variable: particle number in the detection volume, N, diffusion coefficient, D, size of beam waist in the direction of the propagation of light, ω_{xy}, and size of beam waist in the perpendicular direction, ω_z. N is set to be 2, 20, 200, respectively.

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