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New fluorescence correlation spectroscopy (FCS) suitable for the observation of anomalous diffusion in polymer solution: Time and space dependences of diffusion coefficients

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Abstract

We propose a new method of fluorescence correlation spectroscopy (FCS) enabling direct detection of anomalous diffusion as the distance dependence of diffusion coefficients (DDDC), namely, sampling-volume-controlled (SVC)-FCS. In discussing the results of SVC-FCS measurement of molecular diffusion in aqueous hyaluronan (HA) solutions in this paper, we suggest the use of "local anomalous diffusion" based on the differential of the mean-square displacement (MSD) which is convenient for understanding the total lineshapes of the observed diffusion coefficient as a function of the diffusion distance or the diffusion time.

Keywords: Diffusion; Anomalous diffusion; Extracellular matrix; Hyaluronan; Fluorescence correlation spectroscopy; Confocal microscope; Inhomogeneous system; Soft matter; Time dependence; Distance dependence

1. Introduction

Material transport in various inhomogeneous media is an essential physical process especially in biological systems [1]. Cell–cell communication through extracellular matrices (ECMs) is one example where various signaling molecules and nourishing substances are exchanged to support life. Each material transport should be driven by a simple accumulation of physical processes, the details of which have not been clarified thus far.

The theories about the diffusion process have a long history, including Fick's early work [2] and Einstein's later work [3]. The analytical solution of the diffusion equation was successfully connected with the random process of Brownian motion under the friction of surrounding media by Einstein's relation. However, most of the achievements in this history concern diffusion in *homogeneous* systems, so-called normal diffusion (ND) in which the mean-square displacement (MSD) of diffusing particles proportionally increases with time t. On the other hand, the majority of the material transports, which are important as a key process in the maintenance of life, occur in *inhomogeneous* media where MSD is not proportional to t. This kind of diffusion is comprehensively called "anomalous diffusion" (AD). Although AD is found in many categories of materials, such as glasses [4], polymer solutions [5,6], colloidal solutions [7], and gel solutions [1], only a few experimental approaches are available.

In this paper, we briefly describe a new instrument, which we have developed recently, [8,9] that is suitable for the investigation of AD in various systems. The technique used is a modification of fluorescence correlation spectroscopy (FCS) [10], in which the size of the confocal volume (CV) is changed by changing the numerical aperture (NA) of the objective lens.

2. Theory

Various particles exhibit random (stochastic) motions. Random motions should be recoded as a function of time $r(t)$ and if a particle is sufficiently large to be detected by optical observation, the data is obtained as a video image of real-time motion. The MSD of this particle can be defined after a huge number (N) of statistical samplings as

$$\langle r^2 \rangle = \frac{1}{N} \sum_{i=1}^{N} (r(t_{1i}) - r(t_{2i}))^2. \quad (1)$$

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However, most of the particles taking part in the communication occurring in bio-systems are smaller than the observation limit of optical microscopes and $r(t)$ is not observable. FCS is a technique for detecting the displacement of such small invisible particles using the time correlation function of fluorescence fluctuation assuming Brownian motion. A fitting method for the FCS time correlation function (TCF), [10] in which ND is assumed, is used to obtain the diffusion coefficient D as

$$G(\tau) = \frac{1}{N}\left\{\left[1+\frac{\tau}{\tau_D}\right]^{-1}\left[1+\frac{\tau}{q^2\tau_D}\right]^{-0.5} + T_r\right\}, \quad (2)$$

where T_r and N are the correction term for triplet and the molecular number, respectively. q is the structure parameter $q = z/w$ (z and w are the axial and radial radii of CV. τ_D is the diffusion time, which is related to D with $\tau_D = w^2/4D$).

For ND in homogenous media, the MSD after a long-term average is expressed by a linear function of time as

$$\langle r^2\rangle = 2\,\mathrm{d}Dt \quad (3)$$

for d-dimensional diffusion [1]. D is defined as a proportionality factor in Eq. (3).

For AD in which Eq. (3) is not valid, various descriptions are possible. One can regard D as a variable function of t or the diffusion distance $L = \sqrt{\langle r^2\rangle}$ as

$$\langle r^2\rangle = 2\,\mathrm{d}D(t)t = 2\,\mathrm{d}D(L)t. \quad (4)$$

Instead of Eq. (3), some studies [11] treat AD with a power of t as

$$\langle r^2\rangle = 2\,\mathrm{d}Dt^{\alpha} \quad (5)$$

and classify AD into three categories as "anomalous super-diffusion", ND, and "anomalous sub-diffusion" for $\alpha > 1$, $\alpha = 1$, and $\alpha < 1$, respectively.

The MSD is experimentally obtained by light (laser, X-ray) and particle (neutron) scattering methods as a function of t. On the other hand, several spectroscopic methods, each having a proper observation time τ_{obs}, can be used to determine D in single-point observation (we express observed D by single-point measurement as D_{obs}), such as photochemical bimolecular reaction (PCBR), FCS, and pulsed field gradient (PFG) NMR [8,9,12,13]. In these cases, τ_{obs} could be easily defined as in the following methods. For PCBR, the second order rate constant of photochemically excited molecules was used to evaluate D_{obs}. The lifetime of excited molecules can be used as the experimental τ_{obs}. Similarly the duration between marking and reading pulses in PFG NMR was used as τ_{obs}. In FCS, τ_{obs} value in Eq. (2) was identified with τ_{obs}.

In many real observations, however, the function profiles of MSD are not monotonic and the definition given by Eq. (5) can only be locally applied, whereas that given by Eq. (4) is more general and comprehensive. In this paper, we suggest another description using the differentiation of MSD [1,14] as

$$\frac{\mathrm{d}\langle r^2\rangle}{\mathrm{d}t} = 2\,\mathrm{d}D \quad \text{(constant)} \quad (6)$$

for local ND from Eq. (3) and

$$\frac{\mathrm{d}\langle r^2\rangle}{\mathrm{d}t} = 2\,\mathrm{d}(D(t) + tD'(t)) \quad \text{(not constant)} \quad (7)$$

for local AD from Eq. (4) with local anomalous sub-diffusion ($\mathrm{d}\langle r^2\rangle/\mathrm{d}t$ decreases) and local anomalous super-diffusion ($\mathrm{d}\langle r^2\rangle/\mathrm{d}t$ increases). There is another choice of basic equation defining the observable diffusion coefficient which is different from (7) but similar to (6) as

$$\frac{\mathrm{d}\langle r^2\rangle}{\mathrm{d}t} = 2\,\mathrm{d}D(t). \quad (8)$$

However, we suppose that this form is inadequate for the present FCS experiment.

The concept of utilizing the differentiation of MSD is not new, as suggested in various literatures. However, our suggestion is that we can classify the local behavior (constant or not) of the MSD function in the neighborhood of each single point of the D measurement that is characterized by certain τ_{obs} or L.

In Fig. 1, we present an example of a general inhomogeneous MSD as a function of t on various scales: (A) MSD in a logarithmic scale against $\log_{10} t$, (B) MSD against t, and (C) the locally observed diffusion coefficient $D_{obs}(t)$ against t. In plot (A), the scales of which are often used in various diffraction experiments, local ND is identified by the line with a slope of 1. In plot (B), the ND is expressed as a straight line from the origin, the slope of which has a value of $6D_{obs}(t)$. In local AD, however, it should be noted that there are two definitions for $D_{obs}(t)$ depending on the choice of the basic equation, (7) or (8). When (7) is chosen, the slope of straight lines (not the slope of MSD curve) connecting the origin and each $\langle r^2\rangle$ point (expressed with dashed lines in Fig. 1B) expresses the value of $6D_{obs}(t)$ at each single-point observation. The differentiation of MSD in plot (B) at single local point is not $6D_{obs}(t)$ but $6(D_{obs}(t) + tD'_{obs}(t))$ as shown in (7). Plot (C) is most convenient for the present experimental approach based on Eq. (4) because $D_{obs}(t)$ is regarded as an observable value. We call this last plot "the time dependence of the diffusion coefficient (TDDC)." Plots against L are also possible, which we call "the distance dependence of the diffusion coefficient (DDDC)." In every plot, there is a transient area between two local ND areas. In other words, $D_{obs}(t)$ seems to transfer from one normal diffusion coefficient (D_1) to another (D_2) along the transient (local AD: local anomalous sub-diffusion in this case) area.

3. Experimental

The preparation of dyes (Alexa 488, Rohdamine123) and protein (Cytchrome *c*: cytc) in aqueous hyaluronan (HA) solutions has been described elsewhere [8,9,12,13]. The details of PCBR and PFG NMR experiment were shown in previous papers [12,13].

To clarify the local AD, we used the sampling-volume-controlled (SVC)-FCS method, the descriptions of which were presented in recent papers [8,9]. In this method, we changed the size of the illumination volume (therefore, also the confocal volume: CV) of laser excitation by changing the diameter of the

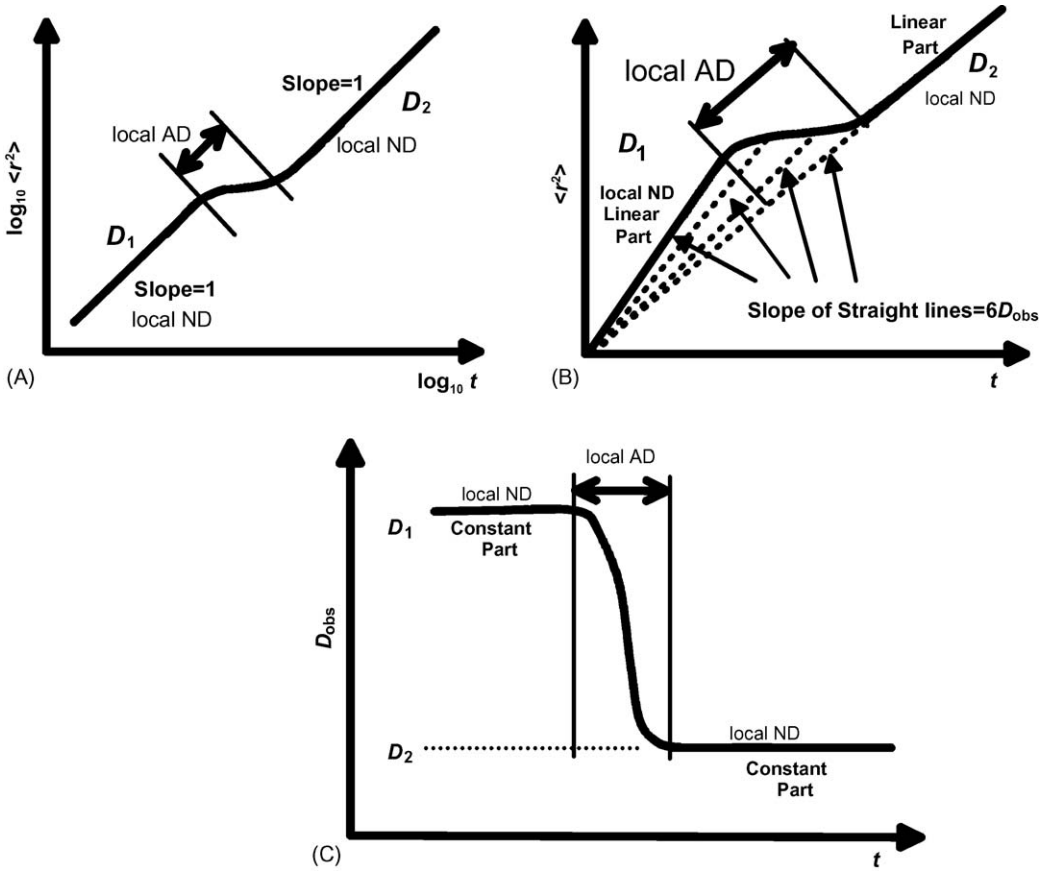

Fig. 1. Various scale presentations for the time dependence of the mean-square displacement (MSD) in which regions of local anomalous (sub) diffusion (AD) are sandwiched by two local normal diffusion (ND) areas ($D_{obs} = D_1$ and D_2). (A) Logarithmic plot of the MSD against $\log_{10} t$. Local ND areas are expressed as a straight line whose slope is equal to 1 where their intersections on the vertical scale is different depending on D_1 and D_2. The local AD area is the transition area between two local ND areas ($D_{obs} = D_1$ and D_2). (B) Linear plot of MSD against t. Local ND areas are expressed as a straight line crossing the origin whose slope is equal to $6D_{obs}$. The local AD area is the transition area between two local ND areas where the slope of the dashed line connecting the origin and each MSD point is equal to $6D_{obs}$. (C) Plot of t dependence of D_{obs} (TDDC) corresponding to (A) and (B).

collimated laser beam. We show a figure to explain the principle underlying this method in Fig. 2. To use the conventional analysis based on Eq. (2), the Gaussian spatial distribution of CV is indispensable and it should be noticed that the collecting efficiency of photons by the detector changes greatly when they propagate through microscope. Our method in Fig. 2A is well designed to sustain the Gaussian optics approximation. Another method, which is inadequate for the present purpose, involves simply inserting an iris before the objective lens (Fig. 2B), at which point, the Gaussian distribution is severely

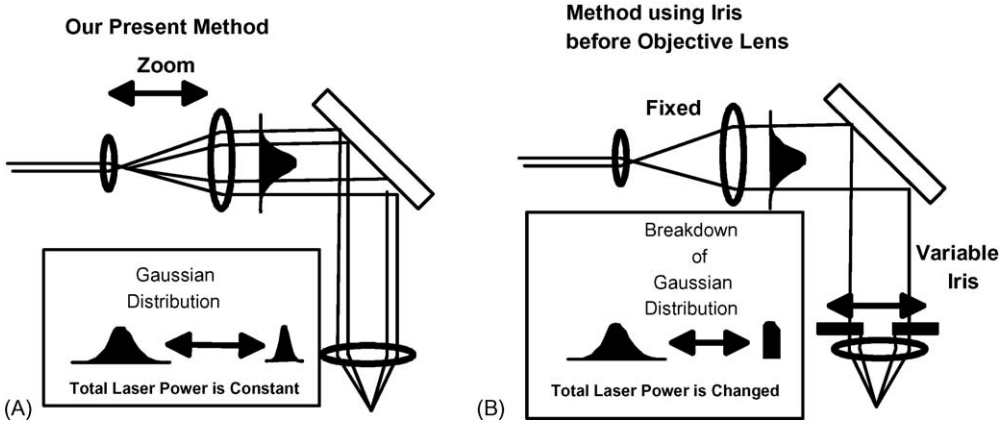

Fig. 2. Comparison of two methods of changing the size of the laser illumination volume. (A) Our present method in which laser beam diameter is changed using a zoom lens, in which a Gaussian distribution of light is sustained. (B) An alternative method in which the beam is cut with an iris before the objective lens. The Gaussian distribution is severely deformed. Fitting results Eq. (2) are not always valid.

deformed because of the over filling effect on the aperture of the iris.

4. Results and discussion

In previous papers, we presented the results of D_{obs} observation for Alexa 488 and cytc in aqueous HA solutions using PCBR, SVC-FCS, and PFG NMR. Since each measurement is a single-point measurement, the envelope curve can be mutually interchanged between $D_{obs}(t)$ and $D_{obs}(L)$ using

$$L = \sqrt{6D_{obs}t}, \tag{9}$$

in other words, between TDDC and DDDC [12,13].

In Fig. 3, the previous results of SVC-FCS [9] for Alexa 488 in HA solution are shown in DDDC and TDDC plots (Fig. 2 of Ref. [9]). Detailed discussion was previously presented, in which we concluded that this change in D originates from the mesh like structure of the HA solution. In the FCS measurement, we still used the general fitting function (Eq. (2)) for TCF. If the extent of AD is severe, TCF should deform greatly and Eq. (2) cannot be applied to it. However, in the present system of the HA solution, the deformation of TCF was not large because the change in D_{obs} was small (10–30%). As a result, AD was detected, not as the deformation of TCF, but as the change of the fitting parameters τ_d, e.g. D_{obs}. We obtained the diffusion distance L [13] from Eq. (9) using D_{obs} leading:

$$L = \sqrt{\frac{3}{2}}w. \tag{10}$$

The diffusing dye molecules are interfered with by the polymer chain and D should be reduced for a long traveling distance of $L > 1\ \mu m$, whereas they have almost no opportunity to interact with the chain for a short traveling distance of $L < 10$ nm. In these two areas, D takes a constant value showing the local ND behavior. In between two local ND areas, there is a local AD area showing a remarkable change in D as shown in Fig. 3. In this AD area, D does not monotonical change, showing a dip at the bottom of each line. We suppose that these dips were caused by the dynamical mesh reorganization induced by the motion of the polymer chains (see Ref. [9]).

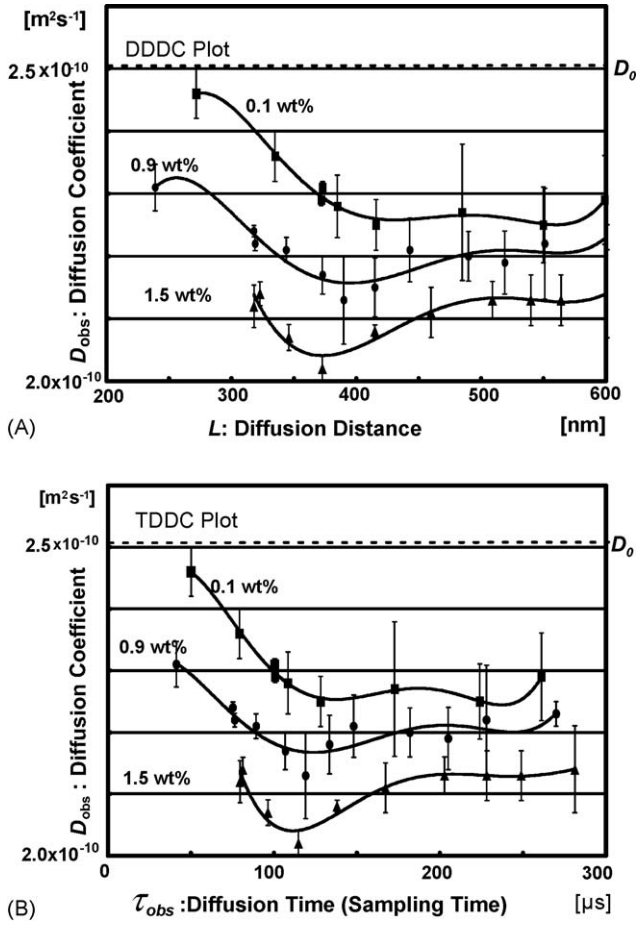

Fig. 3. Plots of the diffusion distance (L) dependence of diffusion coefficient (DDDC) and the observation time ($\tau_{obs} = t$) dependence of diffusion coefficient (TDDC) obtained for Alexa 488 in aqueous HA solution by sampling-volume-controlled (SVC)-FCS. [HA] = 0.1, 0.9, and 1.5 wt.% (reproduced from Fig. 2 in Ref. [9]).

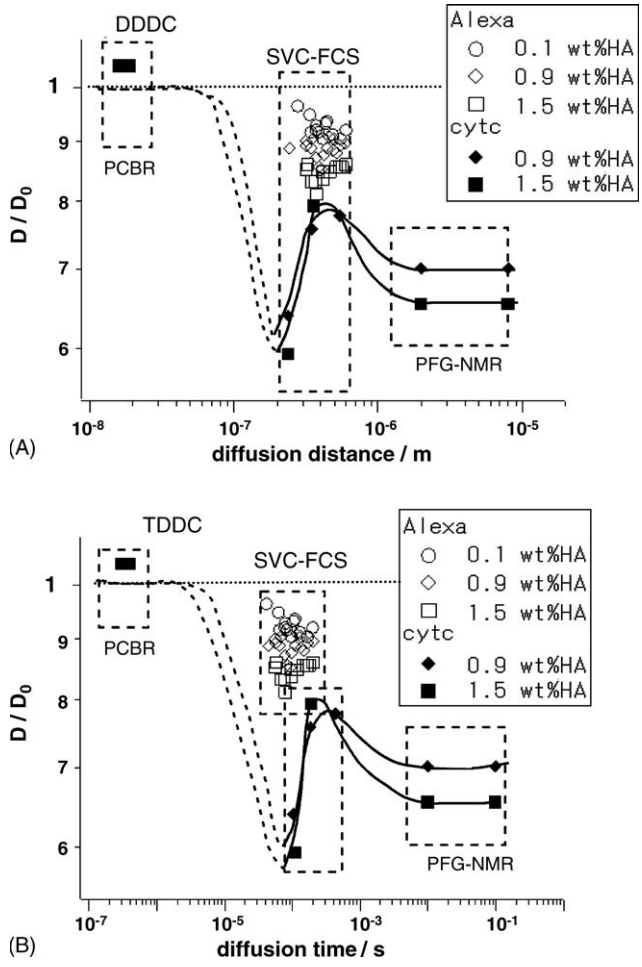

Fig. 4. Relative change in the observed diffusion coefficients of cytc and Alexa 488 observed by using a photochemical bimolecular reaction (PCBR), SVC-FCS, and pulsed field gradient (PFG) NMR. Each area observed by the three methods is indicated with dashed lines. (A) Distance (L) dependence plot (DDDC). (B) Time (t) dependence plot (TDDC).

In Fig. 4, we plotted the relative change in D, measured by PCBR, SVC-FCS, and PFG NMR, for cytc in aqueous HA solutions together with the points of Alexa 488, which are also shown in Fig. 3, for reference. In the SVC-FCS region, a prominent change was also observed whereas no dips were clearly revealed, probably because they are located outside the region where SVC-FCS is applicable. There is a huge blind area between PCBR and SVC-FCS. We also obtained the TDDC plot to indicate that the position of dips seems to be at the same position in the time domain, corresponding to the time constant of the polymer chain motions. We already discussed the mechanism of the appearance of dips in a previous paper [9]. Similar dips were again identified in the same position of TDDC for another molecule (cytc), the molecular weight of which is much larger than Alexa 488. This is another support for our previous conclusion, because the position of the turn-over should be determined, not by the diffusion time of the molecule, but the time constant of the polymer chain motion, that is, probably translational motion and reorganization of the mesh structure.

Fig. 4A shows us another aspect of AD. The local AD positions are different in the DDDC plot depending on the size of diffusing molecules. This means that the HA solution has the ability to sieve molecules. HA is involved in ECM, forming a mesh like structure inside the matrix. ECM has other components, the majority of which form a mesh structure as well. In ECM, various signaling molecules and substances for nutrients diffuse exhibiting AD behavior and ECM should act as a molecular sieve in providing these materials to various cells and organs.

We have performed very wide measurements over three orders of L and six orders of t in order to indicate a rough picture of the entire AD for a single inhomogeneous system (HA solution). Unfortunately in the present study, the experimental changes in D_{obs} were too small (10% for Alexa and 30% for cytc) to totally justify our present treatment using local ND and local AD. All the figures in Fig. 1 are emphasized to show the transient area of each plot. In both DDDC and TDDC, however, the transient area of D_{obs} (locally AD) was very narrow, being sandwiched by two constant areas (locally ND). It is reasonable that the scaling effect of the mesh structure of HA appears in a very narrow region of L and that the molecular diffusion in macroscopic HA appears to be ordinary ND. Since similar cases should be generally found in various inhomogeneous systems, we propose the present classification based on Eqs. (6) and (7) which appears to be more convenient for discussing general AD in inhomogeneous systems. At the same time, SVC-FCS is a suitable tool for probing the local AD as seen in the present experiment.

5. Conclusion

Anomalous diffusion of dye (Alexa 488) and protein (cytc) molecules was observed by SVC-FCS and was discussed using the concept of "local anomalous diffusion", which is convenient for treating the time and distance dependences of diffusion coefficients (TDDC and DDDC) over very wide range; three orders of diffusion distance and six orders of diffusion time.

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